

# The Relationship Between Reelin and Sphingosine-1-Phosphate in the Repositioning of Adult-Generated Dentate Granule Cells

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**Abstract.** Reelin and sphingosine-1-phosphate (S1P) are important in adult neurogenesis, particularly in the repositioning of newly formed dentate granule cells (DGCs). The relationship between Reelin and S1P in the repositioning of new DGCs is investigated in this study, with a specific focus on their functioning pathways and upstream/downstream relationships. Applying gene knockdown and immunostaining methods, this research discovers that Reelin and S1P function in the same signaling pathway during the new DGC's repositioning process, and it appears that Reelin works upstream of S1P. The results could potentially inform therapeutic measures for cognitive disorders associated with neurogenesis impairment.

**Keywords:** Adult neurogenesis, dentate granule cells, Reelin, sphingosine-1-phosphate.

## 1. Introduction

Adult neurogenesis in the hippocampus appears in the subgranular zone (SGZ), where neural stem cells (NSCs) form various kinds of new cells through proliferation, differentiation, and migration [1-13]. In the adult neurogenesis process of dentate granule cells (DGCs), horizontal-to-radial repositioning is crucial to their survival and follow-up circuit integration [2, 13-15]. Reelin and sphingosine-1-phosphate (S1P) are found to facilitate this repositioning process [1-3, 5, 7, 11, 13-18].

Previous research shows that Reelin is encoded by the RELN gene as an extracellular matrix protein. It regulates the repositioning of newborn DGCs and contributes to their dendritic spine development [1, 3, 7, 11, 16, 18, 19]. In the meantime, as a bioactive lipid molecule, S1P's synthesis process is modulated by Sphingosine Kinase 1 and 2, which are encoded by the SPHK1 and 2 genes. The repositioning, neurite growth, and circuit integration of new DGCs can be promoted by S1P signaling [1, 2, 5, 13-15, 17].

The effects of Reelin and S1P on the repositioning of newborn DGCs have significant similarities, suggesting the potential overlapping of functioning pathways. Nevertheless, no studies have investigated whether Reelin and S1P signaling processes share the same pathway. Hence, this study focuses on the relationship between Reelin and S1P in the repositioning of newborn DGCs, using retroviral vectors, immunostaining, electrophysiological recording, and RNA sequencing to investigate the following two research questions:

1) Do Reelin and S1P work cooperatively in the same pathway or independently in two different pathways in the repositioning process of newly generated DGCs in the hippocampus?

2) If they work in the same pathway, what is the upstream/downstream relationship between Reelin and S1P in the repositioning process of newly generated DGCs in the hippocampus?

## 2. Methods and Expected Results

To answer these research questions, two experiments were conducted with adult mice, examining the relationship between Reelin and S1P in the horizontal-to-radial repositioning of newly generated DGCs.

- Experiment 1: Whether Reelin and S1P Work in the Same Pathway

To explore whether Reelin and S1P work cooperatively in the same pathway or independently in two different pathways, newly generated DGCs in adult mice were labeled with green fluorescent protein (GFP) using retroviral vectors to observe their repositioning results [13, 20]. Cells in the mice DG were also infected with retroviral vectors carrying short-hairpin RNA to conduct gene knockdown. Four different vectors were designed, targeting RELN (shReelin), SPHK1/2(shS1P), both RELN and SPHK1/2 (shReelin/shS1P), and luciferase (shLuc) as a control. At 3 dpi, doxycycline (DOX) was added to the drinking water to induce the short-hairpin RNA expression (Figure 1A) [13, 21, 22]. The knockdown results were detectable at 5 and 7 dpi, when the levels of Reelin and S1P in the mice DG cells were analyzed under fluorescence microscopy after immunostaining. While both Reelin and S1P can be detected in the shLuc mice, their expression levels decrease significantly among shReelin, shS1P, and shReelin/shS1P mice, indicating a successful knockdown (Figure 1B, 1C) [23]. These results are consistent with retroviral knockdown conducted in previous research [13].

