

Spatially resolved transcriptomics identifies intercellular signaling post-ischemic stroke that controls neural stem cell proliferation

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SUMMARY

Ischemic stroke mobilizes adult neural stem cells (NSCs), which could be leveraged for recovery. However, the intercellular signals that activate NSCs are poorly understood. Cells located in the cerebral lesion may send signals to the NSC niche to initiate the regenerative responses. Here, we computationally inferred cell-cell communication between the ischemic infarct region and ventricular-subventricular zone (V-SVZ) NSC niche from spatial gene expression profiles and identified ligand-receptor pairs in the signal transduction events at three time points. Further, we detailed the regulatory function of galectin-9 on the proliferation of NSCs as a checkpoint molecule that guards the responses of NSCs under physiological conditions. In addition, we show that TIM-3 may mediate the inhibitory effect of galectin-9 on NSC proliferation. Collectively, these data reveal galectin-9 as an important regulator of NSC proliferation under both physiological and injury conditions.

INTRODUCTION

Globally, stroke is the second most prevalent cause of death and disability (Murphy and Werring, 2020). Ischemic stroke accounts for 85% of the total cases with the remaining 15% being hemorrhagic (Murphy and Werring, 2020), both of which can be further divided into various subtypes according to different classification systems based on the etiologies and phenotypes (Amarenco et al., 2009). Ischemic stroke has been studied extensively using numerous animal models, including middle cerebral artery occlusion (MCAO), craniotomy, photothrombosis, endothelin-1 (ET-1), and embolic stroke models, the details of and pros and cons of which have been thoroughly reviewed (Kleinschnitz et al., 2015).

Ischemic stroke is a brain injury that triggers the responses of neural stem cells (NSCs) (Ruddy and Morshead, 2018). NSCs are characterized by their capability of self-renewal and to produce differentiated neural cells via asymmetric cell division (Gage, 2000; Mummery et al., 2021). In the adult mammalian brain, NSCs exist in specific niches of the ventricular-subventricular zone (V-SVZ) of the lateral ventricle (LV) wall and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Ming and Song, 2005). The multifaceted responses of

endogenous NSCs to ischemic stroke (Tang et al., 2023) involve proliferation (Arvidsson et al., 2002), migration (Arvidsson et al., 2002), differentiation (Morishita et al., 2020), and synaptogenesis (Kanemura et al., 2024). Previous studies have shown that in adult rodent brain, following ischemic insults, neurogenesis occurs from the neural stem and/or progenitor cells in the subventricular zone, or local cortical progenitors, or striatal astrocytes (Lindvall and Kokaia, 2015). However, around 80% of the new neurons formed in the injured striatum die between 2 and 6 weeks post-ischemia (Arvidsson et al., 2002). Postmortem studies on adult human brains of patients after ischemic stroke revealed increased numbers of proliferating cells in ipsilateral SVZ in comparison with the contralateral SVZ (Macas et al., 2006; Marti-Fàbregas et al., 2010) and uninjured control (Macas et al., 2006). Although stem cell therapies have shown promising potential for treating ischemic stroke (Barthels and Das, 2020), some well-recognized risks such as tumorigenesis have been documented (Goldman, 2016). Intuitively, another strategy for promoting recovery after ischemic stroke is to take advantage of the intrinsic mechanisms of endogenous NSC mobilization. However, the intercellular signals which activate NSCs after brain injury are still incompletely understood.



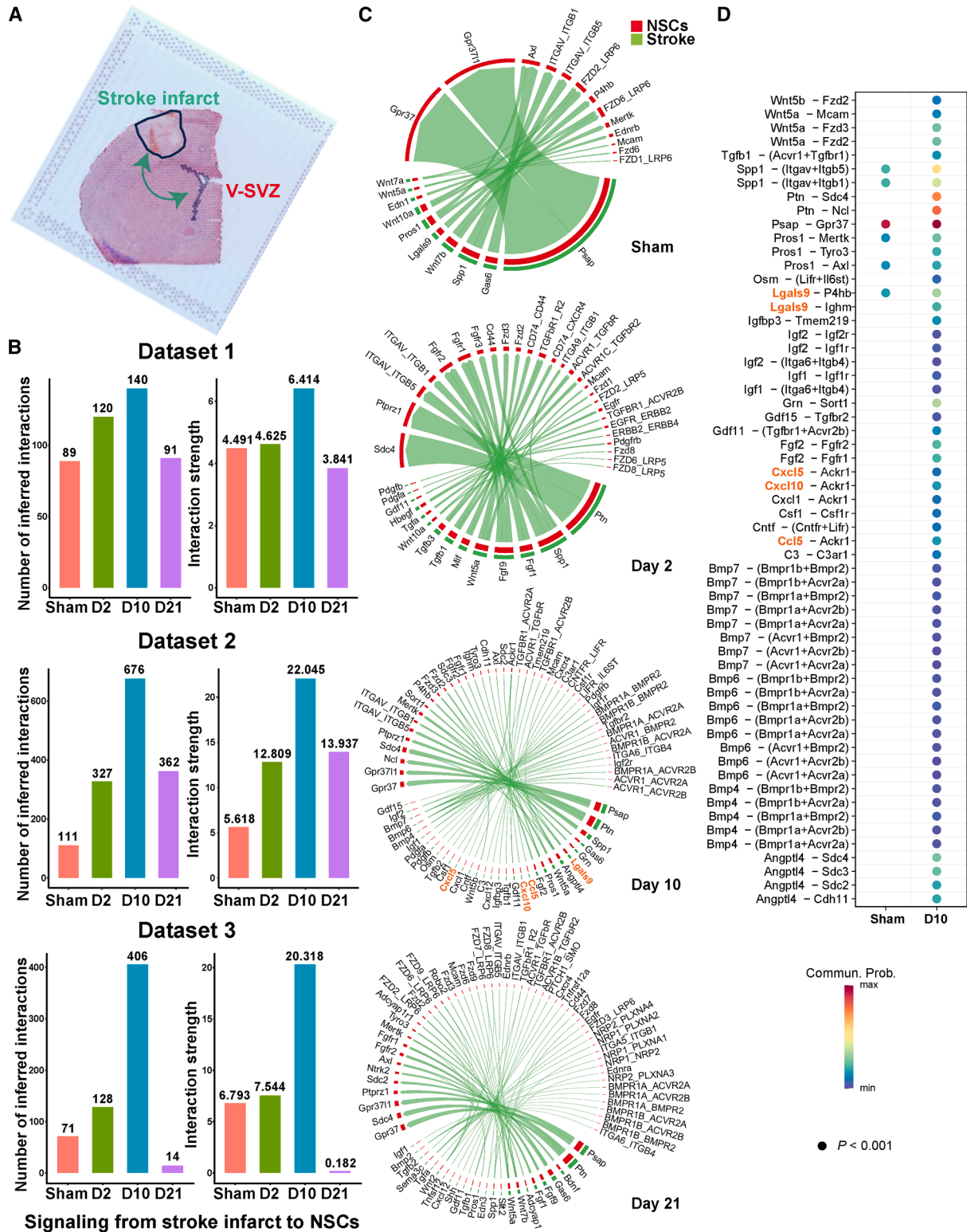


Figure 1. Inference of cell-cell communication from spatial transcriptomics data

(A) Hematoxylin and eosin stained section on a Visium capture area (Scott et al., 2024). Cell-cell communication between cortical ischemic lesion and the V-SVZ NSC niche was inferred by CellChat.

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Intercellular signaling can be inferred by spatial transcriptomics (Almet et al., 2024; Cang et al., 2023) that enables high-throughput quantification of gene expression *in situ*, and therefore, characterization of cell-cell communication via defined ligand-receptor (L-R) pairs (Tian et al., 2023). Many tools and resources have been developed and curated for deciphering cell-cell communication, which were systematically reviewed and compared recently (Dimitrov et al., 2022). In spite of differences in strategies adopted among these methods, all infer cell-cell communication from gene expression, predominantly focusing on L-R pairs (Armingol et al., 2021). We hypothesized that after stroke, the cells located in the cerebral infarct secrete and send signals to the NSC niche to initiate the regenerative response.

Here, we used CellChat (Jin et al., 2025) to computationally infer signaling between the ischemic infarct region and V-SVZ NSC niche. We identified L-R pairs and signaling pathways potentially involved in the signal transduction events 2, 10, and 21 days after ischemic stroke. Many of the L-R pairs have previously been characterized as regulators of NSC proliferation, migration, differentiation, and synaptogenesis. After screening several candidate genes of interest with limited known roles in the V-SVZ NSC niche, our study presents the first evidence, to our knowledge, of the negative regulatory function of galectin-9 on the proliferation of NSCs from both *in vitro* and *in vivo* assays as well as the galectin-9 knockout (*Lgals9*^{-/-}) mouse. Galectin-9 is a β -galactoside binding lectin (Hirashima et al., 2002) that has not only been extensively studied as an immune response modulator (Cao et al., 2018; Gossink et al., 2025; Zhu et al., 2005) but also associated with post-stroke recovery (Wei et al., 2012; 2015; He et al., 2017; Liang et al., 2021; Han et al., 2024). Thus, our present work depicts galectin-9 as a checkpoint signaling molecule that under physiological conditions controls NSC proliferation, and this physiological pathway is likely leveraged in the later recovery phase post-ischemic stroke to restore the quiescent state of NSCs. We further investigate the potential receptors for galectin-9 identified by our spatial transcriptomics data and suggest that known receptors toll-like receptor 4 (TLR4) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) are expressed in

the V-SVZ NSC niche. Of these, TIM-3 potentially mediates the inhibitory effect of galectin-9 on NSC proliferation while TLR4 may be leveraged in nearby ependymal cells.

