

Dysfunctional S1P/S1PR1 signaling in the dentate gyrus drives vulnerability of chronic pain-related memory impairment

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This study investigates the molecular mechanisms underlying chronic pain-related memory impairment by focusing on S1P/S1PR1 signaling in the dentate gyrus (DG) of the hippocampus. Through behavioral tests (Y-maze and Morris water maze) and RNA-seq analysis, the researchers discovered that S1P/S1PR1 signaling is crucial for determining susceptibility to memory impairment, with decreased S1PR1 expression linked to structural plasticity changes and memory deficits. This work has **important** significance and a **convincing** level of evidence, thus offering new insights into the mechanisms underlying chronic pain-related memory impairment.

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Abstract

Memory impairment in chronic pain patients is substantial and common, and few therapeutic strategies are available. Chronic pain-related memory impairment has susceptible and unsusceptible features. Therefore, exploring the underlying mechanisms of its vulnerability is essential for developing effective treatments. Here, combining two spatial memory tests (Y-maze test and Morris water maze), we segregated chronic pain mice into memory impairment-susceptible and -unsusceptible subpopulations in a chronic neuropathic pain model induced by chronic constrictive injury of the sciatic nerve. RNA-seq analysis and gain/loss-of-function study revealed that S1P/S1PR1 signaling is a determinant for vulnerability to chronic pain-related memory impairment. Knockdown of the S1PR1 in the DG promoted a susceptible phenotype and led to structural plasticity changes of reduced excitatory synapse formation and abnormal spine morphology as observed in susceptible mice, while overexpression of the S1PR1 and pharmacological administration of S1PR1

agonist in the DG promoted an unsusceptible phenotype and prevented the occurrence of memory impairment, and rescued the morphological abnormality. Finally, GO enrichment analysis and biochemical evidence indicated that down-regulation of S1PR1 in susceptible mice may impair DG structural plasticity via interaction with actin cytoskeleton rearrangement-related signaling pathways including Itga2 and its downstream Rac1/Cdc42 signaling and Arp2/3 cascade. These results reveal a novel mechanism and provide a promising preventive and therapeutic molecular target for vulnerability to chronic pain-related memory impairment.

Introduction

Clinical and preclinical studies have demonstrated that chronic pain impairs memory or accelerates memory decline (1–3). Memory impairment is also a potential contributing factor to the maintenance of chronic pain and poor treatment response (2, 4, 5). Although memory deficits in chronic pain patients are substantial and common, some clinical observations indicate high heterogeneity (1, 6), suggesting that chronic pain-related memory impairment exhibits susceptible and unsusceptible features. However, few preclinical studies model this clinical scenario and explore its underlying mechanisms.

Findings from both human and animal studies have indicated that cognitive dysfunction associated with chronic pain is linked to structural and functional deficits within the hippocampus (7–11). In particular, the dentate gyrus (DG), as part of the hippocampus, plays a crucial role in memory formation processing (12, 13). The DG has been postulated to perform a variety of mnemonic tasks, such as pattern separation (14), novelty detection (15), and processing information related to spatial contexts (16). Nevertheless, the impact of pain-related cognitive syndromes on the dendritic morphologies of DG neurons, such as dendritic complexity, is inconsistent across different studies (7, 8, 17), and the molecular mechanisms remain minimally understood. The principal cell type of the DG is the dentate granule cells (DGCs), which are divided into immature and mature ones (18). Immature newborn DGCs undergo neurogenesis and play key roles in learning and memory due to their high excitability and enhanced synaptic plasticity (19, 20). Our previous study found that mice with chronic pain-related memory impairment showed significantly reduced adult neurogenesis in the DG (9). Contrarily, mature DGCs are less excitable and exhibit reduced synaptic plasticity to an extent, but emerging evidence suggests that this cell population is equally recruited in memory formation (21–25). It is intriguing to understand how mature DGCs would change in the state of chronic pain and by what molecular mechanism they facilitate chronic pain-related memory impairment.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite, functioning as a key signaling molecule in a variety of cellular processes, such as cell division, adhesion, migration, and death (26–28). S1P acts both through extracellular and intracellular modes (27–29). In its extracellular mode, increasing evidence has suggested that the S1P system is a modulator of pain and memory processing pathways through S1P receptors 1–5 (S1PR1–5), particularly S1PR1. For instance, in models of traumatic nerve injury, astrocyte-mediated S1PR1 neuroinflammation contributes to central sensitization through increased S1P production and IL-1 β release in the spinal cord, and beneficial effects of pain alleviation can be observed following IL-10-dependent S1PR1 antagonism (30). While there is evidence on S1P signaling regulating pain perception in the spinal cord, its role in higher pain centers remains largely unexplored. In regard to S1P function in memory processing, a recent study has hinted at the possibility of CNS S1PR1 agonism in the cellular repositioning of new DGCs and in regulating the integration of new neurons into pre-existing circuits, which may govern the process of memory formation (31). Additionally, activation of S1PR1 after traumatic brain injury in rats can significantly enhance neurogenesis and neurocognitive function (32). Importantly, increasing evidence suggests that the S1P receptor signaling pathway has profound effects on the regulation of synaptic strength, including

modulating synaptic architecture and plasticity, and mediating excitatory synaptic transmission in the hippocampus (33–35). However, there is limited understanding regarding the involvement of S1P signaling in chronic pain-related memory impairment, as well as the interactive pathways that may underlie the effects.

In the present study, by employing an array of techniques including rodents-based behavioral tests, RNA-Seq, imaging, pharmacological and biochemical approaches, we elucidated a pivotal role of S1PR1 within the hippocampal DG in the context of chronic pain associated memory impairment. Memory impairment-susceptible mice exhibited decreased S1PR1 expression in the hippocampal DG. Knockdown of S1PR1 in the DG facilitated the development of a vulnerable phenotype and led to abnormal structural plasticity in DGCs. Conversely, overexpression of S1PR1 or pharmacological administration of an S1PR1 agonist in the DG, promoted an unsusceptible phenotype, thereby averting the onset of memory impairment, and alleviated morphological abnormalities in DGCs. Subsequent mechanistic investigation demonstrated that, loss of S1PR1 in the DG results in actin dysregulation via interaction with integrin $\alpha 2$ (ITGA2) and its downstream Rac1/Cdc42 signaling and Arp2/3 cascade, leading to abnormal structural synaptic plasticity and ultimately causes memory impairment. Taken together, this study identified potential molecular mechanisms and promising therapeutic targets for preventing and treating vulnerability to memory impairment associated with chronic pain.

Results

Segregation of chronic pain mice into memory impairment-susceptible and -unsusceptible subpopulations

We utilized sciatic nerve chronic constriction injury (CCI) in C57BL/6J mice to model chronic pain state and tested the memory performance of mice with chronic neuropathic pain. CCI induced long-lasting mechanical allodynia (Supplemental Figure 1A) and thermal hyperalgesia (Supplemental Figure 1B). No significant change in locomotor functions was observed in these injured mice (Supplemental Figure 1C and 1D). Consistent with our recent study (9), only chronic (21~28 days, referred to as CCI-Chronic) and not acute (5~7 days, referred to as CCI-Acute) exposure to constrictive injury-induced neuropathic pain impaired spatial memory formation in both Y-maze test (Figure 1A, B, D, E) and Morris water maze (MWM) test (Figure 1A, C, F, G).

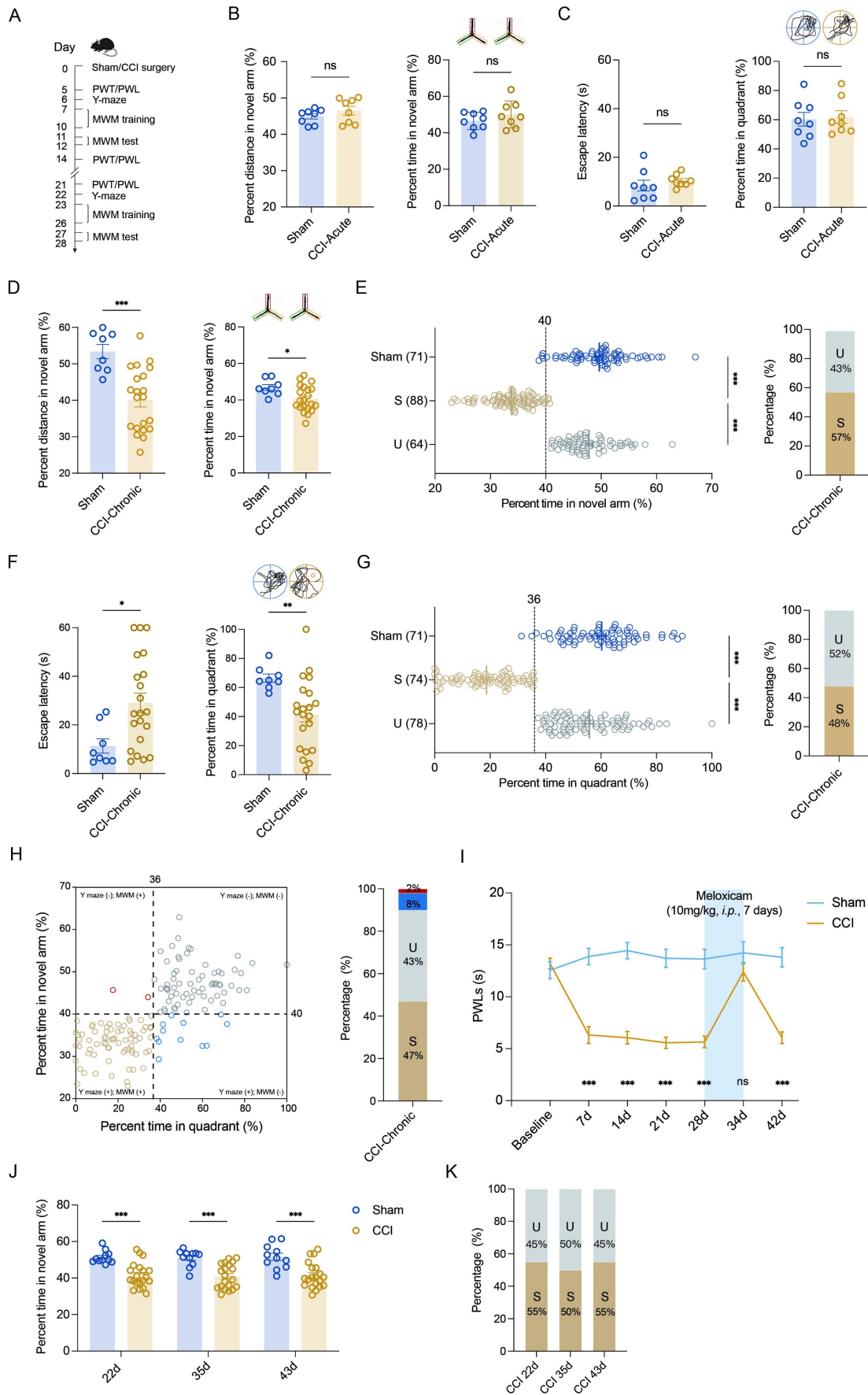


Figure 1.

Segregation of mice with chronic pain into susceptible and unsusceptible subpopulations to memory impairment.

(A) Timeline of CCI surgery, pain threshold tests, Y-maze test and MWM training. (B) Representative travelling traces and statistical results of Y maze test showing distance and percent time in the novel arm (Red) in CCI-Acute mice (6d post CCI, n = 8-21). (C) Representative travelling traces and statistical results of MWM training showing escape latency and percent time in the quadrant in CCI-Acute mice (11d post CCI, n = 8-21). (D) Representative travelling traces and statistical results of Y maze test showing distance and percent time in the novel arm (Red) in CCI-Chronic mice (22d post CCI, n = 8-21). (E) Horizontal scatterplot depicting the distribution of ratio of time in novel arm for Sham, susceptible (S), and unsusceptible (U) mice in Y maze test. Bar graph represents the ratio of S and U mice in CCI-Chronic mice (22d post CCI, n = 71-152). (F) Representative travelling traces and statistical results of MWM training showing escape latency and percent time in the quadrant in CCI-Chronic mice (27d post CCI, n = 8-21). (G) Horizontal scatterplot depicting the distribution of ratio of time in the quadrant for Sham, S, and U mice in MWM training. Bar graph represents the ratio of S and U mice in CCI-Chronic mice (27d post CCI, n = 71-152). (H) Time in novel arm versus percent time in quadrant, for 152 CCI-Chronic mice. Each dot corresponds to one mouse. Colors of dots correspond to the groups of U in Y maze test but S in MWM (Red), U in both Y maze and MWM (Grey), S in Y maze test but U in MWM (Blue), and S in both Y maze and MWM (Yellow), respectively. Bar graph represents the ratio of each group in CCI-Chronic mice (n=152). (I) PWLs before and after administration of meloxicam (10mg/kg, *i.p.*, n = 10-20). (J) Statistical results of MWM training showing escape latency and percent time in the quadrant in 27d CCI mice before meloxicam administration (10mg/kg, *i.p.*, n = 10-20). (K) Performance of CCI mice in Y maze test before and after meloxicam administration (10mg/kg, *i.p.*). Bar graph (right) represents the ratio of U and S on 22d, 36d and 43d after CCI (n = 10-20). Data were analyzed by unpaired t test or one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups when appropriate. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001. CCI, chronic constrictive injury; d, day; MWM, Morris water maze; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; U, unsusceptible; S, susceptible.

To investigate whether memory impairment has susceptible and unsusceptible features in CCI-induced chronic neuropathic pain mice, we analyzed results from Y-maze and MWM tests using the k-means clustering algorithm and segregated into two clusters in a large number of CCI mice ($k=2$). After assigning each data point to its closest k-center, we drew a median between both the centroids as a cutoff value. One cluster, including mice displaying a ratio of time more than the cutoff value, was defined as the unsusceptible mouse cluster. The other cluster, including mice exhibiting a ratio of time less than the cutoff value, was defined as the susceptible mouse cluster. Using this algorithm, a ratio of 40% (percent time spent in the novel arm) and a ratio of 36% (percent time in the target quadrant) were set as cutoff values for Y-maze test (Figure 1E, left) and MWM test (Figure 1G, left), respectively. Accordingly, in the Y maze test, 43% of CCI-Chronic mice were unsusceptible, and the remaining 57% were susceptible (Figure 1E, right). In the MWM test, 52% of CCI-Chronic mice were unsusceptible, and 48% were susceptible (Figure 1G, right).

We then used a four-quadrant chart to plot the data of CCI-Chronic mice memory performance for examining the consistency between the two behavioral assays. One variable, the percent time in the quadrant, was represented on the x-axis, and another variable, the percent time in the novel arm, was represented on the y-axis. The quadrants are determined by dividing the chart into four parts based on the cutoff values of Y-maze and MWM tests. As shown in the chart (Figure 1H, left), quadrant 1 (top right) and 3 (bottom left) respectively displayed unsusceptible and susceptible mice in both Y-maze and MWM tests, while quadrant 2 (top left) and 4 (bottom right) displayed mice unsusceptible in one test but susceptible in the other one. The bar graph (Figure 1H, right) showed that the majority of CCI-Chronic mice exhibited consistent memory

performance in the two behavioral tests (Susceptible: 47%; unsusceptible: 43%), and only 10% of mice displayed susceptibility in a single test (Y maze: 8%; MWM, 2%), suggesting good agreement between the two different assays. Given that the MWM test cannot be conducted once a week for multiple repeated measurements for the same batch of mice, the Y maze test was used to investigate the duration of memory impairment induced by chronic pain. The results showed that memory impairment can last at least 63 days post CCI surgery, providing a workable time window for further investigations (Supplemental Figure 2A, B).

We next examined whether the susceptibility or insusceptibility to memory impairment in mice with chronic pain is associated with the pain threshold. Linear regression, followed by a goodness-of-fit measure of R-squared (r^2), was used to determine the correlation between the two variables: percent time spent in the novel arm and pain threshold, or percent time in the target quadrant and pain threshold. The statistics revealed no correlation between memory performance for each mouse against its thermal pain threshold ($r^2=0.04$, Supplemental Figure 3A; $r^2=0.05$, Supplemental Figure 3B). Furthermore, we administered the CCI-Chronic mice with the *selective COX-2 inhibitor* NSAID analgesic meloxicam. Recommended doses for meloxicam in mice range from 1 to 10 mg/kg *i.p.* (36), and the duration of action (10mg/kg) lasts at least 24 hrs on day 7 post intraperitoneal injection once daily (Supplemental Figure 4). Here, CCI-Chronic mice were subjected to pain treatment by meloxicam (10mg/kg) for 7 days (once daily from day 28 to 34 post CCI) (Figure 1I). During the duration of analgesic effects (day 34~35 post CCI) (Figure 1I), we conducted the Y-maze test and found that the analgesia could not relieve the memory impairment (Figure 1J). Consistently, the percentage of susceptible and unsusceptible mice remained stable (Figure 1K), further indicating that the susceptibility or insusceptibility to memory impairment is marginally correlated with the pain tolerance of mice.