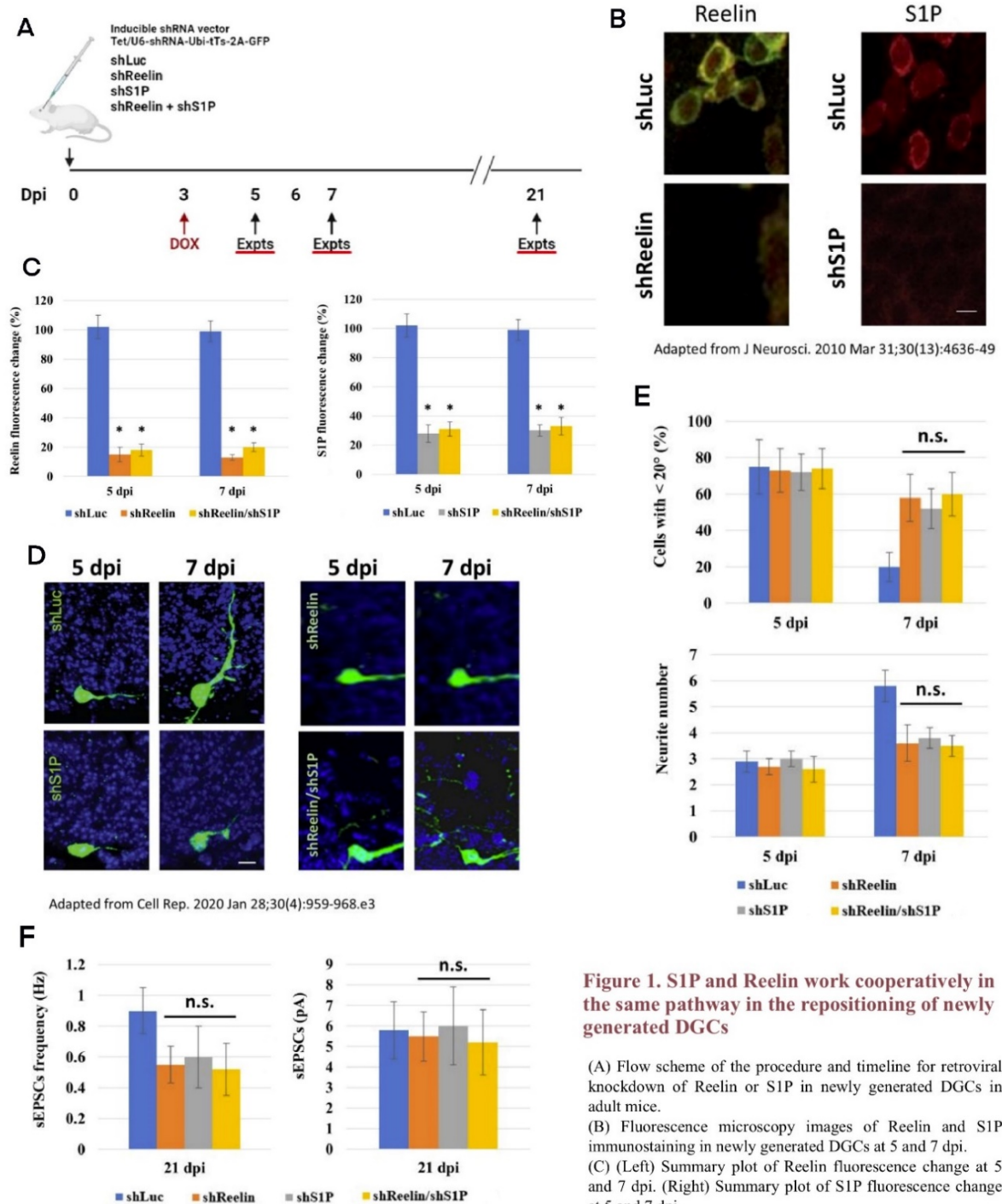
To determine the effects of shReelin, shS1P, and shReelin/shS1P on the repositioning and growth of newly generated DGCs, GFP-labeled newborn DGCs were observed under fluorescence microscopy. For new DGCs in shLuc mice, the horizontal-to-radial repositioning proceeds normally from 5 dpi to 7 dpi; however, in shReelin, shS1P, or shReelin/shS1P mice, the cells remain largely horizontal at 7 dpi, suggesting that the lack of Reelin or S1P may lead to deficiencies in newborn DGC repositioning, which is similar to findings from previous studies (Figure 1D) [13, 19]. To further investigate whether the absence of Reelin, S1P, or both results in the same or similar phenotypes, the percentage of newborn DGCs within 20 degrees and their neurite number at 5 and 7 dpi, as well as the spontaneous excitatory post-synaptic currents (sEPSCs) recording results in their glutamatergic synaptic transmission were summarized with histograms (Figure 1E, 1F) [13]. While shReelin, shS1P, and shReelin/shS1P mice all have more newborn DGCs remaining horizontally positioned, less neurite development, and lower sEPSCs frequency compared to shLuc mice, no significant difference is detected among these three groups, indicating that shReelin, shS1P, and shReelin/shS1P may result in similar phenotypes. These results suggest that Reelin and S1P possibly work in the same pathway during the repositioning of newly generated DGCs, as the knockdown of both does not lead to a greater deficiency than the knockdown of any one of these two genes.