RESULTS

Intercellular communication inference from spatial transcriptomic profiling

To characterize signal transduction between the infarct of ischemic stroke and the V-SVZ NSC niche (Figure 1A), we started by computationally inferring intercellular communication from spatial transcriptomics data. Three 10x Visium platform datasets from three different experiments ($N = 3$), respectively, were analyzed, each of which had three time points—acute (day-2), sub-acute (day-10), and chronic (day-21) post-ischemic stroke, along with a control sham injury (day-2 sham), respectively, as we described previously (Scott et al., 2024). The stroke infarct regions and the saline injection sites of sham controls were manually delineated with the 10x Genomics Loupe Browser. To identify NSCs, the barcoded spots in the V-SVZ were hand-selected with the 10x Genomics Loupe Browser. The sum of the normalized counts of the NSC marker genes—*Sox2*, *Nes*, *Gfap*, *Prom1*, and *Fabp7* was calculated and then ranked across all spots. The identity of NSC was assigned to spots in the first quartile of the ranks. We used CellChat (Jin et al., 2025) to infer cell-cell communication. To get an overview of the changes of intercellular signaling during the time course post-ischemic stroke in our animal model, we began with comparing the total numbers of interactions and interaction strength for samples of each time point and the sham control. The total numbers of intercellular interactions and interaction strength were increased in day-2 and further increased in day-10 samples but then decreased by day-21 compared with the sham control (Figure 1B). All three datasets from three experiments ($N = 3$) demonstrated the exact same trends consistently. This pattern agreed with the previous characterization of endothelin-1 (ET-1) induced stroke in rodents where the most neurological deficits were observed 1 week post-ischemic stroke followed by a significant recovery phase (Abeyasinghe et al., 2014; Roome et al., 2014). The subsequent narratives in this results section were based on the representative analysis from the second dataset. To

(B) Comparison of the total number of interactions and interaction strength among sham control, day-2, day-10, and day-21 post-ischemic stroke. Three 10x Visium datasets collected from three different experiments ($N = 3$) were analyzed.

(C) The L-R pairs and intercellular signaling from the stroke infarct site to the V-SVZ NSC niche inferred from spatial transcriptomics data in sham control and three time points post-ischemic stroke from the second dataset.

(D) The upregulated intercellular signaling L-R pairs from stroke infarct to V-SVZ NSCs in 10 days post-ischemic stroke compared to sham control by comparing the communication probabilities ($p < 0.001$) from the second dataset. Also see Table S1.



reveal the signaling molecules and pathways that play important roles in mobilizing NSCs located in the V-SVZ niche, we focused on the interactions (L-R pairs) from the stroke infarct site to NSCs (Figure 1C). The enhancement of cell-cell communication post-ischemic stroke was visually striking using chord diagrams to represent individual L-R pairs.

Next, we conducted a pairwise comparison of the upregulated and downregulated signaling L-R pairs based on the communication probabilities (Table S1). Compared with sham, the numbers of upregulated L-R pairs were 128, 387, and 199 in day-2, day-10, and day-21 sample, respectively. Moreover, 342 L-R pairs were upregulated in day-10 in comparison with day-2 sample, whereas 385 L-R pairs were downregulated in day-21 compared to day-10. These numbers suggested that the signal transduction from stroke infarct to NSC rose in the acute phase after stroke (day-2), peaked around sub-acute phase (day-10), and started to decline in the recovery/chronic phase (day-21). To pinpoint the critical regulators of NSCs post-stroke, we focused on the increased signaling from the stroke lesion to NSCs in 10 days post-ischemic stroke when compared to sham (Figure 1D). Particularly, the L-R pairs upregulated in day-10 compared with both sham and day-2 but downregulated compared with day-21 were considered for further analyses because both the numbers of inferred interactions and interaction strength peaked at 10 days post-ischemic stroke during which more critical signaling molecules could be potentially identified.

Many of the intercellular signaling events identified computationally have been previously regarded as regulators of NSC proliferation, migration, differentiation, and synaptogenesis, such as *Psap* (Labusch et al., 2024), *Ptn* (Tang et al., 2019), and *Gm* (Liu et al., 2021) which validated our bioinformatic analysis. To expand the knowledge of cell signaling from stroke infarct to NSCs, we explored four ligands inferred from our computational analysis but not previously well characterized in this context, including *Lgals9* (galectin-9), *Ccl5* (C-C motif chemokine ligand 5), also known as RANTES (regulated upon activation normal T cell expressed and secreted), *Cxcl5* (C-X-C motif chemokine ligand 5), also known as ENA-78 (epithelial cell-derived neutrophil-activating peptide-78), or LIX (LPS-induced CXC chemokine), or GCP-2 (granulocyte chemotactic protein-2), or AMCF-II (alveolar macrophage chemotactic factor-II), and *Cxcl10* (C-X-C motif chemokine ligand 10), also known as IP-10 (interferon γ -induced protein 10 kDa). We hand-picked these four ligands because they were potentially involved in the injury responses, for example, galectin-9 is an immune modulator (Cao et al., 2018; Gossink et al., 2025; Yang et al., 2021; Zhu et al., 2005), and CCL5, CXCL5, and CXCL10 are all chemokines that might guide the NSCs to the infarct site. In addition,

the spatial expression patterns of these four ligands demonstrated the same trends as the overall numbers and strength of intercellular communication, with salient increase in 10 days post-ischemic stroke and focal expression at the stroke infarct site in day-10 sample (Figure 2A). The galectin-9 expression over the time course of stroke was further evaluated by immunostaining in the mouse brain tissue collected 20 days after ET-1 induced stroke (Figure 2B). Although the *Lgals9* RNA level started to decline from day-10 to day-21 (Figure 2A), the galectin-9 protein level remained significantly higher in the ipsilateral infarct sites (Figure 2B, the two upper rows) compared to the contralateral controls (Figure 2B, the two lower rows) as shown in four mouse brains (Figure 2C, paired *t* test, $t_{(3)} = 10.38$, $p = 0.0019$). The colocalization of galectin-9 and microglia suggested that the predominant source of galectin-9 post-ischemic stroke at the infarct site was microglia (Figure 2B). To identify the types of cells that generated these signaling molecules, we analyzed an existing published single-cell RNA-sequencing dataset where the cell types were annotated by known cell type markers (Figure 2D) (Cebrian-Silla et al., 2021). As a well-known immunomodulator (Cao et al., 2018; Gossink et al., 2025; Yang et al., 2021; Zhu et al., 2005), in agreement with other data sources (Lee et al., 2024; Zhang et al., 2025), *Lgals9* was primarily expressed in microglia (Figures 2B and 2D). *Lgals9* was also expressed in endothelial cells, which could imply the important functions, for example angiogenesis (O'Brien et al., 2018), in the post-ischemic stroke recovery. Notably, *Lgals9* expression presented in ependymal cells as well (Figure 2D). *Ccl5* was mainly expressed in microglia. *Cxcl10*, another chemokine, had relatively higher expression levels in both microglia and ependymal cells. *Cxcl5* expression was highlighted in the oligodendrocyte lineage.

Intercellular communication inference identifies potential regulators of NSC proliferation

One of the possible functions of these signaling molecules elicited post-ischemic stroke could be to enhance NSC proliferation in the V-SVZ niche. To ask whether these ligands might be unrecognized modulators of NSC proliferation post-ischemic stroke, we first surveyed the impacts of the four ligands identified computationally *in vitro* using neurospheres (Figure 3). We evaluated the effects of the four ligands on the proliferation of the secondary spheres, including galectin-9, CCL5, CXCL5, and CXCL10. NSC proliferation was evaluated indirectly using the resazurin reduction assay and quantified by the fluorescence intensity of resorufin (Larson et al., 1997). We observed significant impacts of the ligands on NSC proliferation (Friedman test, $\chi^2_{(4)} = 14.63$, $p = 0.0055$, $N = 7$). Galectin-9 decreased NSC proliferation by nearly 2-fold compared to the control (Dunn's multiple comparisons test, Adj. $p = 0.0029$),

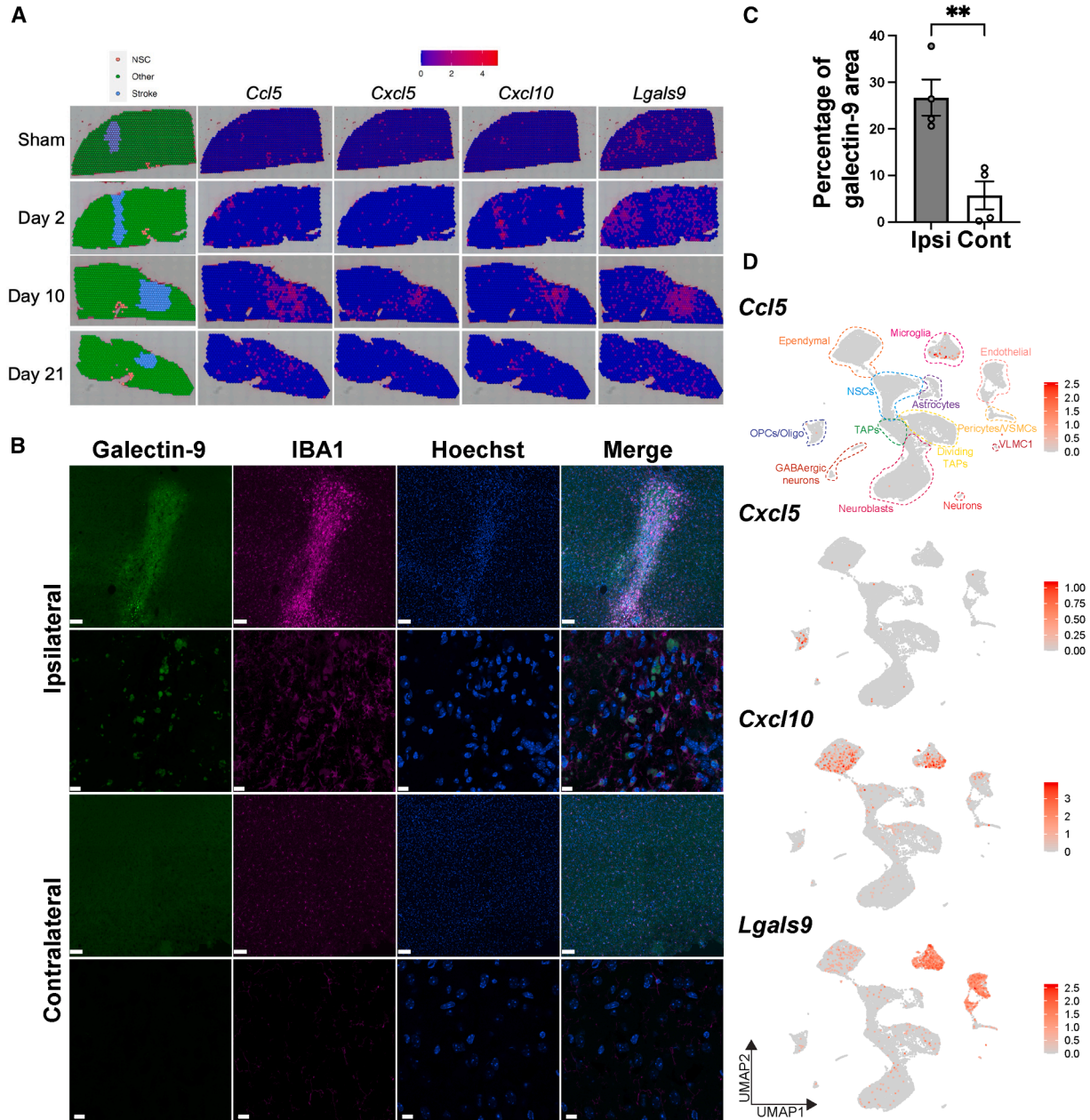


Figure 2. The expression pattern of selected genes of interest

(A) Expression patterns of selected genes of interest from 10x Visium spatial transcriptomics data from sham control, day-2, day-10, and day-21 post-ischemic stroke.