S1PR1 expression is decreased in the hippocampal DG of susceptible mice

Hippocampal DG plays a vital role in learning and memory formation. To identify molecular mechanisms possibly contributing to susceptibility to memory impairment, we analyzed the hippocampal DG by RNA-Seq on day 28 after CCI when the mice with chronic pain were segregated into unsusceptible and susceptible subpopulations by Y-maze and MWM tests. We detected a total of 510 differentially expressed genes (DEGs) between Sham and susceptible mice, with 330 genes (65%) upregulated and 180 genes (35%) downregulated (Figure 2A). We noticed that KEGG analysis revealed significant enrichment of six downregulated genes in the lipid metabolism pathway for Sham versus susceptible mice (Figure 2B and 2C). Combining the trend analysis of Sham versus unsusceptible versus susceptible mice, we found in the trend pattern of Figure 2D (Left: Sham; middle, unsusceptible; right: susceptible), sphingolipid metabolism was significantly enriched (Figure 2E). In particular, transcription of *Sptlc3* was downregulated (Figure 2C and 2E). *Sptlc3* encodes the subunit of the serine palmitoyltransferase (SPT) which catalyzes the rate-limiting step in sphingolipid biosynthesis (37). Defective SPT leads to disturbed sphingolipid homeostasis and failure of the subsequent production of metabolites such as glucosylceramide (38) and sphingosine 1-phosphate (S1P) (39), contributing to the occurrence of biological disorders. Previous studies have found that S1P/S1PR1 signaling is highly involved in hippocampus-engaged behaviors (31), we then verified the expression of S1PR1 in the hippocampal DG by Western blotting (WB), with no change of S1PR1 observed on day 7 after CCI (Figure 2F and 2H) but significant downregulation in susceptible mice compared with Sham and unsusceptible ones (Figure 2G and 2I).

Furthermore, we characterized the expression profile of S1PR1 in the hippocampal DG. Immunofluorescence staining results showed that S1PR1 was mostly co-expressed with neuronal nuclear protein (NeuN) in neurons and merely co-expressed with glial fibrillary acidic protein (GFAP) in astrocytes or ionized calcium-binding adaptor molecule 1 (Iba1) in microglia (Supplemental Figure 5A and 5C). We also detected S1PR1 was highly co-expressed with calcium-

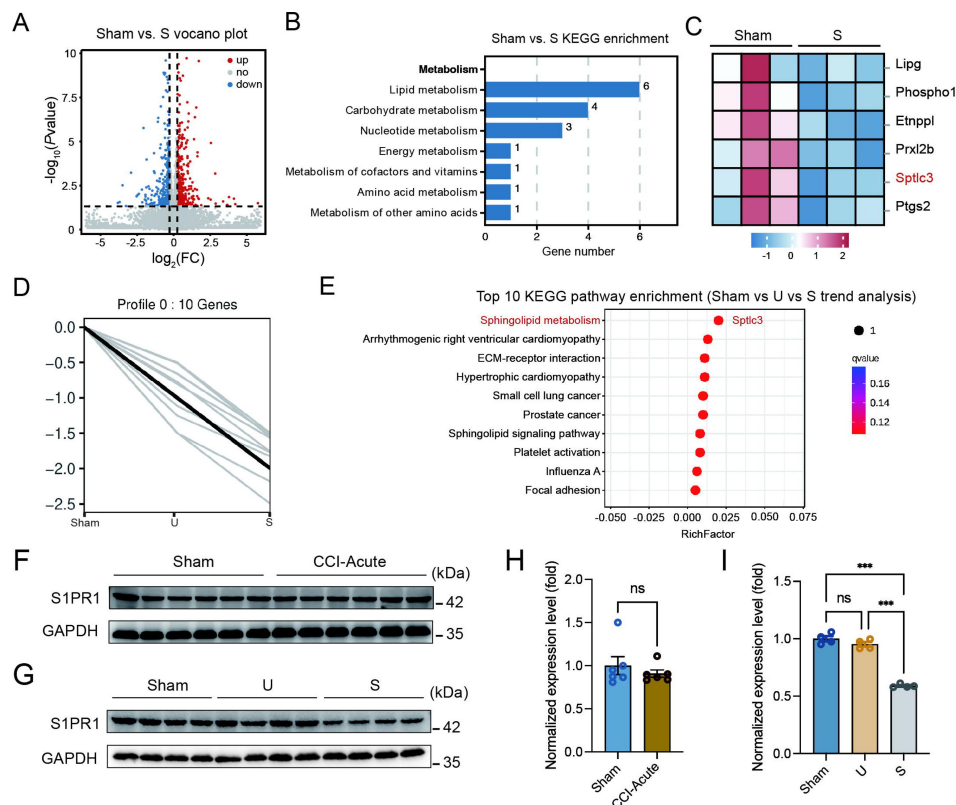


Figure 2.

S1PR1 expression is decreased in the hippocampal DG of susceptible mice.

(A) Volcano plot showing RNA-Seq data for DG from Sham versus susceptible mice. DEGs are designated in red (upregulation [up]) and blue (downregulation [down]) and defined as having an FDR of less than 0.05. (B) Bar plot showing significant enrichment of DEGs in various pathways related with metabolism for Sham versus susceptible mice. (C) Relative expression levels are shown for genes related with lipid metabolism upon susceptible as compared with Sham. (D) Trend pattern used for analysis of Sham versus U versus S. (E) Bubble diagram represents the top 10 enrichment of KEGG pathways. (F-I) Example Western bands (F) and densitometric comparison (H) of the average expression of S1PR1 in DG lysates from Sham and CCI-Acute mice (7d post CCI). Lane1-6 represent Sham, Lane 7-12 represent CCI-Acute ($n = 6$); Example Western bands (G) and densitometric comparison (I) of the average expression of S1PR1 in DG lysates from Sham, U and S mice. Lane1-4 represent Sham, Lane 5-8 represent U, and Lane 9-12 represent S ($n = 4$). Data were analyzed by unpaired t test or one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups when appropriate. All data are presented as the mean $\pm$ s.e.m. ns, not significant; *** $p < 0.001$. CCI, chronic constrictive injury; d, day; U, unsusceptible; S, susceptible.

calmodulin (CaM)-dependent protein kinase II (CaMKII)-expressing excitatory neurons but sparsely with glutamic acid decarboxylase 67 (GAD67)-expressing inhibitory neurons (Supplemental Figure 5B and 5D). Taken together, these findings raised the possibility that S1P/S1PR1 may participate in the occurrence of chronic pain-related memory impairment.

Knockdown of S1PR1 in the hippocampal DG promotes memory impairment susceptibility

To figure out the causal link between S1PR1 and chronic pain-related memory impairment, we generated recombinant adeno-associated virus 2/9 (AAV2/9) expressing a small hairpin RNA targeting S1PR1 (rAAV-CaMKIIa-EGFP-5'miR-30a-shRNA(S1pr1)-3'-miR30a-WPREs, the shRNA sequence is provided in Supplemental Table 1). Following the schematic experimental procedure shown in [Figure 3A](#), intra-DG injection was conducted ([Figure 3B](#) and Supplemental Figure 6A). We first confirmed the knockdown efficiency of the virus in the hippocampal DG using WB ([Figure 3C](#)). We then examined the effects of knockdown of S1PR1 in the DG on pain threshold. Compared with mice expressing scramble shRNA, mice expressing shS1pr1 in the DG had no effects on pain sensation ([Figure 3D](#)). Next, we assessed the effects of loss of S1PR1 in the DG on memory-related behaviors. In the Y-maze test, reduction of S1PR1 worsened the performance of Sham-treated mice by reducing the distance travelled and time spent in the novel arm, as well as enhancing the memory impairment of CCI-treated mice ([Figure 3E](#)). The results were consistent in the MWM test ([Figure 3F](#)). Consequently, knockdown of S1PR1 in the CCI-treated mice led to more susceptible mice (up to 86%) to memory impairment ([Figure 3G](#)). Thus, the above results suggest that DG S1PR1 exerts a negative regulatory effect on chronic pain-related memory impairment.

Upregulation of S1PR1 in the hippocampal DG prevents the development of memory impairment susceptibility

To gain a deeper understanding of the functional consequences of enhanced S1P/S1PR1 signaling in the hippocampal DG, we generated recombinant adeno-associated virus 2/9 (AAV2/9) expressing the S1PR1 coding sequence (rAAV-CaMKIIa-S1pr1-P2A-EGFP-WPRE-hGH-polyA, sequence referred to Supplemental Table 1). Following the experimental flowchart depicted in [Figure 4A](#), intra-DG injection was conducted ([Figure 4B](#) and Supplemental Figure 6B). We first confirmed the overexpression efficiency of the virus in the hippocampal DG using WB ([Figure 4C](#)). Next, we assessed how S1PR1 overexpression in the DG affected pain threshold. Compared with the mice expressing the scramble shRNA, mice overexpressing S1PR1 in the DG had no effects on pain sensitization ([Figure 4D](#)). We then examined whether overexpression of S1PR1 in the DG influences chronic pain-related memory impairment according to behavioral paradigms of Y-maze and MWM tests. In the Y-maze and MWM tests, overexpression of S1PR1 in CCI-treated mice significantly improved the spatial memory formation by promoting insusceptibility to memory impairment (up to 82%), but had no obvious effects on Sham-treated mice ([Figure 4E-G](#)).

A previous study demonstrated that chronic administration of the selective S1PR1 agonist SEW2871 for 14 days inhibited the reduction of S1PR1 expression and improved impaired spatial memory in rats ([40](#)). The dose used in the study ([40](#), [41](#)) was effective to induce biological responses without causing kidney and liver injuries ([41](#)). We then investigated whether a continuous 14-day local infusion of SEW2871 in the DG from day 7 post CCI could inhibit the reduction of S1PR1 expression and confer insusceptibility to memory impairment. For this purpose, a cannula was implanted into the DG of mice, and the S1PR1 agonist SEW2871 at a dose of 0.7mg/kg/day ([40](#)) was administered into the DG from day 7 to 21 post CCI surgery ([Figure 5A](#) and [5B](#)). WB analysis revealed that bilateral DG injection of S1PR1 agonist SEW2871 inhibited the reduction of S1PR1 expression ([Figure 5C](#)), but did not alter the basal nociception of thermal stimuli ([Figure 5D](#)). In contrast, intra-DG administration of SEW2871 in CCI-treated

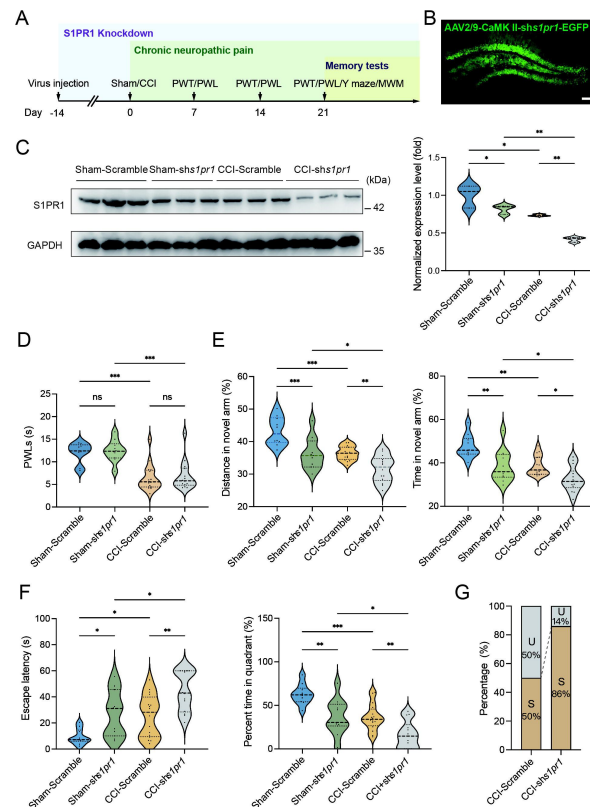


Figure 3.

S1PR1 knockdown in the DG induces memory impairment.

(A) Timeline of intra-DG virus injection, CCI surgery, pain threshold tests, Y maze test and MWM training. **(B)** A confocal image showing virus expression in the DG (Scale bar, 100 μ m). **(C)** Example Western bands showing efficient S1PR1 knockdown in the DG lysates from Sham-Scramble, Sham-shs1pr1, CCI-Scramble, and CCI-shs1pr1. Densitometric comparison of the average expression of S1PR1 ($n = 6$). **(D)** Pain threshold in Sham- and CCI-treated mice subjected to Scramble/shs1pr1 in the DG ($n = 8-10$). **(E)** Quantitative summary of Y-maze showing distances traveled and time spent in the novel arm in Sham- and CCI-treated mice subjected to Scramble/shs1pr1 in the DG ($n = 10-16$). **(F)** Quantitative summary of MWM training showing escape latency and time spent in the quadrant in Sham- and CCI-treated mice subjected to Scramble/shs1pr1 in the DG ($n = 10-16$). **(G)** Ratio of U and S in CCI-Scramble and CCI-shs1pr1 mice. Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CCI, chronic constrictive injury; MWM, Morris water maze; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; U, unsusceptible; S, susceptible.

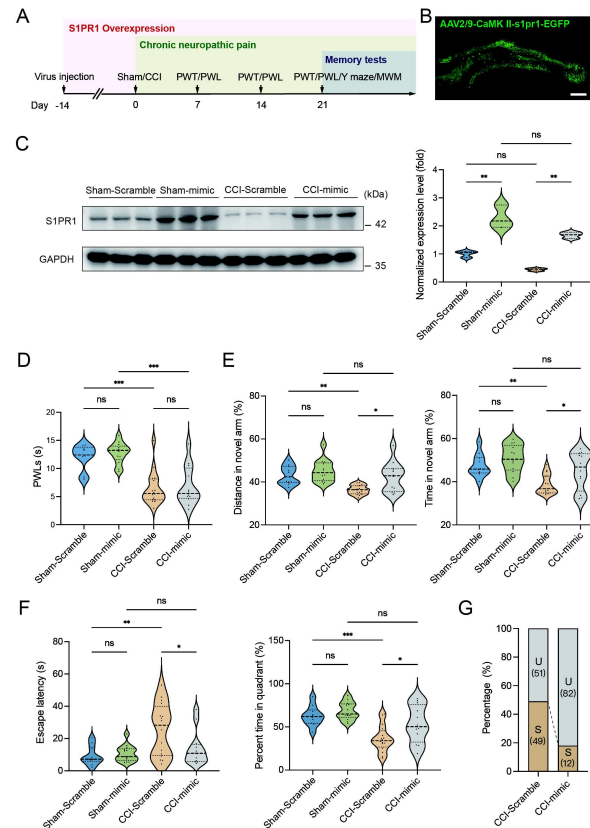


Figure 4.

Overexpression of S1PR1 in the DG rescues chronic pain-induced memory impairment.

(A) Timeline of intra-DG virus injection, CCI surgery, pain threshold tests, Y maze test and MWM training. **(B)** A confocal image showing virus expression in the DG (Scale bar, 100 μ m). **(C)** Example Western bands showing efficient S1PR1 overexpression in the DG lysates from Sham-Scramble, Sham-mimic, CCI-Scramble, and CCI-mimic. Densitometric comparison of the average expression of S1PR1 ($n = 6$). **(D)** Pain threshold in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 8-10$). **(E)** Quantitative summary of Y-maze showing distances traveled and time spent in the novel arm in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 10-16$). **(F)** Quantitative summary of MWM training showing escape latency and time spent in the quadrant in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 10-16$). **(G)** Ratio of U and S in CCI-Scramble and CCI-shs1pr1 mice. Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CCI, chronic constrictive injury; MWM, Morris water maze; PWT, paw withdrawal latency; PWT, paw withdrawal threshold; U, unsuspensible; S, susceptible.