- Experiment 2: The Upstream/Downstream Relationship of Reelin and S1P

A similar method of retroviral knockdown was employed to examine the upstream/downstream relationship between Reelin and S1P in the horizontal-to-radial repositioning of newborn DGCs. Mice were infected with DOX-inducible retroviral vectors targeting RELN (shReelin) or SPHK1/2(shS1P), and the knockdown was induced at 3 dpi (Figure 2A) [13, 21, 22].

Immunostaining shows that both shReelin and shS1P lead to decreased fluorescence levels of the corresponding molecule at 5 and 7 dpi. However, while the fluorescence level of S1P also decreases under shReelin conditions, no significant difference is observed between the fluorescence level of Reelin under shS1P conditions and that under shLuc conditions, which suggests that the lack of Reelin may have an adverse impact on the expression level of S1P-related genes (Figure 2B) [23]. These observation results are further quantified in Figure 2C, where the fluorescence changes in Reelin and S1P are graphed in histograms respectively. For shS1P mice, only the fluorescence level of S1P changes significantly compared to shLuc mice; however, for shReelin mice, the fluorescence levels of both Reelin and S1P decrease greatly at 5 and 7 dpi, indicating Reelin's influence on S1P signaling. Similar results were

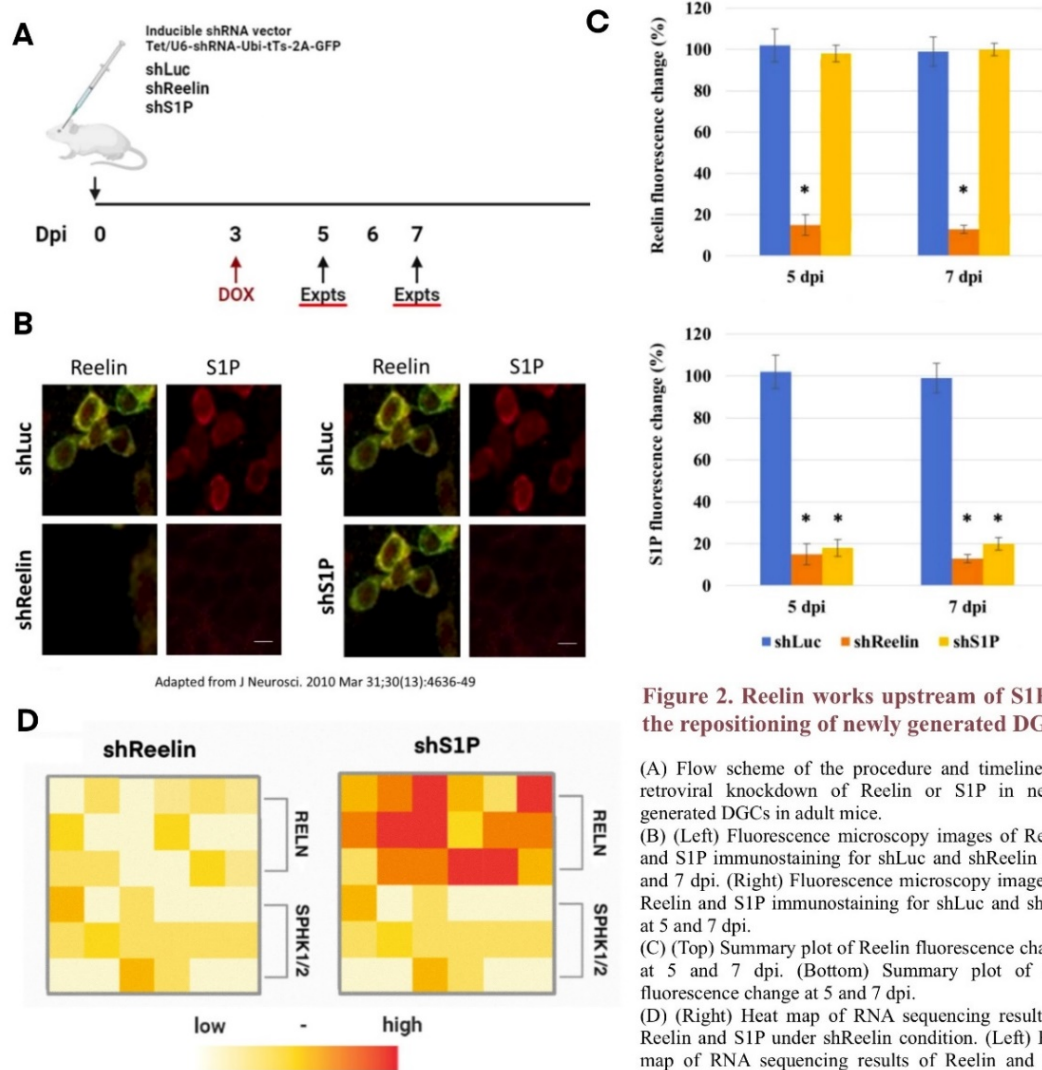
found in the RNA sequencing of the mRNA of RELN, which produces Reelin, and Sphingosine Kinase 1/2, which produces Sphk 1/2 that regulates the synthesis of S1P, further confirming that the expression level of Reelin potentially plays a role in S1P regulation (Figure 2D) [14]. Overall, these results suggest that Reelin may work upstream of S1P in the repositioning and circuit integration of new DGCs, as Reelin exhibits a regulation effect on S1P level and S1P does not show a similar impact on Reelin.