(B) Immunostaining of mouse brain tissue collected on day-20 after stroke for galectin-9 and IBA1. The two upper panels show the ipsilateral infarct site. The two lower panels display the corresponding contralateral region. The scale bars in the first and third row represent 100 μ m, and in the second and fourth represent 10 μ m.

(C) Quantification of the percentages of galectin-9 positive area of the ipsilateral infarct sites and the corresponding contralateral regions from four mouse brains ($N = 4$). Data are represented as mean $\pm$ S.E.M. Error bars indicate S.E.M. $^{**}p < 0.01$.

(D) The expression patterns of select genes of interest on a UMAP plot of V-SVZ whole-cell single-cell analysis from [Cebrian-Silla et al. \(2021\)](#).

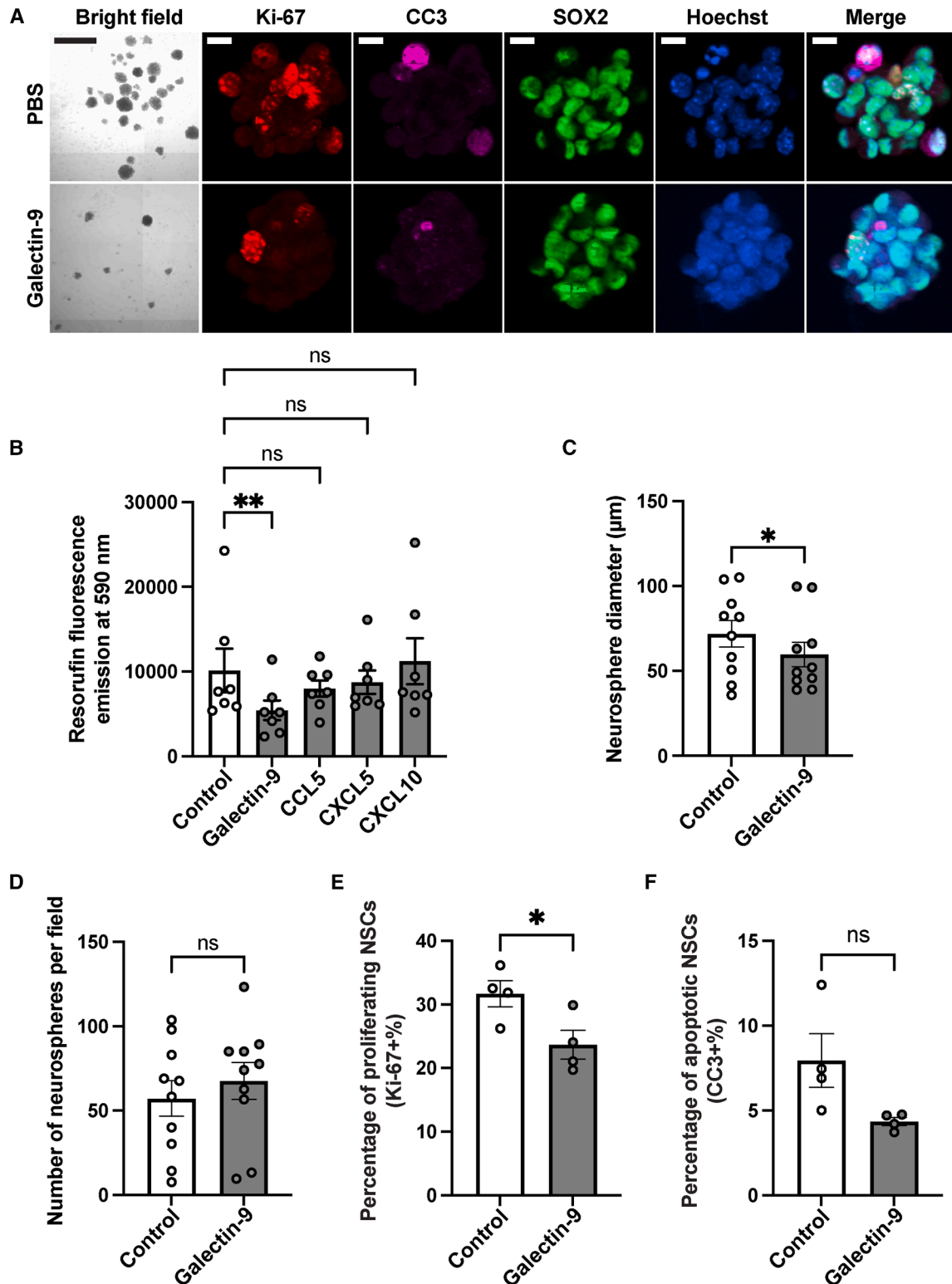


Figure 3. Galectin-9 inhibits NSC proliferation *in vitro*

(A) Bright-field images and immunostaining of neurospheres treated with PBS or galectin-9 (2 μg/mL) for Ki-67, cleaved-caspase-3 (CC3), SOX2, and merged with Hoechst to label nuclei. The scale bar in the bright field images represents 50 μm and in the immunostaining images represent 10 μm.

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whereas the other three ligands tested had no significant effects on NSC proliferation (Figures 3A and 3B). To confirm the inhibitory effects of galectin-9 on NSC proliferation, we quantified the sizes of neurospheres. The diameters of neurospheres cultured with galectin-9 showed approximately 20% reduction compared with those of control (paired t test, $t_{(9)} = 2.663$, $p = 0.0259$) (Figure 3C), which when converted to volume reflects a ~ 2 -fold reduction of the volume of neurospheres (Figure 3A), which compared favorably to the resazurin reduction assay data (Figure 3B). To further examine how galectin-9 influenced the stemness of NSC, we quantified the counts of these secondary neurospheres. Galectin-9 trended toward slightly increased numbers of neurospheres (paired t test, $t_{(9)} = 1.824$, $p = 0.1014$) compared with the control (Figure 3D) but this did not reach significance, suggesting relatively little impact on the stemness of NSCs. The effects of galectin-9 were further evaluated by immunostaining of neurospheres for Ki-67, SOX2, and cleaved-caspase-3 (CC3) (Figures 3A, 3E, and 3F). Galectin-9 significantly reduced the percentage of proliferating cells in neurospheres (unpaired t test, $t_{(6)} = 2.624$, $p = 0.0394$) compared with the control (Figures 3A and 3E) but did not significantly affect the percentage of apoptotic cells in the neurosphere (unpaired t test, $t_{(6)} = 2.251$, $p = 0.0653$) compared to the control (Figures 3A and 3F). Nearly all neurospheres in both control and galectin-9 treated groups displayed uniformly strong expression of NSC marker SOX2 (Figure 3A). Collectively, our *in vitro* experiments demonstrated that galectin-9 inhibited NSC proliferation but preserved NSC stemness.

Another potential role of the signaling molecules from the stroke infarct site to NSCs is to induce NSC migration to the injured area as previously demonstrated (Arvidsson et al., 2002). Given that the results of our computational

inference identified many chemokines, we next evaluated the chemoattractant property for NSCs of these four ligands. We performed quantitative analysis of migration by Boyden chamber assay (Widera et al., 2004). 10,000 cells in single-cell suspension disassociated from secondary neurospheres were seeded into each transwell. The cells migrated to the side facing the medium containing galectin-9 (2 $\mu\text{g/mL}$), CCL5, CXCL5, or CXCL10 (200 ng/mL), along with control, were counted, and expressed as cell numbers per square millimeter (mm^2). We did not observe significant chemotactic effects of these four ligands on NSCs (Friedman test, $\chi^2_{(4)} = 2.841$, $p = 0.585$, $N = 7$) (Figure S1). Although CXCL10 mobilized slightly more NSCs than the control medium did, this did not reach significance. After characterizing the effects of these signaling molecules *in vitro*, our subsequent *in vivo* studies focused on the negative regulatory effects of galectin-9 on NSC proliferation.

Characterization of the origins and targets of galectin-9 in mouse brain

To further illustrate the mechanism underpinning the inhibitory effect of galectin-9 on NSC proliferation *in vivo*, we investigated the sources and receptors of galectin-9, with a focus on the V-SVZ NSC niche in the adult mouse brain. From single-cell data, the two most predominant sources of *Lgals9* expression were microglia and endothelial cells, followed by ependymal cells (Figure 2D). We acquired direct evidence by immunostaining to characterize the cell-type expression of galectin-9. Our data showed extensive expression of galectin-9 colocalized with microgliosis at the infarct site compared to the contralateral side (Figure 2B). Galectin-9 was also found in the microglia sparsely scattered along the V-SVZ in

(B) The effects of selected signaling molecules on NSC proliferation, which was evaluated by resazurin reduction assay (Larson et al., 1997). Neurospheres cultured from the combined tissue from the V-SVZ of two mouse brains were pooled together as one biological replicate (defined as one N , i.e., one data point in the figure). Each biological replicate was seeded with technical triplicates in 96-well plates (i.e., cells from one biological replicate were seeded into three wells as technical triplicates). Two independent batches of cells that had seven biological replicates ($N = 7$, i.e., 14 mice were used) in total were analyzed, where the first batch of cells had four biological replicates ($N = 4$, i.e., 8 mice) and the second batch of cells had three biological replicates ($N = 3$, i.e., 6 mice).