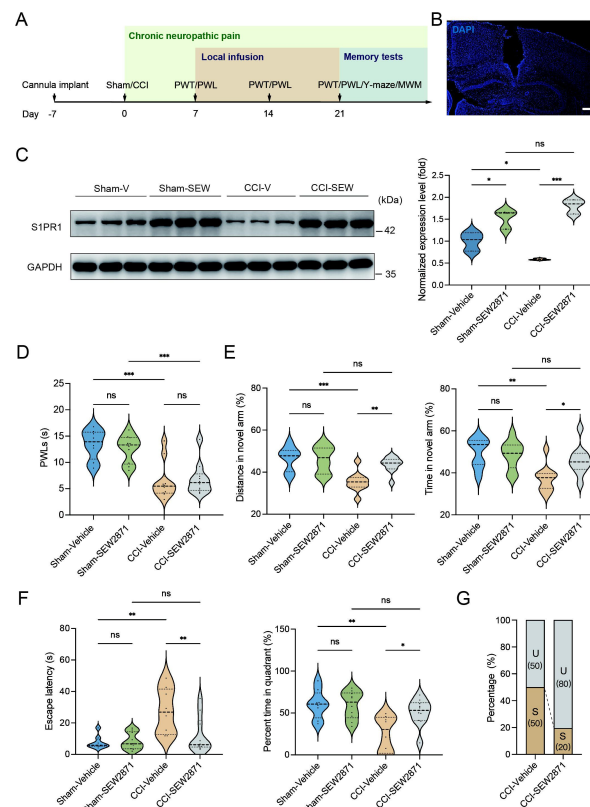


Figure 5.

Local infusion of SEW2871 in the DG prevented the presence of chronic pain-induced memory impairment.

(A) Timeline of cannula implant, CCI surgery, pain threshold tests, Y maze test and MWM training. **(B)** Confocal image showing cannula implanted in the DG (Scale bar, 100 μ m). **(C)** Example Western bands showing expression of S1PR1 in DG lysates from Sham-Vehicle, Sham-SEW2871, CCI-Vehicle, and CCI-SEW2871. Densitometric comparison of the average expression of S1PR1 ($n = 6$). **(D)** Pain threshold in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 8-10$). **(E)** Quantitative summary of Y-maze showing distances traveled and time spent in the novel arm in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 10-16$). **(F)** Quantitative summary of MWM training showing escape latency and time spent in the quadrant in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 10-16$). **(G)** Ratio of U and S in CCI-vehicle and CCI-SEW2871 mice. Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CCI, chronic constrictive injury; DG, dentate gyrus; U, unsusceptible; S, Susceptible.

mice significantly increased the distance traveled and time in the novel arm in the Y-maze (**Figure 5E** [↗](#)), decreased escape latency and increased percent time in the quadrant in the MWM (**Figure 5F** [↗](#)), resulting in an increased ratio (up to 80%) of unsusceptible mice (**Figure 5G** [↗](#)). Thus, it can be inferred from the above observations that upregulation of S1PR1 in the hippocampal DG promotes insusceptibility to chronic pain-related memory impairment.

S1PR1 deficiency in the hippocampal DG modulates structural plasticity of DGCs

We next questioned how DG S1P/S1PR1 signaling modulates memory impairment. Adult neurogenesis enhances the plasticity of the hippocampus ([42](#) [↗](#)), as well as preexisting granule neurons of the DG undergo dynamic alterations that include dendritic extension and retraction, synapse creation, and elimination ([43](#) [↗](#)). Given that almost all the excitatory inputs from all sources towards DGCs are situated on their dendritic spines while the inhibitory connections are distributed in different layers ([18](#) [↗](#)), we decided to examine the morphological changes of excitatory synapses which technically facilitates our observation. First, we utilized transmission electron microscope (TEM) to observe changes in the number of excitatory synapses and postsynaptic densities (PSD) in mice expressing shs1pr1 and scrambled shRNA (**Figure 6A** [↗](#)). In line with the behavioral tests, Sham-shS1pr1 mice showed a decreased number of excitatory synapses (**Figure 6B** [↗](#)), accompanied by a shorter PSD length and width compared with Sham-Scramble mice (**Figure 6C** [↗](#)). Furthermore, Golgi staining (**Figure 6D** [↗](#)) revealed that Sham-shS1pr1 mice had a lower dendritic intersection number (**Figure 6E** [↗](#)), shortened total dendritic length (**Figure 6F** [↗](#)), decreased mushroom/stubby type (**Figure 6G** [↗](#), left) spines, and no change in thin/filopodia type (**Figure 6G** [↗](#), right) dendritic spines. Additionally, CCI-shS1pr1 mice showed even more pronounced phenotypes as described above compared with CCI-Scramble mice (**Figure 6A** [↗](#)-**6G** [↗](#)). Additionally, overexpression of S1PR1 in the DG significantly restored structural synaptic plasticity by increasing the number of excitatory synapses, PSD length and width (**Figure 7A-C** [↗](#)). Golgi staining results revealed that the intersection number of dendritic branches (**Figure 7D-E** [↗](#)), the total length of dendrites (**Figure 7F** [↗](#)), and the number of mushroom/stubby type dendritic spines (**Figure 7G** [↗](#), left) increased in response to the intervention. Consistently, TEM images and Golgi staining revealed that continuous activation of S1PR1 in the DG significantly prevented the occurrence of defective synaptic plasticity (**Figure 7H-N** [↗](#)), indicating that activation of S1PR1 in the DG confers insusceptibility to memory impairment.

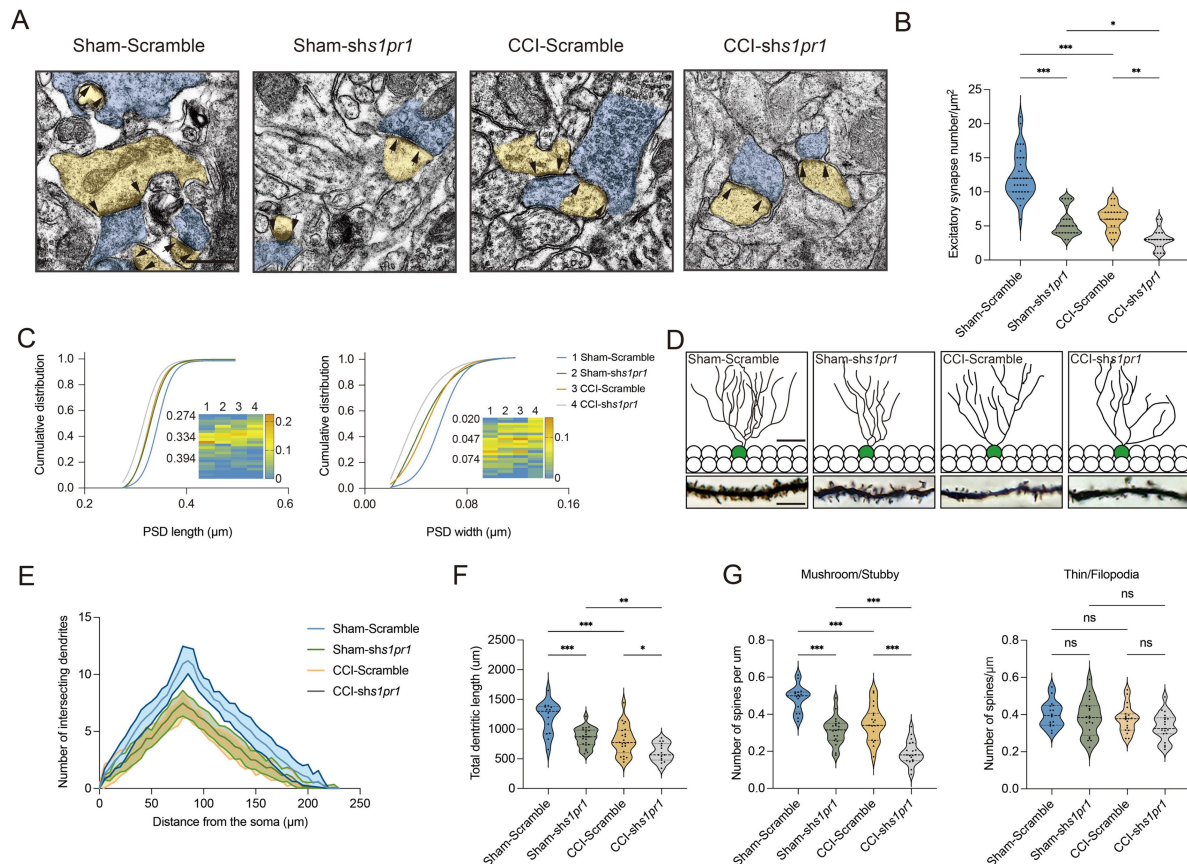


Figure 6.

Susceptible mice exhibit altered excitatory synaptic plasticity in the hippocampal dentate gyrus.

(A) Representative TEM images of synapses in the DG in Sham and CCI-Chronic mice (27d post CCI). Blue indicates presynaptic site and yellow indicates postsynaptic sites of excitatory synapses, respectively. Synaptic densities are bracketed by arrows (Scale bar, 500 nm). **(B)** Mean number of excitatory synapses per μm^2 of DG in Sham, unsusceptible and susceptible mice ($n = 18-24$ from 4 mice/group). **(C)** Cumulative distribution plots for the lengths and widths of postsynaptic density in the DG in Sham, unsusceptible and susceptible mice ($n = 102-156$ from 4 mice/group). **(D)** Representative Golgi-staining images of dendritic spine morphology from the DG in Sham, unsusceptible and susceptible mice (Scale bar, top:50 μm ; bottom:10 μm). **(E)** The number of intersections of all dendritic branches in Sham, unsusceptible and susceptible mice ($n = 15-18$ from 4 mice/group). **(F)** Violin plots indicate the total dendritic length. **(G)** Violin plots indicate the number of mushroom/stubby type dendritic spines (left), and the number of thin/filopodia type dendritic spines (right) in Sham, unsusceptible and susceptible mice ($n = 15-18$ from 4 mice/group). Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups when appropriate. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CCI, chronic constrictive injury; TEM, transmission electron microscope.

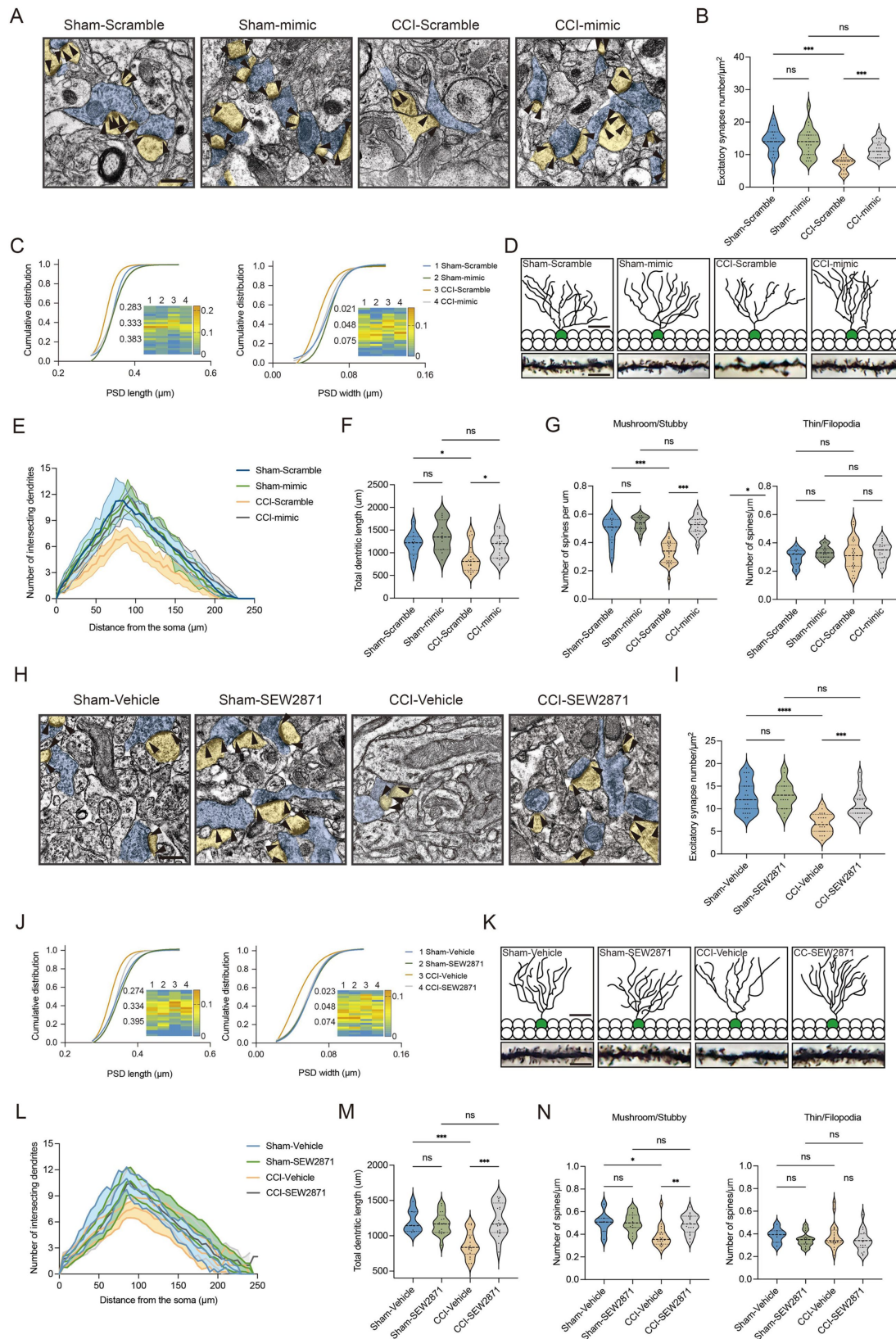


Figure 7.

Overexpression of S1PR1 or local infusion of SEW2871 in the DG maintained the synaptic structural plasticity.

(A) Representative TEM images of synapses in the DG in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG. Blue indicates presynaptic site and yellow indicates postsynaptic sites of excitatory synapses, respectively. Synaptic densities are bracketed by arrows (Scale bar, 500 nm). (B) Mean number of excitatory synapses per μm^2 of DG in Sham- and CCI-treated mice subjected to Scramble/mimic ($n = 18-24$ from 4 mice/group). (C) Cumulative distribution plots for the lengths and widths of postsynaptic density in the DG in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 121-162$ from 4 mice/group). (D) Representative Golgi-staining images of dendritic spine morphology from the DG in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG (Scale bar, top: 50 μm ; bottom: 10 μm). (E) The number of intersections of all dendritic branches in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 18-24$ from 4 mice/group). (F) Violin plots indicate the total dendritic length ($n = 18-24$ from 4 mice/group). (G) The number of mushroom/stubby type dendritic spines (left), and the number of thin/filopodia type dendritic spines (right) in the DG of in Sham- and CCI-treated mice subjected to Scramble/mimic in the DG ($n = 18-24$ from 4 mice/group). (H) Representative TEM images of synapses in the DG in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG. Blue indicates presynaptic site and yellow indicates postsynaptic sites of excitatory synapses, respectively. Synaptic densities are bracketed by arrows (Scale bar, 500 nm). (I) Mean number of excitatory synapses per μm^2 of DG in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 ($n = 18-24$ from 4 mice/group). (J) Cumulative distribution plots for the lengths and widths of postsynaptic density in the DG in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 121-162$ from 4 mice/group). (K) Representative Golgi-staining images of dendritic spine morphology from the DG in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG (Scale bar, top: 50 μm ; bottom: 10 μm). (L) The number of intersections of all dendritic branches in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 18-24$ from 4 mice/group). (M) Violin plots indicate the total dendritic length ($n = 18-24$ from 4 mice/group). (N) The number of mushroom/stubby type dendritic spines (left), and the number of thin/filopodia type dendritic spines (right) in the DG of in Sham- and CCI-treated mice subjected to local infusion of vehicle/SEW2871 in the DG ($n = 18-24$ from 4 mice/group). Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CCI, chronic constrictive injury; DG, dentate gyrus; TEM, transmission electron microscope.

Next, we assessed whether the above structural plasticity changes can be observed in susceptible mice. First, we examined the number of neurons between susceptible and unsusceptible mice by staining for NeuN. The statistical result showed that the number is not grossly different between the two populations (Supplemental Figure 7). We then found that the synaptic structural plasticity in the hippocampal DG is disrupted in susceptible mice, but not in Sham and unsusceptible ones. TEM was also used to observe changes in the number of excitatory synapses and postsynaptic densities (PSD) (Supplemental Figure 8A and 8H). The results showed that 7-day CCI mice exhibited a similar number of excitatory synapses (Supplemental Figure 8B), as well as PSD length and width, to Sham mice (Supplemental Figure 8C). On day 21 post CCI, susceptible mice displayed a decreased number of excitatory synapses (Supplemental Figure 8I), accompanied by shortened PSD length and width compared with unsusceptible and Sham mice (Supplemental Figure 8J). Furthermore, Golgi staining was utilized to identify the dendritic formation and spine morphology (Supplemental Figure 8D and 8K). 7-day CCI mice showed no altered dendritic intersection number (Supplemental Figure 8E), total dendritic length (Supplemental Figure 8F), mushroom/stubby type (Supplemental Figure 8G, left) and thin/filopodia type (Supplemental Figure 8G, right) dendritic spines. On day 21 post CCI, susceptible mice displayed a decreased dendritic intersection number (Supplemental Figure 8L), shortened total dendritic length (Supplemental Figure 8M), and a decreased number of mushroom/stubby type dendritic spines (Supplemental Figure 8N, left) compared with unsusceptible and Sham mice. However, there were no changes in the number of

thin/filopodia type (Supplemental Figure 8N, right) dendritic spines. Overall, these findings suggest that the variation in structural plasticity of the hippocampal DG underlies susceptibility and insusceptibility to chronic pain-related memory impairment, and it is modulated by S1P/S1PR1 signaling.