**Figure 1. S1P and Reelin work cooperatively in the same pathway in the repositioning of newly generated DGCs**

(A) Flow scheme of the procedure and timeline for retroviral knockdown of Reelin or S1P in newly generated DGCs in adult mice.  
(B) Fluorescence microscopy images of Reelin and S1P immunostaining in newly generated DGCs at 5 and 7 dpi.  
(C) (Left) Summary plot of Reelin fluorescence change at 5 and 7 dpi. (Right) Summary plot of S1P fluorescence change at 5 and 7 dpi.

(D) Fluorescence microscopy images of newly generated DGCs at 5 and 7 dpi.  
(E) (Top) Summary plot of the proportions of cells with an angle less than 20 degrees at 5 and 7 dpi. (Bottom) Summary plot of the numbers of neurites of newly generated DGCs at 5 and 7 dpi.  
(F) Glutamatergic synaptic transmission recorded from newly generated DGCs at 21 dpi.



**Figure 2. Reelin works upstream of S1P in the repositioning of newly generated DGCs**

(A) Flow scheme of the procedure and timeline for retroviral knockdown of Reelin or S1P in newly generated DGCs in adult mice.

(B) (Left) Fluorescence microscopy images of Reelin and S1P immunostaining for shLuc and shReelin at 5 and 7 dpi. (Right) Fluorescence microscopy images of Reelin and S1P immunostaining for shLuc and shS1P at 5 and 7 dpi.

(C) (Top) Summary plot of Reelin fluorescence change at 5 and 7 dpi. (Bottom) Summary plot of S1P fluorescence change at 5 and 7 dpi.

(D) (Right) Heat map of RNA sequencing results of Reelin and S1P under shReelin condition. (Left) Heat map of RNA sequencing results of Reelin and S1P under shS1P condition.

### 3. Conclusion

In conclusion, this study investigates the relationship between Reelin and S1P in the horizontal-to-radial repositioning of newly generated DGCs. It is found that Reelin and S1P function cooperatively in the same signaling pathway during the new DGC repositioning process. Through retroviral knockdown experiments, it was observed that the absence of either Reelin or S1P results in similar deficiencies in DGC repositioning, neurite development, and synaptic activity, suggesting their interdependent roles. Additionally, the findings indicate that Reelin may act upstream of S1P, as knockdown of Reelin significantly affects S1P expression levels, whereas S1P knockdown does not alter Reelin expression.

While the study presents expected results suggesting a cooperative relationship between Reelin and S1P in the same pathway, it is important to acknowledge that disruptions in molecules within the same pathway do not always lead to identical phenotypes due to potential compensatory mechanisms or context-dependent effects. Consequently, the actual outcomes of this study may differ from the anticipated findings. Nevertheless, the insights from this study enhance the current understanding of the molecular mechanisms underlying adult neurogenesis and may inform future therapeutic strategies targeting cognitive disorders associated with impaired neurogenesis.

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