(C and D) Comparison of the diameters (C) and numbers (D) of neurospheres treated with PBS or galectin-9 (2 $\mu\text{g/mL}$) per microscopic field of the wells in a 96-well plate. In (C) and (D), neurospheres cultured from the combined tissue from the V-SVZ of two mouse brains were pooled together as one biological replicate (defined as one N , i.e., one data point in the figure). Each biological replicate was seeded with technical triplicates in 96-well plates (i.e., cells from one biological replicate were seeded into three wells as technical triplicates). Three independent batches of cells that had 10 biological replicates ($N = 10$, i.e., 20 mice) in total were analyzed, where the first batch of cells had four biological replicates ($N = 4$, i.e., 8 mice), the second batch of cells had two biological replicates ($N = 2$, i.e., 4 mice), and the third batch of cells had four biological replicates ($N = 4$, i.e., 8 mice).

(E) Quantification of the percentages of proliferating Ki-67+ cells in the neurospheres treated with PBS or galectin-9 (2 $\mu\text{g/mL}$).

(F) Quantification of the percentages of cleaved-caspase-3 positive (CC3+) cells, in neurospheres treated with PBS or galectin-9 (2 $\mu\text{g/mL}$). In (E) and (F), neurospheres cultured from the combined tissue from the V-SVZ of two mouse brains were pooled together as one biological replicate (defined as one N , i.e., one data point in the figure). Neurospheres of four biological replicates ($N = 4$, i.e., 8 mice) in total were analyzed. Ten to twelve neurospheres were imaged for each biological replicate, respectively, for quantification.

Data are represented as mean $\pm$ S.E.M. Error bars indicate S.E.M. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

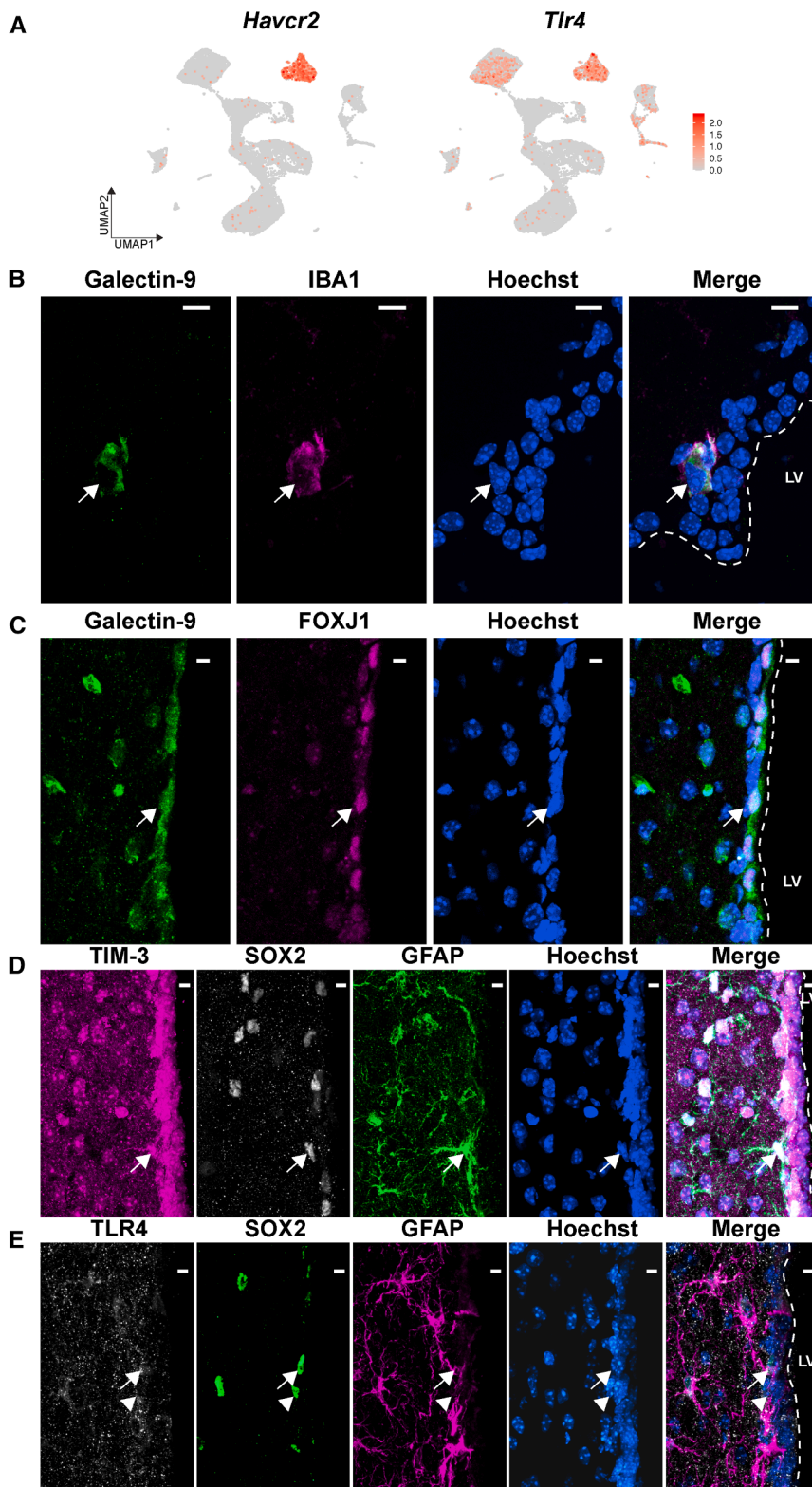


Figure 4. Cell-type expression of galectin-9 and its known receptors in mouse brain

(A) The expression patterns of *Havcr2* (TIM-3) and *Tlr4* on a UMAP plot of V-SVZ whole-cell single-cell analysis (Cebrian-Silla et al., 2021).

(B) Immunostaining of the lateral wall of 8-week-old C57BL/6 mouse brains for galectin-9, IBA1, and merged with Hoechst to label nuclei. Arrows indicate the colocalization of galectin-9 and IBA1.

(C) Immunostaining of the lateral wall of 8-week-old C57BL/6 mouse brains for galectin-9, FOXJ1, and merged with Hoechst to label nuclei. Arrows indicate the colocalization of galectin-9 and FOXJ1.

(D) Immunostaining of the lateral wall of 8-week-old C57BL/6 mouse brains for TIM-3, SOX2, GFAP, and merged with Hoechst to label nuclei. SOX2+GFAP+ cells allow identification of NSCs. Arrows indicate the representative NSC that expresses TIM-3.

(E) Immunostaining of the lateral wall of 8-week-old C57BL/6 mouse brains for TLR4, SOX2, GFAP, and merged with Hoechst to label nuclei. SOX2+GFAP− cells allow identification of ependymal cells. Arrows indicate the representative NSC that expresses TLR4. Arrowheads indicate the representative ependymal cell that expresses TLR4.

Scale bars represent 10 μ m. Dotted lines delineate the lateral wall of the lateral ventricle (LV).

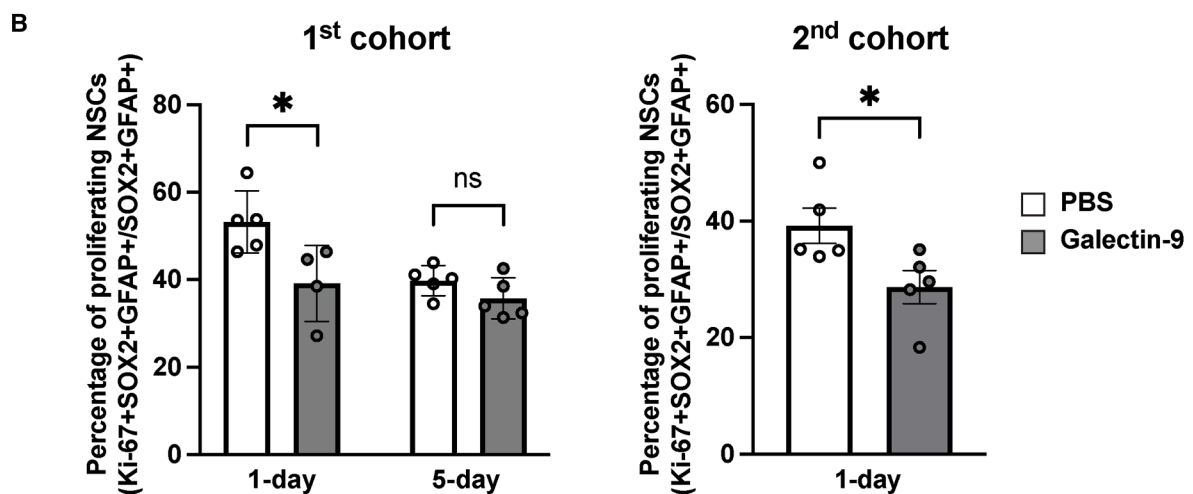
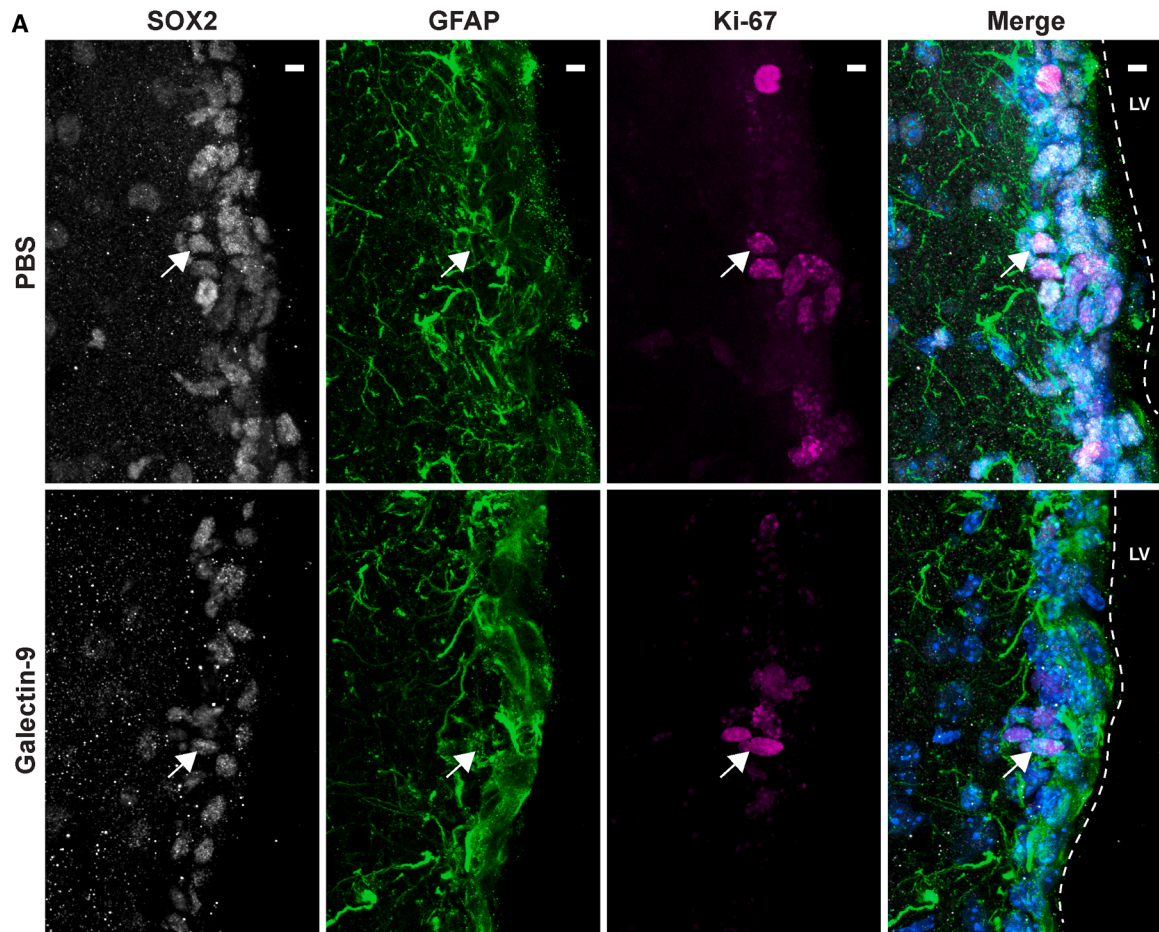