Defective S1P/S1PR1 signaling induced dysregulation of actin cytoskeleton organization in susceptible mice

Neuronal structural plasticity, such as morphogenesis of dendrites and dendritic spines, is primarily regulated by the actin cytoskeleton (44, 45). Disruption of normal actin organization has been associated with numerous neurological and psychiatric diseases (46). It has been demonstrated that S1P/S1PRs modulate significant cytoskeletal rearrangements in various cellular systems through actin regulatory proteins such as Rho GTPases, including Rac1 and Cdc42 (28, 47). These proteins promote filopodia formation by stimulating actin polymerization through WAVE and the Arp2/3 complex (47, 48). Additionally, we analyzed the RNA-Seq data to identify genes that may be involved in S1PR1-regulated cytoskeletal dynamics in our animal model. In the KEGG analysis comparing Sham versus susceptible mice and the trend analysis (Sham versus unsusceptible versus susceptible mice), integrin $\alpha 2$ (encoded by *Itga2*) was found to be enriched and downregulated among cell adhesion molecules and regulation of actin cytoskeleton pathway genes (Figure 8A and 8B). Integrins serve as linkers between the extracellular matrix and intracellular actin cytoskeleton, mediating cytoskeletal organization (49). Therefore, we asked whether S1P/S1PR1 induces structural plasticity in susceptible mice by regulating actin dynamics in the DG. To address this, we verified the expression levels of actin regulatory proteins, including Rac1, Cdc42, Arp2, Arp3 and *Itga2*, in 7-day CCI mice (Figure 8C and 8D), unsusceptible mice, and susceptible mice (Figure 8E and 8F). To figure out the regulation is specifically S1PR1-dependent, but not through other S1PRs and Rho GTPase, we also checked the expression level of S1PR2 and Rho GTPase RhoA which interacts with S1PR2 (28, 47, 50) (Supplemental Figure 9). We observed a significant decreased in the levels of Rac1, Cdc42, Arp2, Arp3 and *Itga2* in susceptible mice, but not in Sham and unsusceptible mice. As expected, there is no change in the expression level of S1PR2 and RhoA in CCI animals. To determine whether the reduction in levels of these proteins is associated with downregulated S1PR1 signaling, we examined lysates of hippocampal DG tissue extracted from S1PR1 DG conditional knockdown mice, revealing decreased levels of Rac1, Cdc42, Arp2,

Arp3 and *Itga2* as assayed by WB (Figure 8G and 8H). Conversely, S1PR1 overexpression in the DG led to increased levels of Rac1, Cdc42, Arp2, Arp3 and *Itga2* (Figure 8I and 8J). These data indicate that S1PR1 functions upstream of Rac1, Cdc42, Arp2/3 and *Itga2*. Given a previous study suggesting the involvement of an isoform of integrin ITGB4 in S1PR1-mediated Rac1 activation (51), our results raise the possibility that dysregulated actin rearrangement elicited by S1PR1 downregulation in the DG may be influenced by *Itga2*-dependent activation of the Rac1/Cdc42 signaling cascade and Arp2/3 dependent actin polymerization.

S1P/S1PR1 regulates actin polymerization by interaction with ITGA2

We then investigated the regulatory role of S1P/S1PR1 signaling in actin polymerization. The polymerization of monomeric actin (G-actin) into actin filaments (F-actin) to form the actin cytoskeleton frequently occurs primarily at or near the plasma membrane. The organization of actin filaments determines the shape, stiffness, and movement of the cell surface and also facilitates spine morphology and function (52). Thus, we first examined actin polymerization by quantifying the transition from G-actin to F-actin transition. Densitometric analysis of F-actin/G-actin Western blots (Figure 9A and 9B) indicated a significant decrease in the relative percentage of F-actin in the DG of susceptible mice compared with that of Sham and unsusceptible

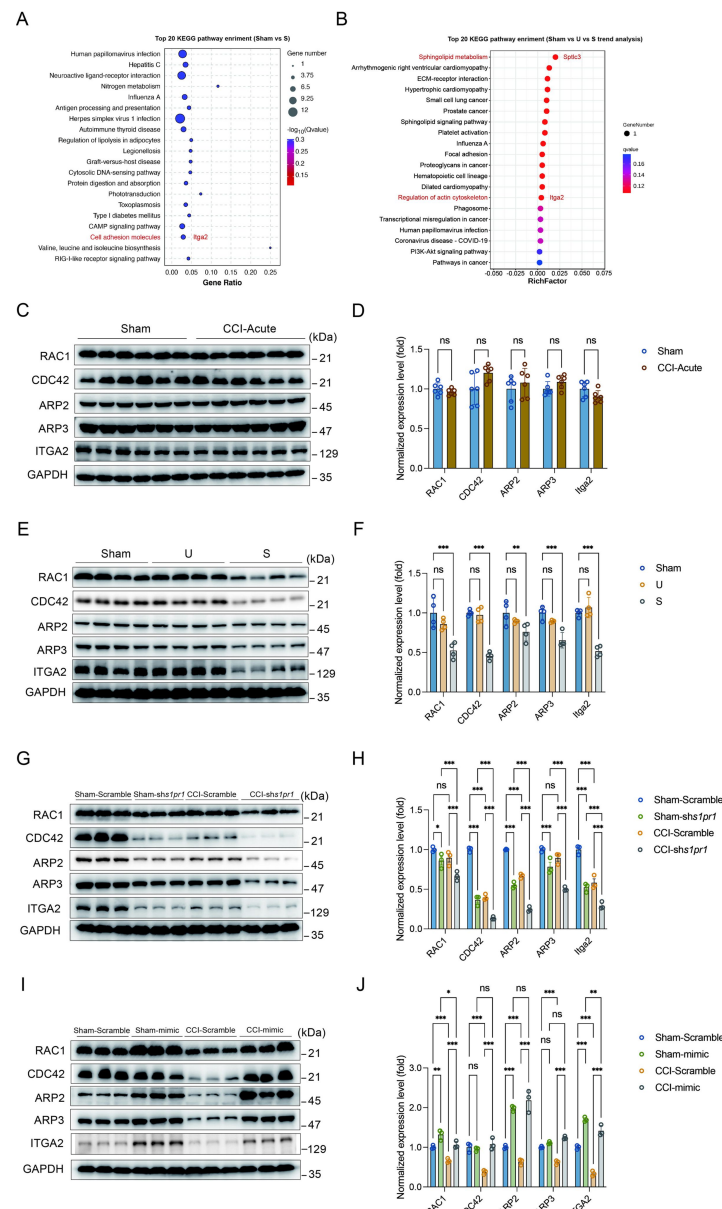


Figure 8.

Susceptible mice, S1PR1-knockdown mice and S1PR1 overexpression mice exhibit altered CDC42/RAC1 activity, ARP2/3-dependent actin signaling and engage ITGA2 in the DGcs.

(A) Bubble diagram showing significant enrichment of DEGs in top 20 KEGG pathways for Sham versus susceptible mice. (B) Bubble diagram represents the top 20 enrichment of KEGG pathways using analysis of Sham versus U versus S. (C) Example Western bands showing expression of RAC1, CDC42, ARP2, ARP3, and ITGA2 in DG lysates from Sham and CCI-Acute mice (7d post CCI). (D) Densitometric comparison of the average expression of RAC1, CDC42, ARP2, ARP3 and ITGA2 (n = 6). (E) Example Western bands showing expression of RAC1, CDC42, ARP2, ARP3, and ITGA2 in DG lysates from Sham, U and S mice. (F) Densitometric comparison of the average expression of RAC1, CDC42, ARP2, ARP3 and ITGA2 (n = 6). (G) Example Western bands showing expression of RAC1, CDC42, ARP2, ARP3, and ITGA2 in DG lysates from Sham- and CCI-treated mice subjected to Scramble/shs1pr1 in the DG. (H) Densitometric comparison of the average expression of RAC1, CDC42, ARP2, ARP3 and ITGA2 (n = 6). (I) Example Western bands showing expression of RAC1, CDC42, ARP2, ARP3, and ITGA2 in DG lysates from Sham- and CCI-treated mice subjected to Scramble/s1pr1-mimic in the DG. (J) Densitometric comparison of the average expression of RAC1, CDC42, ARP2, ARP3 and ITGA2 (n = 6). Data were analyzed by unpaired t test or one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups when appropriate. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001. CCI, chronic constrictive injury; DG, dentate gyrus; U, unsusceptible; S, Susceptible.

mice. Immunofluorescence observations using a fluorescently conjugated phalloidin, which binds only to F-actin, revealed that HT-22 mouse hippocampal neuronal cells with knockdown of S1PR1 had accumulated F-actin aggregates mostly around the nuclei and lost the majority of the thinner filament bundles (**Figure 9C**) compared with control cells. Similarly, we utilized primary hippocampal cells to investigate the neuron morphology and distribution of F-actin. We observed less branches and accumulated F-actin aggregates rather than diffused distribution compared with control cells. Furthermore, we investigated whether S1PR1 regulates actin polymerization via interaction with ITGA2. We conducted yeast two-hybrid screening *in vitro* and a co-immunoprecipitation assay *in vivo* to examine the interaction between the two proteins. To identify putative interaction between S1PR1 and ITGA2, the full sequences of S1PR1 (cloned into the pBT3-STE vector) and ITGA2 (cloned into the pPR3-C vector) were used as bait and prey, respectively (Supplemental Figure 10A and 10B). The auto-activation test showed that positive controls (the pNubG-Fe65 and pTSU2-APP vector together) grew on the DDO (SD/-Trp/-Leu), TDO (SD/-Trp/-Leu/-His) and QDO (SD/-Trp/-Leu/-His/-Ade) plates. Meanwhile, the negative control (the pPR3-N and pTSU2-APP vectors together), the pPR3-N empty vector, and the pBT3-STE vector with *s1pr1* grew on the DDO and TDO but not on the QDO plate (Supplemental Figure 11). These results indicated that S1PR1 did not exhibit auto-activation activity in yeast. Next, a yeast two-hybrid assay was performed by co-transforming pBT3-STE-*s1pr1* and pPR3-C-*itga2* in the NMY51 yeast strain. Yeast cells harboring both *s1pr1* and *itga2* grew vigorously on both DDO media and QDO/X-gal media (**Figure 9D**). Additionally, in a CoIP assay, total protein extracts from the mice DG were immunoprecipitated by the anti-S1PR1-specific antibody and analyzed by immunoblotting probed with the anti-S1PR1 and anti-ITGA2 antibodies with immunoglobulin G as the negative control. The *in vivo* CoIP assay showed that S1PR1 interacts with ITGA2 in the DG (**Figure 9E**). The above results indicated that S1PR1 may physically interact with ITGA2. To determine the functional interaction between S1PR1 and ITGA2 in regulating chronic pain-related memory impairment, a recombinant adeno-associated virus 2/9 (AAV2/9) expressing a small hairpin RNA targeting ITGA2 was generated (rAAV-CaMKIIa-EGFP-5'-miR-30a-shRNA (*Itga2*)-3'-miR30a-WPREs, the sequence of shRNA referred to Supplemental Table 1). Following the schematic experimental procedure shown in **Figure 9F**, intra-DG injection of the ITGA2 knockdown virus was conducted (**Figure 9G** and Supplemental Figure 6C). We first confirmed the knockdown efficiency of the virus in the hippocampal DG using WB (**Figure 9H**). We then examined the effects of knockdown of ITGA2 in the DG on pain threshold and memory performance. Consistent with the effects of S1PR1 knockdown in the DG, compared with mice expressing scramble shRNA, mice expressing sh*Itga2* in the DG had no effects on the pain sensation (**Figure 9I**) and worsened memory-related behaviors in the Y-maze and MWM tests (**Figure 9J** and **9K**). To further demonstrate that S1PR1 and ITGA2 participate in the same pathway, we knocked down the two proteins at the same time (**Figure 9L** and **9M**). As expected, it did not elicit additive effects on behavioral tests of Y-maze and MWM tests compared to the knockdown of each one of them in isolation (**Figure 9N** and **9O**). Overall, these findings suggest that DG S1PR1 may govern the susceptibility of memory impairment by regulating actin polymerization via interaction with ITGA2.

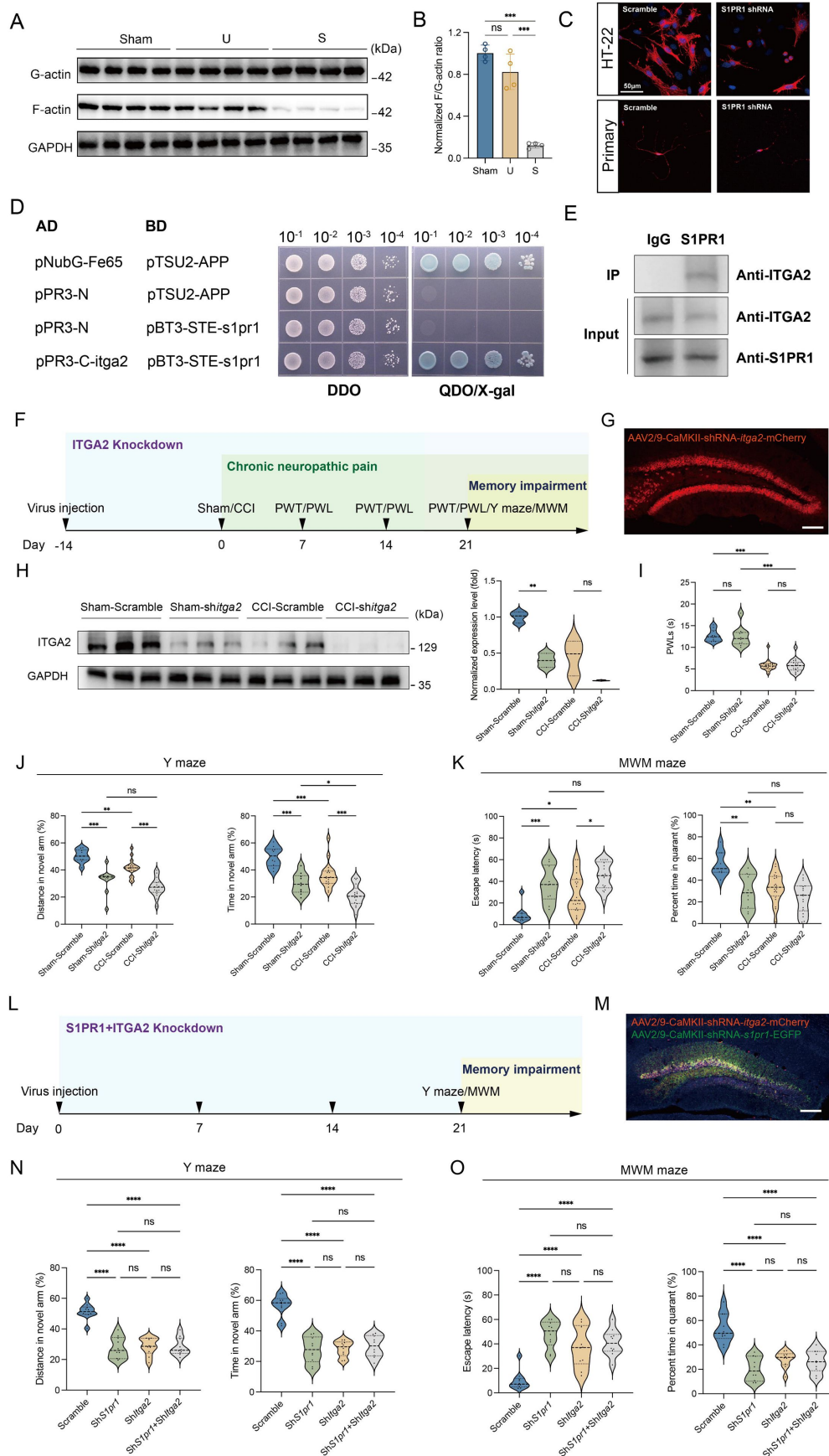


Figure 9.

S1PR1 regulates actin polymerization by interaction with ITGA2. (A-B)

Quantification of F/G-actin ratio in dentate gyrus of Sham, U and S mice by Western blot. **(C)** Phalloidin staining of F-actin showing the simple cytoskeleton of s1pr1^{-/-} knockdown HT-22 cells and primary hippocampal neurons in comparison to highly organized actin fibers present within scramble HT-22 cells and primary hippocampal neurons (Scale bar, 50 μ m). **(D)** Interaction between s1pr1 and itga2 in a yeast two-hybrid system. pNubG-Fe65 and pTSU2-APP were used as a pair of positive control. pPR3-N and pTSU2-APP were used as a negative control. DDO, SD/-Trp/-Leu; QDO, SD/-Trp/-Leu/-His/-Ade. **(E)** In vivo co-immunoprecipitation assay shows that S1PR1 interacts with ITGA2 in the dentate gyrus of mice. Total protein extracts were immunoprecipitated by the anti-S1PR1-specific antibody and analyzed by immunoblot probed with the anti-S1PR1 and anti-ITGA2 antibodies. Immunoglobulin G was used as the negative control. **(F)** Timeline of intra-DG virus injection, CCI surgery, pain threshold tests, Y maze test and MWM training. **(G)** A confocal image showing virus expression in the DG (Scale bar, 100 μ m). **(H)** Example Western bands showing efficient ITGA2 knockdown in the DG lysates from Sham-Scramble, Sham-shitga2, CCI-Scramble, and CCI-shitga2. Densitometric comparison of the average expression of S1PR1 ($n = 3$). **(I)** Pain threshold in Sham- and CCI-treated mice subjected to Scramble/shitga2 in the DG ($n = 10-20$). **(J)** Quantitative summary of Y-maze showing distances traveled and time spent in the novel arm in Sham- and CCI-treated mice subjected to Scramble/shitga2 in the DG ($n = 10-19$). **(K)** Quantitative summary of MWM training showing escape latency and time spent in the quadrant in Sham- and CCI-treated mice subjected to Scramble/shitga2 in the DG ($n = 9-20$). **(L)** Timeline of intra-DG virus injection, Y maze test and MWM training. **(M)** A confocal image showing virus expression in the DG (Scale bar, 100 μ m). **(N)** Quantitative summary of Y-maze showing distances traveled and time spent in the novel arm in WT mice subjected to Scramble/shs1pr1/shitga2/shs1pr1+shitga2 in the DG ($n = 10$). **(O)** Quantitative summary of MWM training showing escape latency and time spent in the quadrant in WT mice subjected to Scramble/shs1pr1/shitga2/shs1pr1+shitga2 in the DG ($n = 10$). Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Tukey's multiple comparisons between multiple groups. All data are presented as the mean $\pm$ s.e.m. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. CCI, chronic constrictive injury; d, day; MWM, Morris water maze; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; DG, dentate gyrus; U, unsusceptible; S, susceptible.

Discussion

The current study aimed to explore a disease model of chronic pain-related memory impairment and uncover the molecular underpinnings of both susceptibility to chronic pain-related memory impairment and factors that promote susceptibility to such variations. We established a paradigm to segregate mice with chronic pain into memory impairment-susceptible and -unsusceptible subpopulations. Susceptible mice displayed long-lasting memory impairment 21 days after CCI surgery, while unsusceptible mice continued to maintain normal memory function. Importantly, TEM and Golgi staining assays revealed that susceptible mice exhibited signs of reduced excitatory synapse formation and abnormal spine morphology in the brain area of the hippocampal DG involved in cognition/memory. Interestingly, the phenotypic variability in mice is attributed to dysregulation of S1P/S1PR1/integrin α 2 signaling-induced disorganization of actin cytoskeleton through the Rac1/Cdc42 signaling and Arp2/3 cascade (**Figure 10**). These are significant findings that demonstrate with certainty that comorbidity of memory impairment in complicated chronic pain syndromes has a pathophysiological substrate in the brain that could be a key therapeutic target for intervention.

Several studies have shown that neuronal plastic changes in the hippocampus are highly relevant to chronic pain-induced memory impairment. For example, the hippocampal extracellular matrix (ECM) exhibits aberrated structural synaptic plasticity connected to deficiencies in working

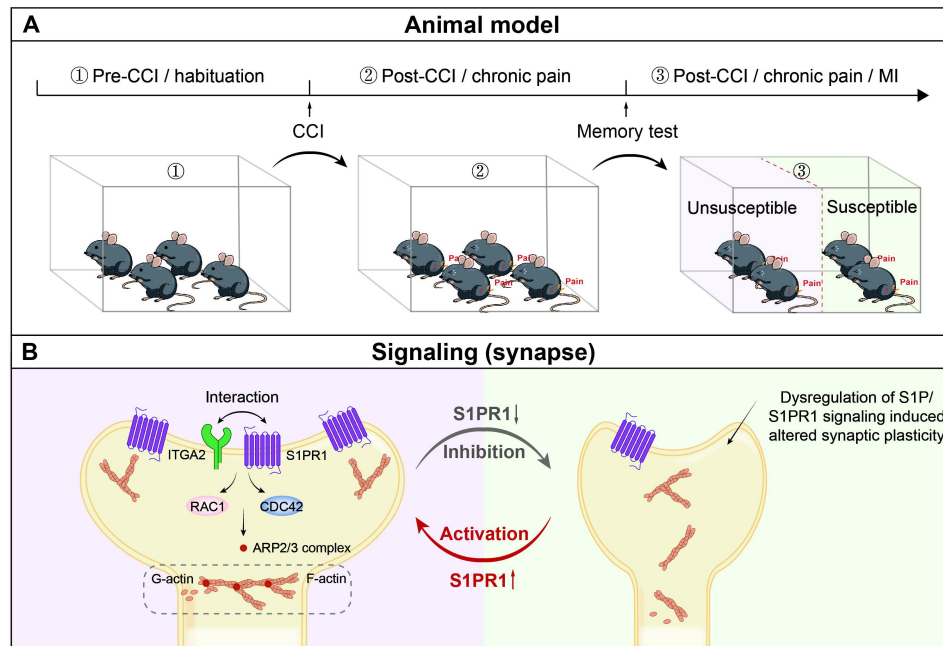


Figure 10.

Schematic representation of S1P/S1PR1 mediated susceptibility to chronic pain related memory impairment.

(A) Mice subjected to chronic pain can be separated into memory impairment susceptible and unsusceptible subpopulations 21 days post CCI. (B) Structural synaptic plasticity is mainly regulated by the actin cytoskeleton organization. In the DGCs of Sham/unsusceptible mice, S1P/S1PR1 signaling mediates actin dynamics via Itga2-dependent activation of the Rac1/Cdc42 signaling cascade and Arp2/3 dependent actin polymerization, whereas in susceptible mice dysregulation of S1P/S1PR1 signaling in the DGCs leads to defective actin cytoskeleton organization which alters the synaptic plasticity. CCI, chronic constrictive injury; DGCs, dentate granule cells.

location memory in a mouse model of chronic pain. These deficits are also correlated with decreased hippocampus dendritic complexity (7 [↗](#)). Moreover, rodents with neuropathic pain show altered short-term synaptic plasticity related to the decrease in hippocampus volume detected in patients. The anomaly may be the cause of the typical learning and emotional deficiencies seen in people with chronic pain (11 [↗](#)). In addition, the hippocampus regions involved in the processing of pain information show abnormalities in neurite arborization, dendritic length, and dendritic spine architecture (53 [↗](#)). Consistently, we found that hippocampal DG neurons displayed decreased excitatory synapses and altered spine morphology in susceptible mice. Although extensive investigations have proven the link between hippocampal DG function with memory formation, it remains unclear what regulates the plastic changes in the hippocampal DG, a well-studied brain area responsible for memory formation, and how it participates in the modulation of memory impairment in chronic pain. Interestingly, using RNA-Seq, we detected significant transcriptional downregulation of sphingolipid metabolism in the DG, which we verified at protein levels due to dysregulation of S1P/S1PR1 signaling. A previous study found that the expression of S1PR1 is upregulated in freshly formed DG cells, which is required for neurite arborization and horizontal-to-radial repositioning of these cells (31 [↗](#)). They also raised a question of whether S1PR1 regulates mature DGC activity. In the current study, we mainly focused on S1P function in mature DGCs. We knocked down DG S1PR1 and found that the loss of S1PR1 in the DG induced more susceptible mice to memory impairment without affecting pain threshold. Additionally, we also overexpressed DG S1PR1 and noticed that it promoted mice to be unsusceptible to memory impairment, similarly irrelevant to sensitivity to thermal pain stimuli. These findings imply that S1PR1 in the DG may exclusively negatively regulate chronic pain-related memory impairment. Nevertheless, CNS S1PR1 activation has also been reported in conditions with cisplatin-induced cognitive impairment (54 [↗](#)), and peripheral administration of either agonist or functional S1PR1 antagonist can ameliorate spatial memory impairment (40 [↗](#), 55 [↗](#)). Herein, the mechanisms of action of S1PR1 signaling (agonism or antagonism) with regard to the memory performance remain controversial.

Further, our study pointed out that S1PR1 activation in the DG may not be involved in the processing of pain. To date, a growing body of evidence has shown that activation of S1P axis at spinal cord triggers the occurrence of peripheral sensitization of pain. Activation of S1PR1 in astrocytes in the spinal cord contributes to neuropathic pain (30 [↗](#)), and mice with astrocyte-specific alterations of S1PR1 in the spinal cord did not experience neuropathic pain (56 [↗](#)). Additionally, elevated S1P levels at spinal cord injury sites attract macrophages and microglia, and their activation worsens the inflammatory response (57 [↗](#)). Moreover, an S1PR1 antagonist lessens neuropathic pain induced by spinal cord injury by inhibiting neuroinflammation and glial scar formation (58 [↗](#)). In contrast with the evidence available in the spinal cord, the role of S1P signaling in higher pain centers is poorly understood (59 [↗](#)). The present study provides evidence that in the hippocampal DG, S1P/S1PR1 signaling is irrelevant to pain perception.

Then how does S1PR1 regulate the structural plasticity in the DG and further affect memory formation in chronic neuropathic pain? Our study provides a notable insight by uncovering an integrin $\alpha 2$ -dependent modification of the actin cytoskeleton through the activation of the Rac1/Cdc42 signaling cascade and Arp2/3-dependent actin polymerization. Actin cytoskeleton rearrangement is associated with synapse formation, spine architecture and function, thus affecting numerous processes such as memory formation (60 [↗](#)–62 [↗](#)). Rho GTPases (RhoA, Rac1 and Cdc42) are key regulators of cytoskeleton assembly and are masters in maintaining spine morphology and memory (63 [↗](#)). For example, Rac1 induces branching of actin filaments in lamellipodia by mediating actin polymerization via activating the Arp2/3 complex (64 [↗](#)–66 [↗](#)). Cdc42 is also an important signaling protein for reorganization of actin cytoskeleton and morphogenesis of cells. Loss of Cdc42 causes deficits in synaptic plasticity and remote memory recall (67 [↗](#)). Given the evidence that S1P/S1PR1 modulates significant cytoskeletal rearrangements via actin regulatory proteins of Rho GTPases Rac1 and Cdc42, but not through RhoA which interacts with S1PR2 (28 [↗](#), 47 [↗](#), 50 [↗](#)), we therefore examined the expression of

Rac1 and Cdc42 at protein levels. Our findings revealed a significant downregulation of Rac1 and Cdc42 expression in susceptible mice and mice with S1PR1 knockdown in the DG, suggesting that S1PR1 functions upstream of Rac1 and Cdc42 in the DG. Consistent with this observation, we detected a downregulation of the actin polymerization modulator Arp2/3 complex. Actin remodeling involves the dynamic alterations in actin polymerization that contribute to the structural changes observed at neural synapses. Actin polymerization of the transition of G-actin into F-actin is crucial for orchestrating all the modifications necessary to facilitate synaptic communication by increasing spine volume. Conversely, the inability to form F-actin results in contrasting outcomes of decreased spine volume and diminished synaptic communication (68 [↗](#)). To further elucidate the causal relationship between DG S1PR1 and actin remodeling, we quantitated F-actin and G-actin in the mouse DG, and generated a S1PR1 knockdown HT-22 cell line to visualize F-actin by phalloidin staining. Our findings in susceptible mice indicate a significant inhibition of the transition from G-actin to F-actin, suggesting the involvement of S1P/S1PR1 in DG actin polymerization. RNA-Seq data further revealed that integrin alpha 2 may participate in this regulatory process. Integrins are linkers of the extracellular matrix and intracellular actin cytoskeleton mediating cytoskeletal organization (69 [↗](#)). The concomitant alteration in expression levels of Itga2 with S1PR1, and the physical and functional analysis between the two proteins of S1PR1 and ITGA2, indicate their collaboration in the regulation of cytoskeleton arrangement.

This study has potential limitations. First, the memory performance in the chronic pain model is exclusively evaluated based on spatial cues which mainly rely on the hippocampal functions. Further validation using various types of memory tests would strengthen the evidence for the categorization of mice into susceptible and unsusceptible subgroups. Additionally, the study would also benefit from a comprehensive exploration on the impact of different types of chronic pain on memory impairment. Different chronic pains including chronic nociceptive pain, chronic neuropathic pain, composite pain and chronic psychological pain, have different pathological mechanisms and impacts on memories. It is worth noting that preclinical and clinical research has demonstrated that the influence of pain intensity on memory function in chronic pain patients remains controversial (70 [↗](#)). Thus, future research needs to facilitate the investigation of differences between the various subtypes of chronic pains and comorbid memory impairment. Furthermore, the reliance on animal models may limit the generalizability of the findings to humans. Based on the current data, decreased serum S1P level could be a potential biomarker for evaluating the susceptibility of memory impairment. In future studies, it would be interesting to examine whether S1P levels in the serum of patients with pain is associated with memory impairment. Besides, except for S1PR1 and ITGA2 there are also other potential molecular targets implicated in this study, more work is needed to define their roles on the disease occurrence.

In summary, our study develops a paradigm for separating the susceptible and unsusceptible subgroups to chronic pain-induced memory impairment and delineates the key role of S1P/S1PR1 signaling in vulnerability to memory impairment. Since there are currently few pharmacological alternatives for the treatment of the comorbidity, these findings may serve as a foundation for the development of optimal preventive and therapeutic medications. Additionally, the strategy this study used to categorize memory impairment susceptibility may inspire new methods for stratifying patient populations in clinical settings.

Methods

Animals

Male C57BL/6J mice (8-10 weeks old) were housed 4-6 per cage with ad libitum access to food and water, at a constant temperature (22 to 25°C) and humidity (40% to 60%). They were kept under a 12:12 light/dark cycle (lights on at 8:00 AM). All animal protocols were approved by the Animal

Virus vectors, chemicals, and antibodies

Information for viral vectors, chemicals, and antibodies used in the present study was listed in Supplemental Tables 1-3.

Chronic neuropathic pain model

Chronic constrictive injury (CCI) of the sciatic nerve was used to establish chronic neuropathic pain, as described previously (71). Briefly, mice were anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*) and the left sciatic nerve was exposed by separating the muscles at the mid-thigh level then loosely ligated with three non-absorbable 4-0 silk braided suture proximal to the trifurcation at a 1.0 to 1.5 mm interval. After suturing and sterilizing, animals were placed in a recovery cage on a heating pad. For the following behavioral tests, a control group and an experimental group is identical in all respects of handling and experimental conditions, except for one difference-nerve injury.

Paw withdrawal threshold (PWT)

Mechanical allodynia was measured by PWT in a double-blinded manner by using the up and down method with von Frey filaments. Mice were placed on a wire mesh grid and allowed for 60-minute acclimation. A von Frey hair weighing 0.008 to 2.0 g was used. The filaments (from 0.16g filament) were perpendicularly applied to the plantar surface of the left hind paw through the wire mesh grid. Once a positive response is observed, change the filament to next lowest level. In the absence of a response, move to the next highest filament. Paw withdrawal, flinching, or paw licking was considered a positive response. 50% PWT was determined using the “up-down” method.

Paw withdrawal latency (PWL)

Thermal hyperalgesia was evaluated by PWL in a double-blinded manner following Hargreaves *et al.* (72). Briefly, the mice were placed on a glass platform and allowed for 60-minute acclimation. Thermal stimulation was focused on the plantar surface of the left hind paw through the glass plate. The time taken to withdraw from the heat stimulus is recorded for 3 times at an interval of 5 minutes for rest. The average time was considered as the PWL. Thermal stimulation was no more than 20 seconds.

Behavioral test battery to assess learning and memory

We followed the golden rule that we start with the least stressful test (Y-maze) and leave the most stressful of all for last (Morris Water maze). Additionally, we also ensured that between tests the animals have enough resting time to decrease carryover effects from prior tests. A common order of behavioral tests associated with learning and memory are Y-maze forced alternation (Y maze in this study), novel object recognition, Morris water maze, radial arm water maze and Y-maze spontaneous alternation (73).