Figure 5. ICV injection of galectin-9 inhibits proliferation of NSCs in the V-SVZ niche of adult mouse brain

(A) Immunostaining of the lateral wall of 8-week-old C57BL/6 mouse brains ICV injected with PBS or galectin-9 for SOX2, GFAP, Ki-67, and merged with Hoechst to label nuclei. SOX2+GFAP+ cells allow identification of NSCs. Ki-67+SOX2+GFAP+ cells represent proliferating NSCs. Arrows indicate representative triple positive cells. Scale bars represent 10 μ m. Dotted lines delineate the lateral wall of the lateral ventricle (LV).

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healthy wild-type mice (Figure 4B, arrows). In agreement with the single-cell data, galectin-9 was expressed in vascular endothelial cells as well (Figure 4C). Focusing on the role of galectin-9 in the regulation of NSCs, our data demonstrated that galectin-9 was detected in FOXJ1 positive ependymal cells along the lateral wall of the ventricles (Figure 4C, arrows). Galectin-9 produced by ependymal cells in the V-SVZ could cause immediate impact on NSCs because of their proximity in this niche. Many receptors of galectin-9 have been previously identified, including TIM-3 (*Havcr2*), TLR4, PD-1, and CD44 (Gossink et al., 2025). The bioinformatics inference by CellChat from our spatial transcriptomics data indicated CD44, CD45, TIM-3, IGHM, BCR, and P4HB as the receptors for galectin-9 in the signaling pathway activated post-ischemic stroke (Table S1; Figures 1C and 1D). From the analysis of single-cell data, besides microglia, both TIM-3 and TLR4 were expressed in the cell populations in the V-SVZ, including NSCs, transit-amplifying progenitors (TAPs), dividing TAPs, and neuroblasts (Figure 4A). However, TLR4 showed particularly high expression level in ependymal cells, signifying the important roles of ependymal cells in response to injury, infection, and inflammation in the brain, especially given their location lining the ventricles (Xie and Li, 2024). Our immunohistochemical data confirmed the expression of TIM-3 in NSCs (Figure 4D, arrows, TIM-3+SOX2+GFAP+ cells) and the expression of TLR4 in both NSCs (Figure 4E, arrows, TLR4+SOX2+GFAP+ cells) and ependymal cells (Figure 4E, arrowheads, TLR4+SOX2+GFAP− cells).

Galectin-9 inhibits NSC proliferation *in vivo*

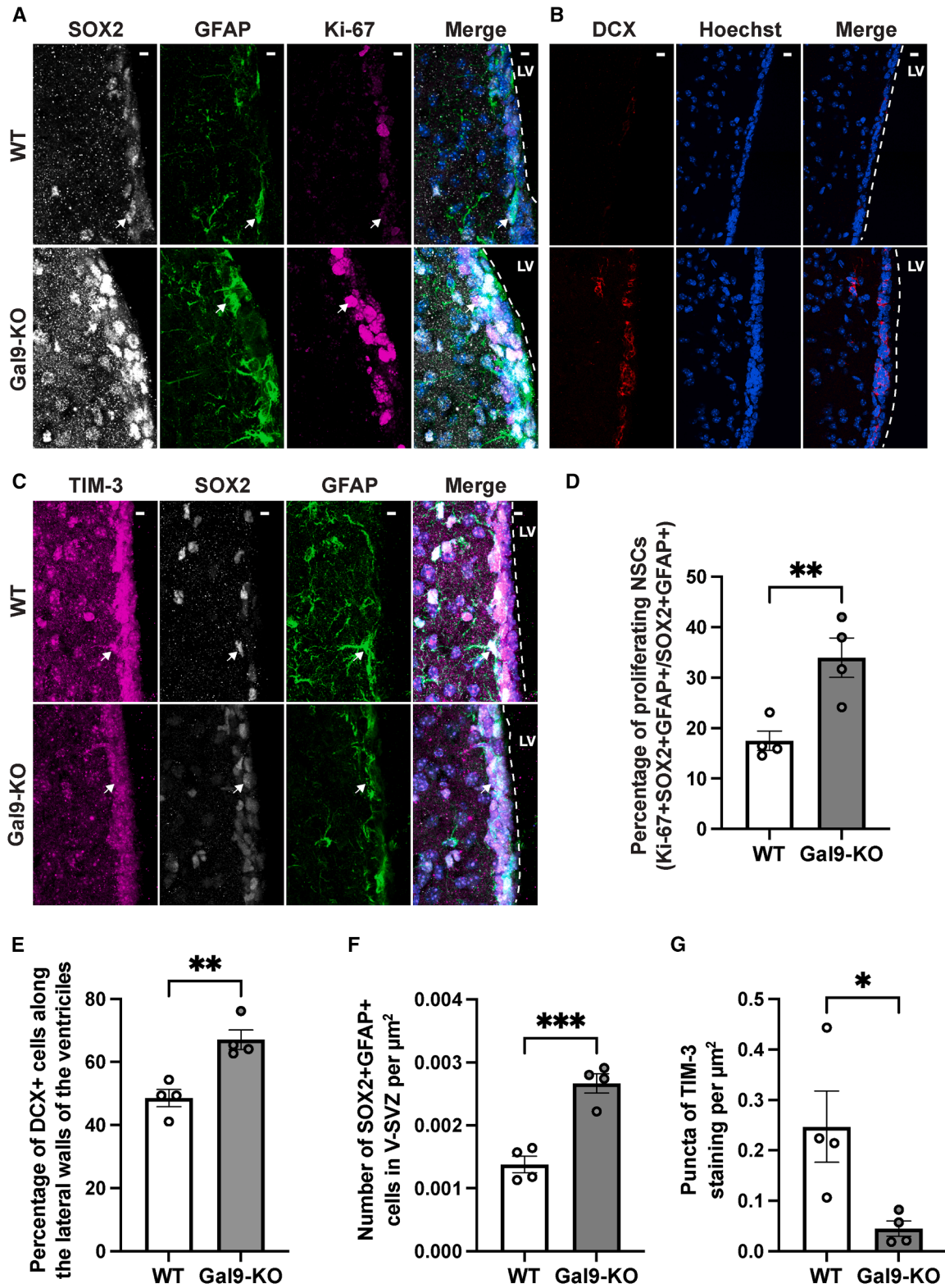
We further extended our *in vitro* observation of the negative impact of galectin-9 on NSC proliferation via intracerebroventricular (ICV) injection. Galectin-9 or PBS was injected into the LV in the right hemisphere of 8-week-old C57BL/6 mouse brains. NSCs were identified by immunohistochemistry as SOX2+ and GFAP+ cells in the V-SVZ region. Ki-67 immunostaining was used as a cell proliferation marker (Figure 5A). The percentage of proliferating NSCs were quantified in two independent cohorts of mice (Figure 5B). For the first cohort, we collected the brains 1 day and 5 days after ICV injection. The percentage of proliferating NSCs was significantly reduced in the mouse brains injected with galectin-9 in 1-day post-ICV compared with PBS injection (unpaired *t* test, $t_{(7)} = 2.678$, $p = 0.032$). At 5 days, this inhibitory effect was no longer significant (unpaired *t* test, $t_{(8)} =$

1.542, $p = 0.16$) compared with the control, possibly due to the elimination of galectin-9 from the LV after injection. We repeated the ICV experiment using a second cohort and collected brains 1 day after injection. The results faithfully recapitulated the significant reduction of the percentage of proliferating NSCs in brains injected with galectin-9 compared to the control (unpaired *t* test, $t_{(8)} = 2.519$, $p = 0.036$). Our data demonstrated that galectin-9 inhibited NSC proliferation *in vivo*, echoing our *in vitro* evidence obtained from the neurosphere assay (Figure 3).

Galectin-9 functions as a negative modulator of the proliferation of NSCs under physiological conditions in the V-SVZ

Based on our findings of the negative impact of galectin-9 on NSC proliferation, we next compared the NSCs in the V-SVZ niche of galectin-9 knockout (Cao et al., 2018; Smith et al., 2021) to wild-type (WT) mouse brains to ask whether galectin-9 controlled NSC proliferation under physiological conditions. The sections of forebrains from 8-week-old *Lgals9*^{−/−} (Gal9-KO) mice and age-matched WT mice were collected and analyzed by immunohistochemistry (Figures 6A–6C). First, we quantified the proliferating NSCs in the V-SVZ niche. The percentage of proliferating NSCs from Gal9-KO brains was significantly higher compared with those from WT (unpaired *t* test, $t_{(6)} = 3.792$, $p = 0.009$) (Figures 6A and 6D). This inhibitory effect of galectin-9 on NSC proliferation was further consolidated by the increased percentage of doublecortin positive (DCX+) cells along the lateral wall of the LV in the Gal9-KO brains compared to the WT (unpaired *t* test, $t_{(6)} = 4.493$, $p = 0.0041$) (Figures 6B and 6E). Moreover, the density of NSCs (marked by SOX2 and GFAP) in the V-SVZ niche of Gal9-KO mice showed a striking increase compared to WT mice (unpaired *t* test, $t_{(6)} = 6.384$, $p = 0.0007$) (Figures 6A and 6F). These data strongly suggested that when galectin-9 was absent, NSCs proliferation was enhanced and therefore more NSCs could be produced over the course of time. Thus, galectin-9 may function as a checkpoint molecule, controlling the amount of NSCs in the brain. Compared to the short-term effects of injected recombinant galectin-9 (Figure 5), our data presented here evaluated the long-term, chronic role of galectin-9 loss of function on the regulation of NSC proliferation (Figure 6). We further examined the expression of TIM-3, a canonical receptor of galectin-9. The density of the puncta of TIM-3 immunostaining was significantly lower

(B) Quantification of the percentages of proliferating Ki-67+ NSCs in the V-SVZ of 8-week-old C57BL/6 mouse brains ICV injected with PBS or galectin-9 (each *N*, corresponding to one data point in Figure 5B, represents one mouse brain. *N* = 5 for PBS control group in the first cohort, *N* = 4 for the galectin-9 injection group in the first cohort; *N* = 5 per group for the second cohort of experiment). Data are represented as mean ± S.E.M. Error bars indicate S.E.M. ns $p > 0.05$, * $p < 0.05$.



(legend on next page)



in the Gal9-KO mouse brains compared to that of WT (unpaired t test, $t_{(6)} = 2.796$, $p = 0.031$) (Figures 6C and 6G). Hence, the negative regulatory effects of galectin-9 on NSC proliferation may be mediated by the TIM-3 receptor.

DISCUSSION

Although a significant body of work has demonstrated enhanced neurogenesis post-ischemic stroke (Lindvall and Kokaia, 2015), signal transduction from the infarct region to the NSC niche has not been systematically investigated. Our study, therefore, set out to examine the intercellular signaling inferred computationally via both *in vitro* and *in vivo* assays. We first computationally inferred intercellular communication based on spatial transcriptomic data. Many of the L-R pairs identified by CellChat (Jin et al., 2021; 2025) have been validated by previous studies, which affirmed the high quality of our spatial transcriptomic data, and the effectiveness and accuracy of our bioinformatic approach. For instance, one of the L-R pairs we identified was the chemokine SDF-1 (Stromal cell-derived factor 1, also known as CXCL12) and its receptor CXCR4. Previous studies have shown that SDF-1 was expressed in reactive astrocytes and activated microglia after stroke and guided the migration of neural progenitor cells and neuroblasts to the injury site (Li and Ransohoff, 2008; Lindvall and Kokaia, 2015). Another example was *Gm* (or Progranulin) that stimulated cell proliferation in the V-SVZ after ischemic stroke and promoted neuronal differentiation in the peri-infarct region (Liu et al., 2021; Purrahman et al., 2023). Although we identified a few chemokines that might play important roles in post-ischemic stroke processes, the lack of statistically significant responses to the chemokines from NSCs disassociated from secondary neurospheres in our assay might be due to the

NSCs not reaching a migration competent stage and is hence a limitation of this work.

Out of several candidate genes of interest, we for the first time, to our knowledge, report the evidence of a regulatory role on NSC proliferation by a signaling pathway centered on galectin-9. We showed that galectin-9 inhibited NSC proliferation both *in vitro* (Figure 3) and *in vivo* (Figure 5). We also demonstrated that knocking out galectin-9 resulted in increased NSC proliferation (Figure 6). The molecular mechanism underlying this inhibitory effect has not been characterized to the best of our knowledge. Perhaps contradictory to the concept of mobilization boosting NSCs proliferation post-ischemic stroke to aid neurogenesis (Arvidsson et al., 2002), we identified galectin-9 as a checkpoint molecule which *inhibited* NSC proliferation. We suggest that galectin-9 might play such a role in the sub-acute and chronic/recovery phase post-ischemic stroke when the proliferation response of NSCs declines.

We first characterized the origins of galectin-9 based on previously published single-cell data (Figures 2D; Cebrian-Silla et al., 2021). Galectin-9 originating from microglia may play an important role in the acute responses to injury, i.e., microgliosis post-ischemic stroke. In addition, due to the potential exposure to the external environment in the LV and the weakened blood-brain barrier, galectin-9 coming from microglia in the V-SVZ and vascular endothelial cells, respectively, may function as an immunomodulator. However, our study points to a new role of galectin-9 derived from ependymal cells that under physiological conditions may maintain the quiescent state of NSCs, and this mechanism could be potentially leveraged in the chronic phase post-ischemic stroke to prevent the long-term overreaction of NSC proliferation (Figure 7).

Figure 6. Gal9-KO results in enhanced proliferation of NSCs in the V-SVZ niche of adult mouse brain

(A) Immunostaining of the lateral wall of 8-week-old WT and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 brains for SOX2, GFAP, Ki-67 and merged with Hoechst to label nuclei. SOX2+GFAP+ cells allow identification of NSCs. Ki-67+SOX2+GFAP+ triple positive cells represent proliferating NSCs. Arrows indicate the representative triple positive cells.

(B) Immunostaining of the lateral wall of 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 brains for doublecortin (DCX) and merged with Hoechst to label nuclei.

(C) Immunostaining of the lateral wall of 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 mouse brains for TIM-3, SOX2, GFAP, and merged with Hoechst to label nuclei. Arrows indicate the representative NSCs that expresses TIM-3.

Scale bars represent 10 μ m in (A–C). Dotted lines delineate the lateral wall of the lateral ventricle (LV) in (A–C).

(D) Quantification of the percentages of proliferating Ki-67+ NSCs in the V-SVZ of 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 mouse brains.

(E) Quantification of the percentages of doublecortin positive (DCX+) cells in the V-SVZ of 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 mouse brains.

(F) Quantification of the densities of SOX2+GFAP+ NSCs in the V-SVZ of 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 mouse brains.

(G) Quantification of the densities of the puncta of TIM-3 staining in the 8-week-old wild-type (WT) and Gal9-KO (*Lgals9*^{-/-}) C57BL/6 mouse brain sections.

Each N , corresponding to one data point in the Figures 6D–6G, represents one mouse brain. $N = 4$ per group for each mouse strain in Figures 6D–6G. Data are represented as mean $\pm$ S.E.M. Error bars indicate S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

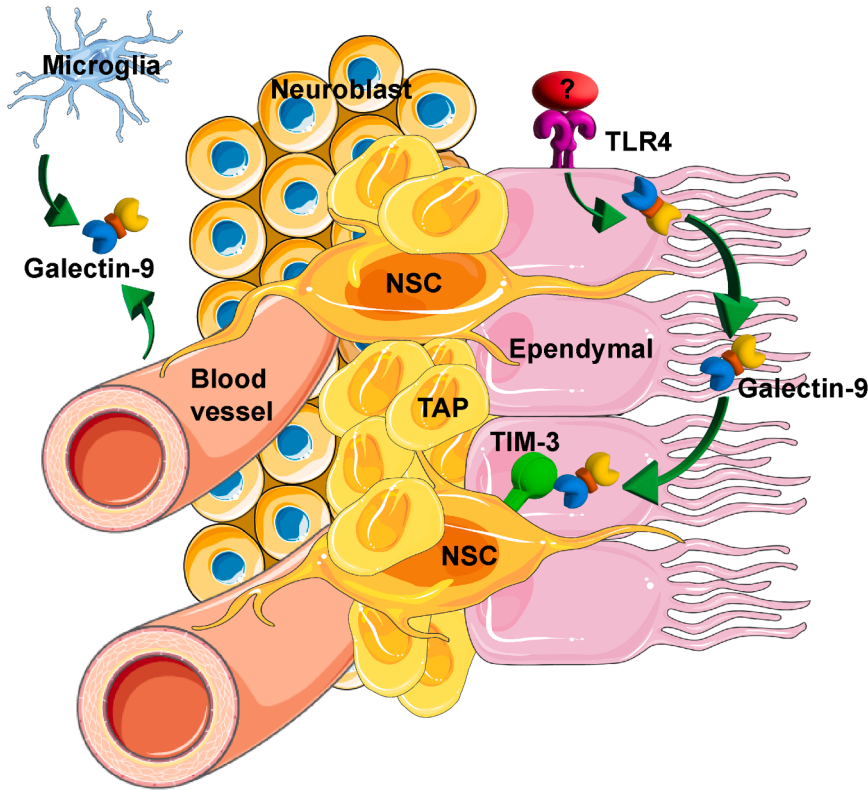


Figure 7. Working hypothesis for the impact of galectin-9 centered signaling pathway on NSCs under physiological conditions and post-ischemic stroke

Galectin-9 from microglia, vascular endothelial cells, and ependyma cells (Figure 2D) may play different roles in distinctive scenarios. Galectin-9 coming from the microglia and vascular endothelial cells may function as an immunomodulator due to the potential exposure to the external environment in the V-SVZ and the weakened blood-brain barrier. Under physiological conditions, galectin-9 produced by ependymal cells may be crucial for maintaining NSCs in the quiescent state by inhibiting their proliferation. This mechanism could be potentially leveraged during the chronic phase post-ischemic stroke. TLR4 receptors on ependymal cells are activated by proinflammatory cytokines (red oval with a question mark) elicited after stroke. The activated TLR4 signaling pathway triggers ependymal cells, which express galectin-9 under physiological conditions, to increase its expression. Galectin-9 can bind TIM-3 on NSCs and inhibits proliferation to prevent over-proliferation following injury. TAP represents transit-amplifying progenitor; NSC represents neural stem cell.