Y maze

Testing occurs in a Y-shaped apparatus consisting of 3 white, enclosed arms with an angle of 120° from each other, each arm measuring 30 cm in length, 6 cm in width, and 15 cm in height, named the arms of the maze A, B and C. The experiment was started after the mice had habituated for half an hour in the behavioral chamber, and within 10 min of training, after the C-arm (novel arm) was occluded with a white plastic plate, mice were gently placed in the A-arm so that they were free to explore in both A and B arms. The mice were then returned to their home cage and allowed

to move freely for 1 hour. During the test period (5 min), the C-arm (novel arm) was opened, and the time and distance of the mice's exploration in the three arms were traced by a video-tracking system. The percent time and distance of mice exploring in the C-arm (novel arm) were analyzed.

Morris water maze (MWM) test

MWM test was performed in a round tank (100 cm in diameter and 51 cm in depth) with a small platform (8.5 cm in diameter, submerged 0.5 cm below water surface). The water was dyed with nontoxic and edible titanium dioxide. A computerized video tracking system (ANY-maze, Stoelting Co., IL, USA) was located 200 cm above the center of the tank to record performance. The tank was divided into 4 equal quadrants, southwest (SW), northwest (NW), northeast (NE) and southeast (SE). The platform is placed in the center of SW quadrant. Different graphic cues were put up on the walls of the four orientations to help the mice to build up spatial memory. Mice were trained for 4 days and tested for 2 days. During acquisition training, four trials were conducted every day, starting from the NE quadrant. Animals were placed in the water with its head facing the wall of the tank. The time (s) when the animal touched the underwater platform was recorded. If the time exceeds 60s, guide the animal to the platform and keep them stay for 10 seconds. Hereafter, the animals were dried and placed into a care cage. The interval between the two training sessions is 15 ~ 20min. On the first test day, escape latency assay and probe trials were given. The animals were placed in the diagonal quadrant (NE) of the platform quadrant, and the time to reach the platform and the swimming path of the mice were recorded. On the second test day, the platform was removed, and the percent time of total 30s of each animal spent in the target quadrant and the number of platform-site crossovers were recorded as the index of spatial memory.

Transmission Electron Microscope

Mice were deeply anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*) and their brains were collected on the ice to extract dentate gyrus of hippocampus (1mm*1mm*0.5mm) within one minute. After primary fixation with 4% paraformaldehyde and 3% glutaric dialdehyde solution at 4°C overnight and post-fixation with 1% osmium tetroxide in 0.1 M sodium phosphate buffer at 37°C for 2 h, tissues were washed with ddH₂O 4 times for 10 minutes each time. Different concentrations of ethanol and acetone are used to dehydrate the tissues gradiently. Then they were embedded with paraffin at different temperatures for 48h. Semithin sections at a 1μm thickness were prepared and stained with toluidine blue as survey sections and are viewed using a light microscope in order to locate and trim the regions of hippocampal granule cell layer. Subsequently, ultrathin sections at a 70nm were obtained and sequentially stained with uranyl acetate and lead citrate for TEM observation. 4-6 images for each animal were recorded at 6300x magnification, and a counting frame (5×5 μm²) was placed on each image for counting the number of excitatory synapses and measuring PSD width and length.

Golgi staining

Golgi staining was performed using FD Rapid Golgi Stain (FD NeuroTechnologies). Mice were anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*). Dissected mouse brains were immersed in solutions A and B in brown bottles for 14 days. The bottles were rotated occasionally to ensure perfect immersion. 14 days later, brains were transferred to solution C for 48 hours at 4 °C in the dark. The brains were sliced using a vibratome (Leica) at a thickness of 100 μm and mounted on gelatin coated slides. After reimmersion in solution C for 2 minutes and subsequent air drying, the brain slices were washed twice for 4 minutes each with ddH₂O and stained with solution D and E for 10 minutes. Stained brain slices were washed again with ddH₂O twice for 4 minutes each and then gone through gradient dehydration with gradient alcohol (50%, 75%, 95%, 100% ethanol, each 4 minutes). Fully dehydrated brain slices were washed with xylene 3 times for 4 minutes each and then mounted by coverslips using neutral gum as mounting media. Slides were stored in room temperature in the dark. Images of dendritic spines were captured using open field fluorescence microscope and analyzed with ImageJ and Sholl ([74](#) ).

Immunohistochemistry

Mice were deeply anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*) and sequentially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde solution. The brains were removed, postfixed with 4% paraformaldehyde solution at 4°C overnight and dehydrated in 30% sucrose solution for 48-h. Brain slices were prepared coronally with freezing microtome (VT1000S, Leica Microsystems) at a 30-μm thickness and were incubated with the blocking buffer containing 1% BSA and 0.1% Triton X-100 in PBS for 2 hours at room temperature and then overnight at 4°C with the primary antibodies. After the slices were washed with TBS 3 times for 10 minutes each time, they were incubated with secondary antibodies for 2 hours at room temperature and followed by washing with PBS and mounting. After staining, slices were visualized with the laser scanning confocal microscopy (LSM880; Zeiss).

Western blotting

Hippocampal dentate gyrus was rapidly stripped and sonicated (Bioruptor™ UCD-200) in RIPA buffer (P0013B, Beyotime Biotechnology) with 1% cocktail (B14001, Bimake) followed by centrifuge (12000rpm, 15 min). Two DGs from one animal were used for each sample. Proteins were then electrophoresed in a 10% SDS polyacrylamide gel (PG112, Shanghai EpiZyme Scientific) and transferred onto a PVDF membrane (IPVH00010, millipore). The membranes were rinsed in triple and blocked with 5% skim milk before incubation with primary antibodies (Supplemental Table 3) at 4°C for 12 hours. After washing with TBST, membranes were incubated with secondary antibodies (Supplemental Table 3) for 2 hrs at room temperature. The final visualization of the membranes was achieved by ECL Chemiluminescent (BLH01S100CN, Bioworld). We then used housekeeping protein normalization for normalizing Western blot data. GAPDH was used as the internal control. The stained blot is imaged, a rectangle is drawn around the target protein in each lane, and the signal intensity inside the rectangle is measured by using ImageJ. The signal intensity obtained can then be normalized by being divided by the signal intensity of the loading internal control (GAPDH) detected on the same blot. The average of the ratios from the control group is calculated, and all individual ratios are divided by this average to obtain a new set of values, which represent the normalized values.

RNA-seq

Sham, unsusceptible and susceptible mice were sacrificed and the DG was rapidly dissected under RNase-free conditions. Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched by Oligo(dT) beads. Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reversely transcribed into cDNA by using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). The purified double-stranded cDNA fragments were end repaired, A base added, and ligated to Illumina sequencing adapters. The ligation reaction was purified with the AMPure XP Beads (1.0X), and polymerase chain reaction (PCR) amplified. The resulting cDNA library was sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Stereotaxic surgeries

Mice were deeply anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*) and mounted on a stereotaxic apparatus (RWD). A midline incision was made on the scalp after disinfection. The skull was leveled and drilled a small hole in the skull with a dental drill. Virus was bilaterally injected into the DG of hippocampus (−1.3 A/P, ±1.95 M/L, and −2.02 D/V) by a 5ul Hamilton syringe at a rate of 0.1 μl/min by a microinjection pump (Harvard Apparatus, Holliston, MA). For the

infusion of S1PR1 agonist, guide cannula (internal diameter 0.34 mm, RWD) was unilaterally implanted into DG of hippocampus (−1.3 A/P, −1.95 M/L, and −2.02 D/V). After surgery, the mice were placed on a hot blanket for 1 hour and returned to the home cages.

Local infusion

To deliver S1PR1 agonist SEW2871 (0.7 μM in 200 nL) to the hippocampal DG, 10 μl syringe (Hamilton) connected to an internal stainless-steel syringe and infusion pump inserted into a guide cannula were used. SEW2871 was delivered into the right DG of hippocampus at a flow rate of 100 nl/min. For multiple infusions, mice were deeply anesthetized by 1% pentobarbital sodium (40 mg/kg, *i.p.*).

Cell culture, transfection and flow cytometry sorting

HT-22 (HT-22 mouse hippocampal neuronal cell line) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen) in 5% CO₂ atmosphere at 37 °C. The cell culture was around 70% confluent before transfection in a 6-well plate. Cells were incubated with transfection solution containing viruses (AAV-U6-shRNA(scramble)-GFP and AAV-U6-shRNA(s1pr1)-GFP) and polybrene in serum-free medium at 37 °C for 8 hours. The media were changed to fresh growth medium with serum post transfection. The transfection efficiency was determined as the percentage of cells expressing GFP in the entire cell population. In order to harvest the stable s1pr1 knock-down virus transfected cell line, we conducted flow cytometry sorting. Briefly, we discarded the original medium of the adherent cultured AAV-U6-shRNA(scramble)-GFP- and AAV-U6-shRNA(s1pr1)-GFP-transfected HT-22 cells, washed with sterilized 1×PBS once, added EDTA-free trypsin to digest for 3 minutes, and terminated the digestion by an equal volume of serum-containing medium. Subsequently, the cell suspension was collected, centrifuged at 600 rpm for 4 min. The supernatant was discarded and resuspended with sterilized 1×PBS into flow tubes. GFP-positive cells (transfected cells) were sorted out by flow cytometry (Beckman CytoFLEX SRT, Beckman) for subsequent experimental processes.

In vivo co-immunoprecipitation assay

Dentate gyrus tissues were carefully dissected, lysed in RIPA buffer (P0013B, Beyotime Biotechnology) with 1× Protease inhibitor cocktail (B14001, Bimake), sonicated (Diagenode, UCD-200) and centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was taken for the co-immunoprecipitation assay using BeaverBeads® Protein A Immunoprecipitation Kit (Beaver, 22202-20) following the manufacturer's instructions. Briefly, 30 μl Protein A beads were mixed with 5 μg/mL anti-S1PR1 antibody (Abcam, Ab259902) and incubated with gentle rotation at room temperature for 15 min. Beads were pelleted and washed twice. Then the beads and the supernatant were incubated with rotation at room temperature for 1 h. The bound proteins were eluted from the beads with 1× Protein Loading Buffer by boiling at 95°C for 5 min. Protein samples were then separated and immunoblotted with anti-ITGA2 antibody (Bioss, bsm-52613R) by Western Blot.

Split-ubiquitin membrane-based Yeast two-hybrid system

In this ubiquitin system, ubiquitin, a protein composed of 76 amino acid residues that can mediate the ubiquitination degradation of target proteins by proteasomes, is split into two domains, namely Cub at the C-terminus and NbuG at the N-terminus, which are fused and expressed with the bait protein "Bait" and the prey protein "Prey", respectively. Here, the coding region of s1pr1 was fused to the "bait" pBT3-STE vector (Clontech) cloning at Sfi IA (5'-GGCCATTACGGCC-3') and Sfi IB (3'-GGCCGCTCGGCC-5') sites. Full-length of itga2 was inserted into the Sfi IA (5'-GGCCATTACGGCC-3') and Sfi IB (3'-GGCCGCTCGGCC-5') sites of the "prey" pPR3-C vector (Clontech). At the same time, Cub is also fused with transcription factor LEXa-VP16. If Bait and Prey proteins could bind, Cub and NbuG would be brought together and a complete ubiquitin

would be formed, which would be recognized by the proteasome and the fused transcription factor would be cut off and enter the cell nucleus to activate the expression of the reporter gene. Series of combinations of bait and prey constructs were cotransformed into the yeast strain NMY51 (Clontech), and different concentrations of bacterial liquid were growing onto SD/-Trp/-Leu plates for 3 d at 30°C, interactions between baits and preys were examined on the selective medium SD/-Leu/-Trp/-His/-Ade. The blue colonies were chosen as candidates for possible interaction. The pNubG-Fe65 and pTSU2-APP vectors were served as positive controls, while the pPR3-N and pTSU2-APP were served as a negative control. Detailed procedures were conducted following the manufacturer's instructions (Clontech).

k-means algorithm

k-means algorithm is used to partition the dataset into pre-defined number of clusters. Each data point belongs to a single group based on the distance between their centroids. The centroid is either the mean or median of all the points within the cluster depending on the characteristics of the data. The main procedures for k-means algorithm are as follows: (1) determine the k, namely group numbers; (2) Randomly assign a centroid to each of the k clusters; (3) Calculate the distance of all data to each of the k centroids; (4) Assign data to the closest centroid; (5) Update the centroid by taking the mean of all the points in each cluster; (6) Repeat steps (3)-(5) until convergence; (7) The algorithm outputs the final cluster centroids and the assignment of each data point to a cluster. For categorization the mice into susceptible and unsusceptible groups (2 groups), we analyzed results from Y-maze and MWM tests using the k-means clustering algorithm and determine the k as 2. After assigning each data point to its closest k-center, we drew a median between both the centroids as a cutoff value. One cluster, including mice displaying a ratio of time more than the cutoff value, was defined as the unsusceptible mouse cluster. The other cluster, including mice exhibiting a ratio of time less than the cutoff value, was defined as the susceptible mouse cluster.

Statistics

The mice were assigned randomly to either the control group or the experimental group for each experiment. Data are presented as mean $\pm$ s.e.m. The summarized data in the violin plots were presented as the median (indicated by the bold dash line) along with the 25th and 75th percentiles (indicated by the slim dash line). K-Means cluster analysis was used to partition a given data set into a set of k groups (In this study, k=2). Unpaired Student's t-test was used to compare the mean of two independent groups. One-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparisons was used to examine the differences in the means of three or more groups. Two-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparisons was employed to determine the mean of a quantitative variable changes according to the levels of two independent variables. Statistical significance is indicated by asterisks as * p < 0.05, ** p < 0.01 and *** p < 0.001. Statistical analyses were performed using GraphPad Prism 9.0. and SPSS V22. Notably, every experiment was carried out a minimum of three times to make sure the results were consistent throughout the manuscript.

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Senior Editor

Lu Chen

Stanford University, Stanford, United States of America

Reviewer #1 (Public review):

This work from Cui, Pan, Fan et al explores memory impairment in chronic pain mouse models, a topic of great interest for the neurobiology field. In particular, the work starts from a very interesting observation, that WT mice can be divided in susceptible and unsusceptible to memory impairment upon modelling chronic pain with CCI. This observation represents the basis of the work where the authors identify the sphingosine receptor S1PR1 as down-regulated in the dentate gyrus of susceptible animals and demonstrate through an elegant range of experiments involving AAV mediated knockdown or overexpression of S1PR1 that this receptor is involved in the memory impairment observed with chronic pain. Importantly for translational purposes, they also show that activation of S1PR1 through a pharmacological paradigm is able to rescue the memory impairment phenotype.

The authors also link these defects to reduced dendritic branching and reduced number of mature excitatory synapses in the DG to the memory phenotype.

They then proceed to explore possible mechanisms downstream of S1PR1 that could explain this reduction in dendritic spines. They identify integrin $\alpha 2$ as an interactor of S1PR1 and show a reduction in several proteins involved in actin dynamic, which is crucial for dendritic spine formation and plasticity.

They thus hypothesize that the interaction between S1PR1 and Integrin $\alpha 2$ is fundamental for the activation of Rac1 and Cdc42 and consequently for the polymerisation of actin; a reduction in this pathway upon chronic pain would thus lead to impaired actin polymerisation, synapse formation and thus impaired memory.

The work is of great interest and the experiments are of very good quality with results of great importance.

Comments on revisions:

The authors have replied satisfactorily to my previous concerns.

<https://doi.org/10.7554/eLife.99862.2.sa2>

Reviewer #2 (Public review):

Summary:

The study investigates the molecular mechanisms underlying chronic pain-related memory impairment by focusing on S1P/S1PR1 signaling in the dentate gyrus (DG) of the hippocampus. Through behavioural tests (Y-maze and Morris water maze) and RNA-seq analysis, the researchers segregated chronic pain mice into memory impairment-susceptible and -unsusceptible subpopulations. They discovered that S1P/S1PR1 signaling is crucial for determining susceptibility to memory impairment, with decreased S1PR1 expression linked to structural plasticity changes and memory deficits.

Knockdown of S1PR1 in the DG induced a susceptible phenotype, while overexpression or pharmacological activation of S1PR1 promoted resistance to memory impairment and restored normal synaptic structure. The study identifies actin cytoskeleton-related pathways, including ITGA2 and its downstream Rac1/Cdc42 signaling, as key mediators of S1PR1's

effects, offering new insights and potential therapeutic targets for chronic pain-related cognitive dysfunction.

This manuscript consists of a comprehensive investigation and significant findings. The study provides novel insights into the molecular mechanisms of chronic pain-related memory impairment, highlighting the critical role of S1P/S1PR1 signaling in the hippocampal dentate gyrus. The clear identification of S1P/S1PR1 as a potential therapeutic target offers promising avenues for future research and treatment strategies. The manuscript is well-structured, methodologically sound, and presents valuable contributions to the field.

Strengths:

- (1) The manuscript is well-structured and written in clear, concise language. The flow of information is logical and easy to follow.
- (2) The segregation of mice into memory impairment-susceptible and -unsusceptible subpopulations is innovative and well-justified. The statistical analyses are robust and appropriate for the data.
- (3) The detailed examination of S1PR1 expression and its impact on synaptic plasticity and actin cytoskeleton reorganization is impressive. The findings are significant and contribute to the understanding of chronic pain-related memory impairment.

Comments on revisions:

The authors have satisfactorily addressed all the issues raised.

<https://doi.org/10.7554/eLife.99862.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

This work from Cui, Pan, Fan, et al explores memory impairment in chronic pain mouse models, a topic of great interest in the neurobiology field. In particular, the work starts from a very interesting observation, that WT mice can be divided into susceptible and unsusceptible to memory impairment upon modelling chronic pain with CCI. This observation represents the basis of the work where the authors identify the sphingosine receptor S1PR1 as down-regulated in the dentate gyrus of susceptible animals and demonstrate through an elegant range of experiments involving AAV-mediated knockdown or overexpression of S1PR1 that this receptor is involved in the memory impairment observed with chronic pain. Importantly for translational purposes, they also show that activation of S1PR1 through a pharmacological paradigm is able to rescue the memory impairment phenotype.

The authors also link these defects to reduced dendritic branching and a reduced number of mature excitatory synapses in the DG to the memory phenotype.

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They thus hypothesize that the interaction between S1PR1 and Integrin $\alpha 2$ is fundamental for the activation of Rac1 and Cdc42 and consequently for the polymerisation of actin; a reduction in this pathway upon chronic pain would thus lead to impaired actin polymerisation, synapse formation, and thus impaired memory.

The work is of great interest and the experiments are of very good quality with results of great importance. I have however some concerns. The main concern I have relates to the last part of the work, namely Figures 8 and 9, which I feel are not at the same level as the results presented in the previous 7 Figures, which are instead outstanding.

In particular:

- In Figure 8, given the reduction in all the proteins tested, the authors need to check some additional proteins as controls. One good candidate could be RhoA, considering the authors say it is activated by S1PR2 and not by S1PR1;

Thanks for your suggestion. We tested the expression level of RhoA in mice 7 days and 21 days post CCI as negative controls (Supplemental Figure 9).

- In addition to the previous point, could the authors also show that the number of neurons is not grossly different between susceptible and unsusceptible mice? This could be done by simply staining for NeuN or performing a western blot for a neuronal-specific protein (e.g. Map2 or beta3-tubulin);

As suggested, we performed immunofluorescence using NeuN antibody to detect the number of neurons in susceptible and unsusceptible mice. The number is not significantly different between the two populations (Supplementary Figure 7).

- In Figure 8, the authors should also evaluate the levels of activated RAC1 and activated Cdc42, which are much more important than just basal levels of the proteins to infer an effect on actin dynamics. This is possible through kits that use specific adaptors to pulldown GTP-Rac1 and GTP-Cdc42;

Thanks for your constructive suggestion. An elevated level and hyperactivation of Rac1 protein are both associated with actin dynamics and dendritic development [1]. We agree that showing the levels of activated RAC1 is better to infer its effect on actin dynamics. Here in Figure 8, the purpose of this experiment is to prove the levels of actin organization related proteins are altered according to the expression level of S1PR1, thus drawing a conclusion that the actin organization was disrupted, but not to specifically emphasize that S1PR1 activated these proteins. We apologize for the confusion made but we think the current data is enough to support the conclusion.

Thanks again for your advice. Your understanding is greatly appreciated.

- In Figure 9C, the experiment is performed in an immortalised cell line. I feel this needs to be performed at least in primary hippocampal neurons;

Thanks for your suggestion. As suggested, we performed the experiment in primary hippocampal neurons. Knockdown of *S1pr1* in primary hippocampal neurons induced reduction in the number of branches and filamentous actin. Please refer to the updated Figure 9C.

- In Figure 9D, the authors use a Yeast two-hybrid system to demonstrate the interaction between S1PR1 and Integrin $\alpha 2$. However, as the yeast two-hybrid system is based on the proximity of the GAL4 activating domain and the GAL4 binding domain, which are used

to activate the transcription of reporter genes, the system is not often used when probing the interaction between transmembrane proteins. Could the authors use other transmembrane proteins as negative controls?

Thanks for your question. We apologize for the unclear description in the method part. Traditional yeast two-hybrid system can only detect protein interactions that occur in the nucleus, but cannot detect ones between membrane proteins. Here, we utilized the split-ubiquitin membrane-based Yeast two-hybrid system. Briefly, in the ubiquitin system, ubiquitin, a protein composed of 76 amino acid residues that can mediate the ubiquitination degradation of target proteins by proteasomes, is split into two domains, namely Cub at the C-terminus and NbuG at the N-terminus, which are fused and expressed with the bait protein “Bait” and the prey protein “Prey”, respectively. At the same time, Cub is also fused with transcription factors. If Bait and Prey proteins could bind, Cub and NbuG would be brought together and a complete ubiquitin would be formed, which would be recognized by the proteasome and the fused transcription factor would be cut off and enter the cell nucleus to activate the expression of the reporter gene. We then determine whether the Bait and Prey proteins interact with each other through the growth of the yeast.

Thanks again for pointing this out. We reworded the method in M&M (Line 678-696).

- In Figure 9E, the immunoblot is very unconvincing. The bands in the inputs are very weak for both ITGA2 and S1PR1, the authors do not show the enrichment of S1PR1 upon its immunoprecipitation and the band for ITGA2 in the IP fraction has a weird appearance. Were these experiments performed on DG lysates only? If so, I suggest the authors repeat the experiment using the whole brain (or at least the whole hippocampus) so as to have more starting material. Alternatively, if this doesn't work, or in addition, they could also perform the immunoprecipitation in heterologous cells overexpressing the two proteins;

Thanks for the question and suggestion. We used DG lysates from both the dentate gyrus of a single mouse as the starting material. We updated the result which showed clearer bands (Figure 9E).

- About the point above, even if the results were convincing, the authors can't say that they demonstrate an interaction in vivo. In co-IP experiments, the interaction is much more likely to occur in the lysate during the incubation period rather than being conserved from the in vivo state. These co-IPs demonstrate the ability of proteins to interact, not necessarily that they do it in vivo. If the authors wanted to demonstrate this, they could perform a Proximity ligation assay in primary hippocampal neurons, using antibodies against S1PR1 and ITGA2.

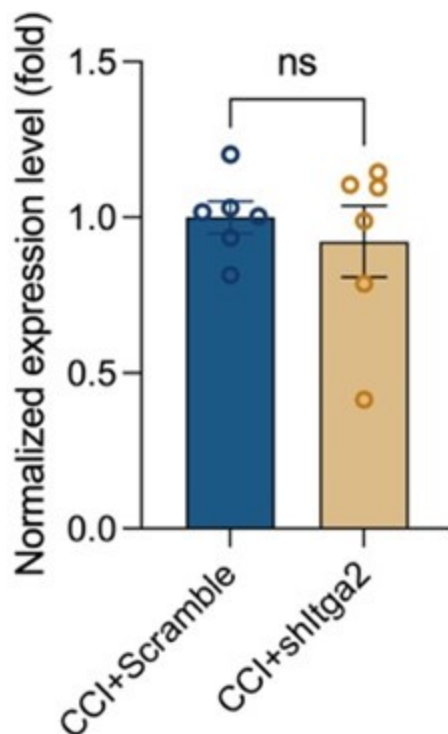
Thanks for your concern. Co-immunoprecipitation (Co-IP) is the gold standard to identify protein-protein interactions [2], and it is one of the most efficient techniques to study these protein-protein interactions in vivo [3]. We repeated the experiment and followed the experimental procedure exactly to avoid the protein interaction due to over-incubation. Over-incubation, particularly at room temperature, may result in non-specific binding and therefore high background, thus we performed Co-IPs at 4°C to preserve protein interactions. We agree that Proximity ligation assay is better suited for studies of endogenously expressed proteins in primary cells [4]. Since we optimized the experiment procedure to avoid non-specific binding and particularly, Co-IP utilized proteins from DG lysates which could validate the specificity of the protein interaction in native tissue, we prefer to keep the Co-IP result in Figure 9E.

Thanks again for your suggestion. We appreciate your understanding on this matter.

- In Figure 9H, could the authors increase the N to see if shItga2 causes further KD in the CCI?

As suggested, we repeated the experiment and increased the N to 6. As shown in the following picture, shItga2 did not cause further KD in the CCI.

Author response image 1.



- To conclusively demonstrate that S1PR1 and ITGA2 participate in the same pathway, they could show that knocking down the two proteins at the same time does not have additive effects on behavioral tests compared to the knockdown of each one of them in isolation.

Thanks for your suggestion. As suggested, we knocked down the two proteins at the same and did not observe additive effects on behavioral tests compared to the knockdown of each one of them in isolation. Please refer to Figure 9L-O.

Other major concerns:

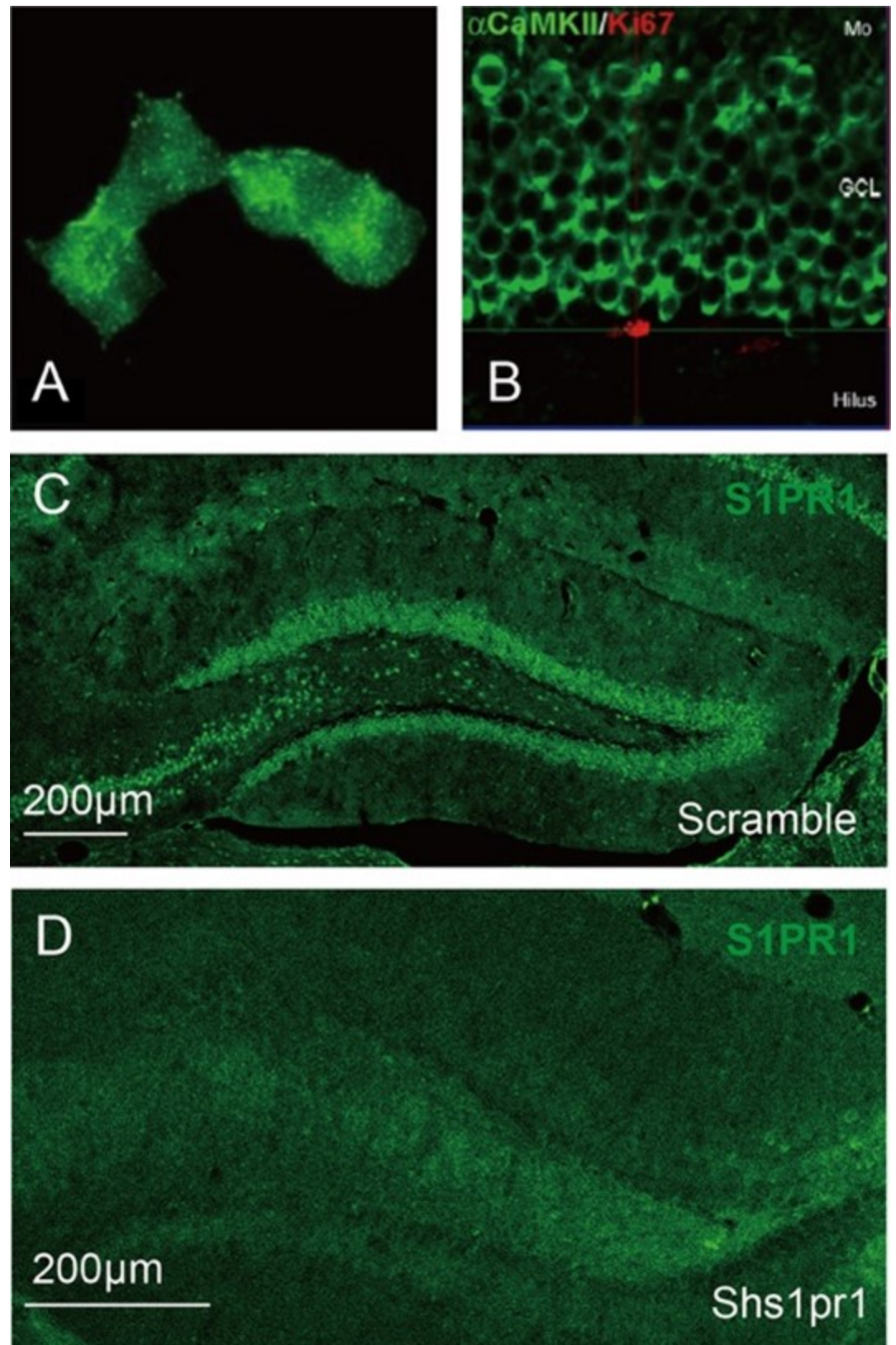
- Supplementary Figure 5: the image showing colocalisation between S1PR1 and CamKII is not very convincing. Is the S1PR1 antibody validated on Knockout or knockdown in immunostaining?;

S1PR1 is a membrane receptor and the S1P1 antibody (PA1-1040, Invitrogen) shows membranous staining with diffuse dot-like signals (Please refer to the image “A” provided by ThermoFisher Scientific). Here, we utilized the antibody to detect the expression of S1PR1 in DG granule cells. We can see the diffuse dot-like signals aggregated in each single granule cell. CaMKII shows intense staining around the border of the granule cell soma (Image “B”) [5].

According to the images shown in Supplementary Figure 5B, we concluded that S1PR1 is expressed in CaMKII⁺ cells.

Besides, as suggested, we validated the S1PR1 antibody on knockdown in immunostaining (Image “C” and “D”). The expression of S1PR1 is significantly decreased compared with the control.

Author response image 2.



- It would be interesting to check S1PR2 levels as a control in CCI-chronic animals;

As suggested, we quantified the S1PR2 levels in Sham and CCI animals, and there is no significant difference between groups (Supplementary Figure 9).

- Figure 1: I am a bit concerned about the Ns in these experiments. In the chronic pain experiments, the N for Sham is around 8 whereas is around 20 for CCI animals. Although I understand higher numbers are necessary to see the susceptible and unsusceptible populations, I feel that then the same number of Sham animals should be used;

Thanks for your concern. In the preliminary experiment, we noticed that the ratio of susceptible and unsusceptible populations is around 1:1. After the behavioral tests, we need to further take samples to investigate molecular and cellular changes of each group. Thus, we set sham around 8 and CCI around 20 to ensure that after characterization into susceptible and unsusceptible groups, each group has relatively equal numbers for further investigations.

- Figures 1E and 1G have much higher Ns than the other panels. Why is that? If they have performed this high number of animals why not show them in all panels?;

Thanks for your concern. For Figure 1B, C, D and F, we showed the data for each batch of experiment, while for Figure 1E and 1G, we used data collected from all batches of experiment. To show the data from a single batch, we would like to demonstrate the ratio of susceptible to unsusceptible is relatively stable, but not only based on a big sample size.

- In the experiments where viral injection is performed, the authors should show a zoomed-out image of the brain to show the precision of the injection and how spread the expression of the different viruses was;

As suggested, we showed the zoomed-out image in Supplementary Figure 6. The viruses are mainly expressed in the hippocampal DG.

- The authors should check if there is brain inflammation in CCI chronic animals. This would be interesting to explain if this could be the trigger for the effects seen in neurons. In particular, the authors should check astrocytes and microglia. This is of interest also because the pathways altered in Figure 8A are related to viral infection.

- If the previous point shows increased brain inflammation, it would be interesting for the authors to check whether a prolonged anti-inflammatory treatment in CCI animals administered before the insurgence of memory impairment could stop it from happening;

- In addition, the authors should speculate on what could be the signal that can induce these molecular changes starting from the site of injury;

- Also, as the animals are all WT, the authors should speculate on what could render some animals prone to have memory impairments and others resistant.

Thanks for the above four suggestions. We have observed inflammation including T cell infiltration and microglia activation in the hippocampal DG in CCI chronic animals and also used S1PR1 modulator which has anti-lymphocyte mediated inflammatory effect to prevent the insurgence of memory impairment from happening. We also examined the alteration in the numbers of peripheral T-lymphocyte subsets and the serum levels of cytokines. Furthermore, we found a neuron-microglia dialogue in the DG which may promote the resilience to memory impairment in CCI animals. Since these are unpublished results, we

apologize that we would not give much detailed information to the public at the current stage. We will publish these data as soon as possible. Thanks for your understanding.

Reviewer #2 (Public Review):

Summary:

The study investigates the molecular mechanisms underlying chronic pain-related memory impairment by focusing on S1P/S1PR1 signaling in the dentate gyrus (DG) of the hippocampus. Through behavioural tests (Y-maze and Morris water maze) and RNA-seq analysis, the researchers segregated chronic pain mice into memory impairment-susceptible and -unsusceptible subpopulations. They discovered that S1P/S1PR1 signaling is crucial for determining susceptibility to memory impairment, with decreased S1PR1 expression linked to structural plasticity changes and memory deficits.