To investigate the mechanism behind the inhibitory effect of galectin-9 on NSC proliferation, we further examined the potential receptors for galectin-9 identified by our spatial transcriptomics data, including CD44, CD45, TIM-3, IGHM, BCR, and P4HB (Table S1; Figures 1C and 1D). Besides our present work, a few previous studies have identified that galectin-9 plays important roles in the response to stroke injury and explored the receptors involved, including TIM-3 (Wei et al., 2012; 2015), CD44 (Han et al., 2024), and TLR4 (Liang et al., 2021). In addition to microglia (Kimura et al., 2025), T cell immunoglobulin and mucin-domain containing-3 (TIM-3, encoded by *Havcr2*) has long been known to be expressed in various CNS tissues and upregulated in response to inflammation and infection (Gielen et al., 2005). Single-cell analysis showed that TIM-3 expression was found in NSCs, TAPs, and dividing TAPs besides microglia (Figure 4A). It has been reported that stroke increased the expression of TIM-3 (Wei et al., 2012; 2015) and galectin-9 (He et al., 2017; Wei et al., 2012; 2015). The protective effects of preconditioning (Wei et al., 2012) and postconditioning (Wei et al., 2015) on stroke were mediated by the downregulation of galectin-9/TIM-3 signaling pathway. These data are supportive of the identification of galectin-9 as an upregulated signaling molecule post-

ischemic stroke from our spatial transcriptomics data (Figure 1). Moreover, our data demonstrated the chronic downregulation of TIM-3 in Gal9-KO mouse brain (Figures 6C and 6G), which upheld the perspective of galectin-9/TIM-3 signaling pathway participating in the recovery process of stroke injury.

Another receptor identified by our spatial transcriptomics data was CD44 (Table S1). A recent study suggested that galectin-9 produced by microglia promoted remyelination after stroke through CD44 receptor on primitive oligodendrocyte progenitor cells (Han et al., 2024). However, our immunohistochemical results showed that besides microglia (Figures 2B and 4B), ependymal cells also produce galectin-9 (Figure 4C), which normally express this molecule under physiological conditions. This is particularly important given the proximity of ependymal cells to the V-SVZ NSC niche. We also demonstrated that galectin-9 suppressed the proliferation of NSCs (Figures 3 and 5) and therefore might potentially negatively impact neurogenesis. One explanation for these seemingly contradictory effects is that our day-10 and day-21 data were collected during a longer time course at different time-points instead of one single time point (at day-3 post-injury) in the study by Han et al. (2024). Although NSC mobilization can be beneficial to recovery during the



time immediately post-stroke, galectin-9 may serve as a break when it is time for the proliferation of NSCs to taper off. Moreover, it is common that one signaling molecule may function in various fashions through divergent pathways at distinct stages of a biological event.

As an immune regulator (Gossink et al., 2025), it is natural to postulate the regulatory effects of galectin-9 on microglia, one important type of immune cells in the CNS (Li and Barres, 2018). A study showed that galectin-9 may switch its roles from promoting differentiation of microglia into M1-type at the acute phase to directing M2-type differentiation in the later stage through TLR4 receptor to promote recovery post intracerebral hemorrhage in the rat brain (Liang et al., 2021). While TLR4 is highly expressed in microglia populations, our analysis of the existing single-cell dataset showed that the expression of TLR4 demonstrated the second highest level in the cluster of ependymal cells after microglia (Figure 4A). The expression of TLR4 in non-hematopoietic cells of CNS, including ventricular ependymal lining has been experimentally confirmed by *in situ* hybridization previously (Chakravarty and Herkenham, 2005) and demonstrated in our current immunohistochemistry data as well (Figure 4E). In addition, the expression of galectin-9 in ependymal cells was also shown in the single-cell analysis (Figure 2D) and experimentally verified by our present study (Figure 4C). It has been shown that the activation of TLR4 by proinflammatory ligand lipopolysaccharide (LPS) could lead to increased galectin-9 expression in human mesenchymal stromal cells (MSCs) (Gieseke et al., 2013) and human periodontal ligament (PDL)-derived cells (Kasamatsu et al., 2005). The galectin-9 synthesized in MSCs in turn inhibited T cell proliferation (Gieseke et al., 2013). Mouse brain infection could cause increased expression of TLR4 and TLR2, noticeably in ependymal cells of the choroid plexus (Wojtkowiak-Giera et al., 2016). In the neurocysticercosis mouse brain, TLR4 expression increased in neurons, and the expression of TLR2 and TLR7 was upregulated in ependymal cells (Mishra et al., 2006). Studies have shown that TLR4 on ependymal cells could respond to systemic inflammatory cytokines (Neal and Gasque, 2013). Not only do Toll-like receptors play important roles in the function of immune cells, many types of cells outside the immune system with immune properties upregulate the expression of TLRs in response to inflammatory stimuli (Typiak and Żurawa-Janicka, 2024). Interestingly, the crosstalk between TIM-3 and TLR4 may occur through the PI3K-Akt-mTOR-NF- κ B axis (Rezabakhsh et al., 2025).

Taken together, we propose a working model for the signaling pathway centered on galectin-9 under injured and physiological states (Figure 7). Galectin-9 originating

from microglia, vascular endothelial cells, and ependymal cells (Figure 2D) may play different roles in distinctive scenarios. Galectin-9 coming from the microglia and vascular endothelial cells may function as an immunomodulator due to the potential exposure to the external environment in the V-SVZ and the weakened blood-brain barrier. Under physiological conditions, galectin-9 produced by ependymal cells could serve to suppress NSC proliferation. Post-ischemic stroke, proinflammatory cytokine(s) are produced at the ischemic stroke infarct site which may bind the TLR4 receptor on ependymal cells and stimulate these cells to increasingly express galectin-9. Galectin-9 can activate the TIM-3 receptor on NSCs and enhance the suppression of NSC proliferation, possibly through mTOR-related pathway (Gonçalves Silva et al., 2016) in the later stage of post-ischemic stroke to shut off the initial responses of NSCs. Our study pinpoints galectin-9 as a modulator of NSC proliferation based on the landscape of cell-cell communication inference from spatial transcriptomics datasets over a time course of distinctive stages post-ischemic stroke. Further research is needed on the detailed molecular mechanisms underlying galectin-9 signaling and development of potential therapeutic approaches which leverage this pathway. Moreover, cell type-specific knockout/knockdown of galectin-9 in microglia, vascular endothelial cells, and/or ependymal cells in future studies would further clarify the origin(s) of galectin-9 responsible for the maintenance of NSC quiescent state under physiological conditions and/or restoration of the baseline proliferation of NSC in the chronic/recovery phase post-ischemic stroke.

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to the lead contact, Scott A. Yuzwa (scott.yuzwa@utoronto.ca).

Materials availability

All materials generated in this study are available from the lead contact.

Data and code availability

All sequencing data used in this study have previously been described and are available in the NCBI SRA and GEO databases as described below. No new code was produced in these studies.

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AUTHOR CONTRIBUTIONS

S.A.Y. and H.H. conceptualized and designed the experiments; H.H. performed the neurosphere, cell proliferation, Boyden chamber, tissue sectioning, immunohistochemistry experiments, and image acquisition and quantification; E.D. performed the intracerebroventricular injection for data in Figure 5B, generated the ET-1 induced stroke in mice, and performed the cryosectioning for data in Figure 2B; H.H., W.C.J.L., and T.T. analyzed the 10x Visium spatial transcriptomics profiling data; H.H. generated visualizations; D.L.C. and M.F. provided the 10x Visium spatial transcriptomics profiling data; H.C. and B.T. provided the Gal9-KO mouse brains; H.H. and S.A.Y. wrote the initial draft of the paper. All authors commented on and approved the final version of the manuscript before submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
goat anti-mouse galectin-9	R&D Systems	Cat#AF3535; RRID: AB_2137240
rabbit anti-doublecortin (DCX)	Cell Signaling Technology	Cat#4604; RRID: AB_561007
rabbit anti-Iba1/AIF-1	Cell Signaling Technology	Cat#17198; RRID: AB_2820254
mouse anti-FOXJ1	eBioscience	Cat#14-9965-80; RRID: AB_1548836
biotin anti-mouse CD366 (Tim-3)	BioLegend	Cat#119720; RRID: AB_2571936
rabbit anti-TLR4	Proteintech	Cat#19811-1-AP; RRID: AB_10638446
rabbit anti-SOX2	Cell Signaling Technology	Cat#3728S; RRID: AB_2194037
goat anti-SOX2	R&D Systems	Cat#AF2018; RRID: AB_355110
mouse anti-GFAP	Cell Signaling Technology	Cat#3670S; RRID: AB_561049
chicken anti-GFAP	Sigma-Aldrich	Cat#AB5541; RRID: AB_177521
mouse anti-Ki-67	BD Biosciences	Cat#550609; RRID: AB_393778
Alexa Fluor® 488-conjugated donkey anti-chicken secondary antibody	Jackson ImmunoResearch Laboratories	Cat#703-545-155; RRID: AB_2340375
Alexa Fluor® 647-conjugated donkey anti-rabbit secondary antibody	Jackson ImmunoResearch Laboratories	Cat#711-605-152; RRID: AB_2492288
Cy TM 3-conjugated donkey anti-mouse secondary antibody	Jackson ImmunoResearch Laboratories	Cat#715-165-150; RRID: AB_2340813
Alexa Fluor® 488-conjugated donkey anti-goat secondary antibody	Jackson ImmunoResearch Laboratories	Cat#705-545-147; RRID: AB_2336933
Cy TM 3 Streptavidin	Jackson ImmunoResearch Laboratories	Cat#016-160-084; RRID: AB_2337244
Donkey serum	Jackson ImmunoResearch Laboratories	Cat#017-000-121; RRID: AB_2337258
Chemicals, peptides, and recombinant proteins		
NeuroCult TM Basal Medium (Mouse & Rat)	STEMCELL Technologies	Cat#05700
NeuroCult TM Proliferation Supplement (Mouse & Rat)	STEMCELL Technologies	Cat#05701
Basic Fibroblast Growth Factors (bFGF)	Corning	Cat#354060
Human Recombinant EGF	STEMCELL Technologies	Cat#78006.1
Heparin	Sigma-Aldrich	Cat#H4784
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
Galectin-9	R&D Systems	Cat#3535-GA
CCL5	PeproTech	Cat#250-07
CXCL5	BioLegend	Cat#573302