Knockdown of S1PR1 in the DG induced a susceptible phenotype, while overexpression or pharmacological activation of S1PR1 promoted resistance to memory impairment and restored normal synaptic structure. The study identifies actin cytoskeleton-related pathways, including ITGA2 and its downstream Rac1/Cdc42 signaling, as key mediators of S1PR1's effects, offering new insights and potential therapeutic targets for chronic pain-related cognitive dysfunction.

This manuscript consists of a comprehensive investigation and significant findings. The study provides novel insights into the molecular mechanisms of chronic pain-related memory impairment, highlighting the critical role of S1P/S1PR1 signaling in the hippocampal dentate gyrus. The clear identification of S1P/S1PR1 as a potential therapeutic target offers promising avenues for future research and treatment strategies. The manuscript is well-structured, methodologically sound, and presents valuable contributions to the field.

Strengths:

(1) The manuscript is well-structured and written in clear, concise language. The flow of information is logical and easy to follow.

(2) The segregation of mice into memory impairment-susceptible and -unsusceptible subpopulations is innovative and well-justified. The statistical analyses are robust and appropriate for the data.

(3) The detailed examination of S1PR1 expression and its impact on synaptic plasticity and actin cytoskeleton reorganization is impressive. The findings are significant and contribute to the understanding of chronic pain-related memory impairment.

Weaknesses:

(1) Results: While the results are comprehensive, some sections are data-heavy and could be more reader-friendly with summarized key points before diving into detailed data.

Thanks for the suggestion. For the first sentence in each part/paragraph, we used statement that summarises what will be investigating in the following experiments to make it more reader-friendly. They are labeled as blue in the main text.

(2) Discussion: There is a need for a more balanced discussion regarding the limitations of the study. For example, addressing potential biases in the animal model or limitations in the generalizability of the findings to humans would strengthen the discussion. Also, providing specific suggestions for follow-up studies would be beneficial.

As suggested, we discussed more on the limitations of this study and outlined some directions for future research (Line 481-498).

(3) Conclusion: The conclusion, while concise, could better highlight the study's broader impact on the field and potential clinical implications.

Thanks. We reworded the conclusion to better highlight the impacts of this study (Line 501-505).

Reviewer #3 (Public Review):

Summary of the Authors' Objectives:

The authors aimed to delineate the role of S1P/S1PR1 signaling in the dentate gyrus in the context of memory impairment associated with chronic pain. They sought to understand the molecular mechanisms contributing to the variability in memory impairment susceptibility and to identify potential therapeutic targets.

Major Strengths and Weaknesses of the Study:

The study is methodologically robust, employing a combination of RNA-seq analysis, viral-mediated gene manipulation, and pharmacological interventions to investigate the S1P/S1PR1 pathway. The use of both knockdown and overexpression approaches to modulate S1PR1 levels provides compelling evidence for its role in memory impairment. The research also benefits from a comprehensive assessment of behavioral changes associated with chronic pain.

However, the study has some weaknesses. The categorization of mice into 'susceptible' and 'unsusceptible' groups based on memory performance requires further validation. Additionally, the reliance on a single animal model may limit the generalizability of the findings. The study could also benefit from a more detailed exploration of the impact of different types of pain on memory impairment.

Assessment of the Authors' Achievements:

The authors successfully identified S1P/S1PR1 signaling as a key factor in chronic pain-related memory impairment and demonstrated its potential as a therapeutic target. The findings are supported by rigorous experimental evidence, including biochemical, histological, and behavioral data. However, the study's impact could be enhanced by further exploration of the molecular pathways downstream of S1PR1 and by assessing the long-term effects of S1PR1 manipulation.

Impact on the Field and Utility to the Community:

This study is likely to have a significant impact on pain research by providing a novel perspective on the mechanisms underlying memory impairment in chronic pain conditions. The identification of the S1P/S1PR1 pathway as a potential therapeutic target could guide the development of new treatments.

Additional Context for Readers:

The study's approach to categorizing susceptibility to memory impairment could inspire new methods for stratifying patient populations in clinical settings.

Recommendations:

(1) A more detailed explanation of the k-means clustering algorithm and its application in categorizing mice should be provided.

As suggested, we explained the k-means clustering algorithm in details (Line 697-711).

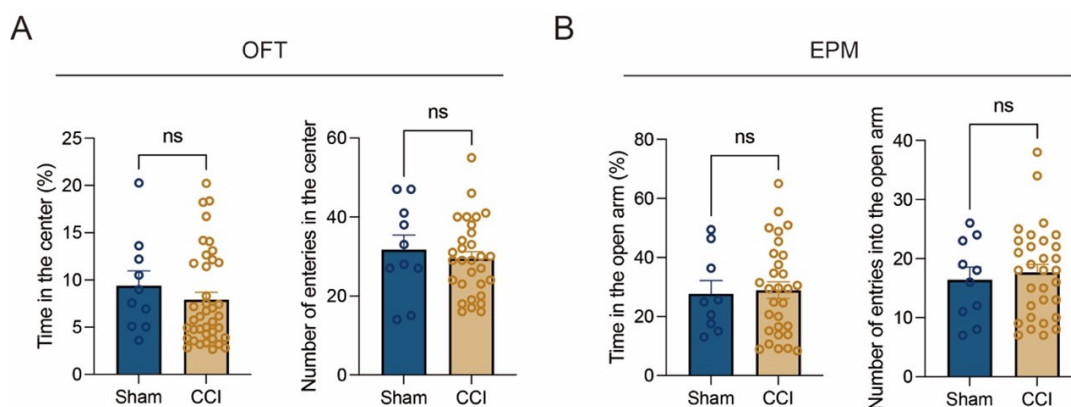
(2) The discussion on the potential influence of different pain types or sensitivities on memory impairment should be expanded.

Thanks for your suggestion. We discussed this point in the limitations of this study (Line 484-491).

(3) The protocol for behavioral testing should be clarified and the potential for learning or stress effects should be addressed.

Thanks for your suggestion. We clarified the order of the battery of behavioral tests in this study (Line 537-542). We start with the least stressful test (Y-maze) and leave the most stressful of all for last (Morris Water maze) [6]. Besides, we also conducted behavioral assays to prove that a one-day rest is enough to decrease carryover effects from prior test (Y-maze). We examined the stress related behaviors one day after Y-maze (23d post CCI) using open field test (OFT) and elevated plus maze (EPM). As shown in Author response image 3, the tests did not reflect the mice were under stressful circumstances. Thus, the order in which the tests were performed are appropriate in this study.

Author response image 3.



(4) Conduct additional behavioral assays for other molecular targets implicated in the study.

We agree that other molecular targets on susceptibility to memory impairment would be interesting to know. Our study was designed to focus specifically on ITGA2 this time and we'd like to keep the focus intact, but we have included your point as a consideration for future study (Lines 496-498). Thank you for the suggestion.

(5) The effective drug thresholds and potential non-specific effects of pharmacological interventions should be discussed in more detail.

As suggested, we emphasized this point of drug SEW2871 in Line 242-245.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Minor concerns:

- In Figure 6E the lines of the different groups are not visible. Showing the errors as error bars for each point would probably be better;

We apologize for the mistake of using mean $\pm$ SD here instead of mean $\pm$ SEM. After changing to mean $\pm$ SEM, the lines of Figure 6E, Figure 7E and 7L become much clearer. It looks a little bit messy to show the error bars since there are numerous points, so we prefer to keep the line style.

- Do the authors have any speculation on why the % time in the quadrant is not further affected in the KD *Itga2* in CCI animals (Figure 9K)?;

In CCI animals, the level of S1PR1 expression is decreased. ITGA2 may participate in the same pathway with S1PR1. Thus, knocking down ITGA2 in CCI animals will not further affect the animal behaviors. This has been proved by knocking down the two proteins at the same time and no additive effects were observed on behavioral tests compared to the knockdown of each one of them in isolation (Figure 9L-O).

- In the methods, it's unclear if in the multiple infusion, the animals were anaesthetised or kept awake;

We have clarified this point in the method. mice were deeply anesthetized by 1% pentobarbital sodium (40 mg/kg, i.p.). (Line 649-650)

- As the DG is quite small, could the authors clarify if, when performing western blots, they used the two DGs from one animal for each sample or if they pulled together the DGs of several animals?;

We used the two DGs from one animal for each sample. The amount of protein extracted from each sample is enough for 20-30 times of Western Blot assays. We have now added this to the method for clarity (Line 612).

- Is it possible to check the correlation between performance in the YM and MWM with S1PR1 levels?;

We would also be interested in this point. The data that we have cannot reveal this for it is difficult to manipulate the S1PR1 levels by using KD and overexpression viruses.

- EM images have a poor resolution in the figures, could the authors show higher-resolution images?;

We have inserted 300 DPI images for high resolution output.

- In line 268 there is a mention of an "*ShLamb1*"?

We apologize for the mistake and it was revised.

Reviewer #3 (Recommendations For The Authors):

This study explored the role of S1P/S1PR1 signaling within the dentate gyrus (DG) in chronic pain-related memory impairment using a murine model. The authors identified decreased expression of S1PR1 in the DG of mice susceptible to memory deficits. They demonstrated that S1PR1 knockdown increased susceptibility to memory deficits, whereas its overexpression or pharmacological activation mitigated these effects. Further biochemical and immunofluorescence analyses indicated that disruptions in S1P/S1PR1 signaling were related to disruptions in actin cytoskeleton dynamics, influenced by molecular pathways involving ITGA2, Rac1/Cdc42 signaling, and the Arp2/3 complex. These findings offer intriguing insights and suggest a potential therapeutic target for treating memory impairment in chronic pain.

Major Concerns:

The following five major concerns are the same with the five recommendations from Reviewer 3 on Page 9-10. Please refer to the answers above.

- (1) The division of subjects into 'susceptible' and 'unsusceptible' categories requires further clarification regarding the methodologies and rationale employed, particularly concerning the use of the k-means clustering algorithm in data analysis. This explanation will strengthen the scientific grounding of the categorization process.*
- (2) The categorization of 'susceptible' and 'unsusceptible' groups might also benefit from a more detailed analysis or discussion concerning the influence of different pain sensitivities or types of pain assessments. Although the study mentions that memory impairment stands independent of pain thresholds, a more nuanced exploration could provide deeper insights.*
- (3) The article could benefit from more clarity on the protocol of behavioral testing, especially regarding the potential effects of repeated testing on performance outcomes due to learning or stress.*
- (4) While the connection between S1P/S1PR1 signaling and the molecular pathways highlighted (ITGA2, Rac1/Cdc42, Arp2/3) is intriguing, only ITGA2 underwent further behavioral validation in vivo. Conducting additional behavioral assays for one or more of the molecular targets could substantially strengthen these findings.*
- (5) Discussions regarding effective drug thresholds and the potential for non-specific effects are essential to fully evaluate the implications of pharmacological interventions utilized in the study.*

Minor Concerns:

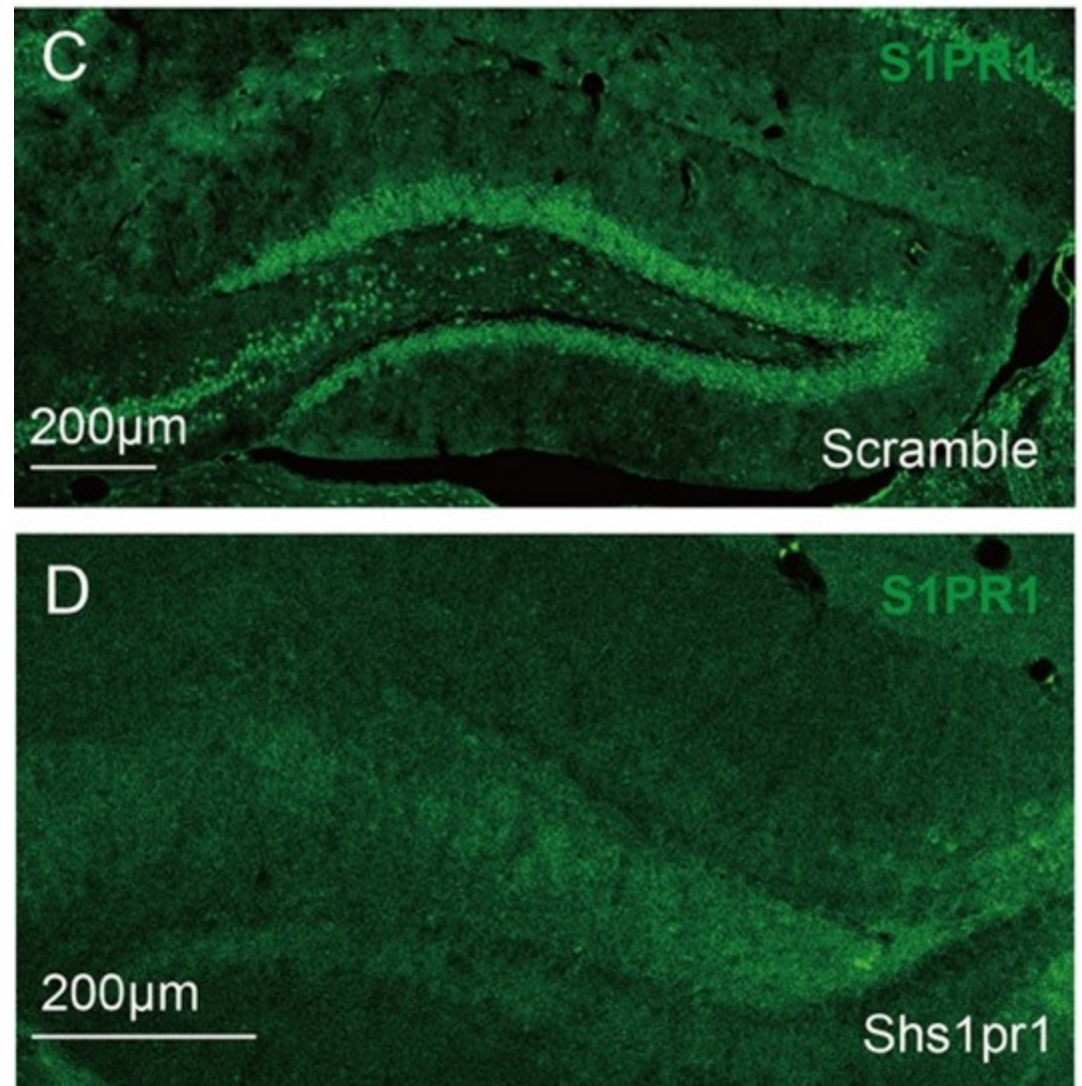
- (1) Clarification of evidence of the specific infusion sites in pharmacological experiments would enhance the transparency and replicability of these methods.*

For the infusion of S1PR1 agonist, guide cannula (internal diameter 0.34 mm, RWD) was unilaterally implanted into DG of hippocampus (-1.3 A/P, -1.95 M/L, and -2.02 D/V) as evidenced by Figure 5B.

- (2) It would be beneficial if the manuscript provided details regarding the efficiency and reach of viral transfection within the neuronal population. This information would help in assessing the impact of genetic manipulations.*

S1PR1 immunostaining showed that the efficiency is quite high and the reach of viral transfection is sufficient.

Author response image 4.



(3) The manuscript should make explicit the normalization techniques used in quantitative assessments such as Western blotting, including the housekeeping genes or proteins used for this purpose.

Here, we used housekeeping protein normalization for normalizing Western blot data. GAPDH was used as the internal control. First, the stained blot is imaged, a rectangle is drawn around the target protein in each lane, and the signal intensity inside the rectangle is measured by using ImageJ. The signal intensity obtained can then be normalized by being divided by the signal intensity of the loading internal control (GAPDH) detected on the same blot. The average of the ratios from the control group is calculated, and all individual ratios are divided by this average to obtain a new set of values, which represent the normalized values (Line 619-625).

(4) Details about the control groups in behavioral assessments were subjected to comparable handling and experimental conditions as the chronic pain groups are crucial, barring nerve injury, for maintaining the integrity of the comparative analysis.

We agree that a control group and an experimental group is identical in all respects except for one difference-nerve injury. We have added this point in the method (Line 520-522).

Minor Recommendations:

The following four minor recommendations are the same with the four minor concerns from Reviewer 3 on Page 12-13. Please refer to the answers above.

- (1) Clarify the specifics of infusion site verification in pharmacological experiments.
- (2) Provide details on the efficiency and neuronal reach of viral transfections.
- (3) Explicitly describe the normalization techniques used in quantitative assessments.
- (4) Ensure that control groups in behavioral assessments undergo comparable handling to maintain analysis integrity.

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