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CXCL10	PeproTech	Cat#250-16
Poly-D-Lysine Hydrobromide	Sigma-Aldrich	Cat#P6407-5MG
Laminin, mouse	Corning	Cat#354232
Hoechst 33258	Sigma-Aldrich	Cat#B2883
PermaFluor™ Aqueous Mounting Medium	Epredia	Cat#TA-030-FM
Bupivacaine Injection 0.25%	Center de distribution de médicaments vétérinaires (CDMV)	Cat#ORK008504
Buprenorphine Slow Release 0.6 mg/mL	Chiron Compounding Pharmacy Inc.	
Resazurin sodium salt	Millipore Sigma	Cat#R7017
Fisher Healthcare Tissue-Plus™ O.C.T. Compound	Fisher Scientific	Cat#23730571
Critical commercial assays		
DC™ Protein Assay Kit II	Bio-Rad	Cat#5000112
Deposited data		
Studying stroke in mouse cortex 10x Visium spatial transcriptomics	Scott et al. (2024)	BioProject: PRJNA952594
Single-cell RNA sequencing dataset of the mouse brain ventricular-subventricular zone	Cebrian-Silla et al. (2021)	GEO: GSM5039270
Experimental models: Organisms/strains		
C57BL/6NCrl inbred mouse	Charles River	RRID: IMSR_CRL:027
<i>Lgals9</i> ^{-/-} mouse of C57BL/6 background	Cao et al. (2018)	
Software and algorithms		
Space Ranger (v.1.0.0)	10x Genomics	https://www.10xgenomics.com/support/software/space-ranger/latest
Seurat (v.5.3.0)	R package	https://satijalab.org/seurat/
Loupe Browser (v.9.0.0)	10x Genomics	https://www.10xgenomics.com/support/software/loupe-browser/latest
CellChat (v.2.2.0)	R package	https://github.com/jinworks/CellChat
ZEN Microscopy Software (v.2.6)	ZEISS	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
Fiji (ImageJ2) (v.2.16.0/1.54p)	National Institutes of Health	https://imagej.net/software/fiji/
GraphPad Prism (v.10.5.0 for macOS)	GraphPad	https://www.graphpad.com
Other		
Millicell 24 well standing inserts	Millipore	Cat#PI8P01250
Fisherbrand Superfrost Plus Microscope Slides	Fisher Scientific	Cat#1255015
Amicon Ultra Centrifugal Filter, 30 kDa MWCO	MilliporeSigma	Cat#UFC503024
10 µL Microliter #701 Syringe	Hamilton Company	Cat#80330
29 Gauge Small Hub Removable Needle	Hamilton Company	Cat#7803-06
Epredia™ HM525 NX cryostat	Fisher Scientific	Cat#956640EC70



EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

8-week-old male C57BL/6 wild type (WT) mice (RRID: IMSR_CRL:027) were obtained from Charles River and housed at the Department of Comparative Medicine at the University of Toronto, following a 14/10 h light/dark cycle and had *ad libitum* access to water and rodent chow. Age-matched male *Lgals9*^{-/-} (Gal9-KO) mice of C57BL/6 genetic background as previously described (Cao et al., 2018), were housed in a specific pathogen-free animal facility at University of Toronto Scarborough, Toronto, Canada. For the ET-1 induced stroke mouse model (Scott et al., 2024) used in Figure 2 and ICV injections in Figure 5, 8-week-old male C57BL/6J mice were ordered from the in-house breeding colony and housed at The Center for Phenogenomics (Toronto, Canada). All procedures complied with policies formed by the Canadian Council of Animal Care and approved by the local Animal Care Committee at the University of Toronto and The Center for Phenogenomics, Toronto, Canada.

METHOD DETAILS

Visium spatial transcriptomics

The 10x Visium spatial transcriptomics profiling data were described previously (Scott et al., 2024) and are available at NCBI SRA database BioProject Accession: PRJNA952594 (Scott et al., 2024). The three datasets analyzed in this work were from three independent experiments ($N = 3$), respectively.

Inference of cell-cell communication

The Space Ranger (version 1.0.0) output were processed by Seurat (version 5.3.0) (Hao et al., 2023) implemented in R (version 4.5.1) (R Core Team, 2025). The data were then normalized and scaled by SCTransform. Principal components were computed, and clustering was performed with 30 principal components and a resolution of 0.8. UMAP plots were created using the DimPlot and FeaturePlot functions implemented in Seurat (version 5.3.0) (Hao et al., 2023). The stroke infarct regions and the saline injection site of sham control were manually delineated from 10x Genomics Loupe Browser (version 9.0.0). To identify NSCs, the barcoded spots in the V-SVZ were hand selected from 10x Genomics Loupe Browser. The sum of the normalized counts of the NSC marker genes – *Sox2*, *Nes*, *Gfap*, *Prom1*, and *Fabp7* was calculated and then ranked for all spots. The identity of NSC was assigned to spots in the first quartile of the ranks. Cell-cell communication was inferred by CellChat (version 2.2.0) (Jin et al., 2025) Comparison analysis was conducted among sham, day-2, 10, and 21 post-ischemic stroke for the second dataset following the CellChat analysis pipeline.

Analysis of scRNA-sequencing data

Whole single-cell RNA sequencing (scRNA-Seq) data of microdissected ventricular-subventricular zone (V-SVZ) on the lateral wall of the lateral ventricle of male (postnatal day 35) and female (postnatal day 29) hGFAP-GFP mice were obtained from NCBI GEO Data (GSE165554). We used the data from the male and female mixed sample (GSM5039270) (Cebrian-Silla et al., 2021). The Seurat object (.rds file) was loaded into R (version 4.5.1) (R Core Team, 2025) and analyzed by Seurat (version 5.3.0) (Hao et al., 2023). DimPlot and FeaturePlot implemented in Seurat (version 5.3.0) (Hao et al., 2023) were used to generate the UMAP of clusters of V-SVZ cell types and the expression patterns of selected genes in Figures 2D and 4A.

Neurosphere cultures

The neurosphere cultures were prepared as described previously (Azari et al., 2010; Ouzikov et al., 2024). Ventricular-subventricular zone (V-SVZ) on the lateral wall of the lateral ventricle was microdissected from the brains of 8-week-old male C57BL/6 mice. To get enough cells for the culture in the experiment, the tissue from the V-SVZ of two mouse brains was pooled together as one biological replicate (i.e., the neurospheres in one biological replicate came from the combined tissue collected from two mice). Neurospheres were grown in NeuroCult Basal Medium (Mouse & Rat) (STEMCELL Technologies, Cat#05700) containing 10% NeuroCult Proliferation Supplement (Mouse & Rat) (STEMCELL Technologies, Cat#05701), 10ng/mL bFGF (Corning, Cat#354060), 20ng/mL EGF (STEMCELL Technologies, Cat#78006.1), 1μL/mL of 0.2% heparin (Sigma-Aldrich, Cat#H4784) and 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco, Cat#15140122). Following 7–10 days of growth of the primary spheres, spheres were disassociated and seeded for cell proliferation and Boyden chamber assays.



Cell proliferation assay

The tissue from the V-SVZ of two mouse brains was pooled together as one biological replicate (defined as one *N*). Two independent batches of cells that had seven biological replicates (*N* = 7, i.e., from 14 mice) in total were analyzed, where the first batch of cells had four biological replicates (*N* = 4, i.e., 8 mice) and the second batch of cells had three biological replicates (*N* = 3, i.e., 6 mice). The secondary spheres were seeded into each well of a 96-well plate and cultured with 2 µg/mL galectin-9 (R&D Systems, Cat#3535-GA), 200 ng/mL of CCL5 (PeproTech, Cat#250-07), CXCL5 (BioLegend, Cat#573302), or CXCL10 (PeproTech, Cat#250-16), along with control (adding corresponding solvents) for 5 days. Each biological replicate was seeded with technical triplicates in 96-well plates (i.e., cells from one biological replicate were distributed into three wells as technical triplicates). Cell proliferation was evaluated indirectly using the resazurin reduction assay and quantified by the fluorescence intensity of resorufin (Larson et al., 1997) using resazurin (Millipore Sigma, Cat#R7017, 44 µM final). The measurements were taken in 40 min after adding resazurin. For the quantification of neurosphere diameter and count for galectin-9, three independent batches of cells that had ten biological replicates (*N* = 10, i.e., 20 mice) in total were analyzed, where the first batch of cells had four biological replicates (*N* = 4, i.e., 8 mice), the second batch of cells had two biological replicates (*N* = 2, 4 mice), and the third batch of cells had four biological replicates (*N* = 4, i.e., 8 mice).

Boyden chamber assay

Cell migration was evaluated by Boyden chamber assay as described previously (Widera et al., 2004). One Millicell cell culture insert with a pore size of 8 µm (Millipore, Cat#PI8P01250) (as the upper chamber) was put into a well of a 24-well cell culture plate (as the lower chamber) to form a Boyden chamber. Both chambers were coated with 40 µg/mL poly-D-lysine hydrobromide (Sigma-Aldrich, Cat#P6407-5MG) and 4 µg/mL laminin (Corning, Cat#354232) dissolved in sterile Milli-Q water. 600 µL coating solution was added to the lower chamber and 400 µL coating solution was added to the upper chamber and then incubated overnight at room temperature. 600 µL of 2 µg/mL galectin-9 (R&D Systems, Cat#3535-GA), 200 ng/mL of CCL5 (PeproTech, Cat#250-07), CXCL5 (BioLegend, Cat#573302), or CXCL10 (PeproTech, Cat#250-16) dissolved in growth factor-free NeuroCult Basal Medium (Mouse & Rat) (STEMCELL Technologies, Cat#05700) with 10% NeuroCult Proliferation Supplement (Mouse & Rat) (STEMCELL Technologies, Cat#05701) were placed in the lower chamber, along with controls (media only). 10,000 cells disassociated from the secondary spheres suspended in 400 µL of growth factor-free NeuroCult Basal Medium (Mouse & Rat) (STEMCELL Technologies, Cat#05700) with 10% NeuroCult Proliferation Supplement (Mouse & Rat) (STEMCELL Technologies, Cat#05701) were seeded in the upper chamber. The cells dissociated from the secondary spheres cultured from the combination of V-SVZ tissue of two mouse brains were used as one biological replicate (defined as one *N*) for each group in one batch of experiments. Two independent batches of cells that had seven biological replicates (*N* = 7, i.e., from 14 mice) in total were analyzed, where the first batch of cells had four biological replicates (*N* = 4, i.e., 8 mice) and the second batch of cells had three biological replicates (*N* = 3, i.e., 6 mice). Each biological replicate was seeded with two technical replicates in the Boyden chamber (i.e., the cells of one biological replicate were derived from tissues collected from two mice combined and then were seeded into two chambers as two technical replicates for one biological replicate). The chambers were incubated for 6 h at 37°C in a humidified incubator with 5% CO₂. After the incubation period, the filter was removed, and the upper side of the insert filter, containing non-migrating cells, was removed with a cotton swab, and washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) three times. The insert filter was then fixed with 4% paraformaldehyde (PFA) in PBS for 20 min, washed with PBS, and stained with Hoechst 33258 (Sigma-Aldrich, Cat#B2883) for 10 min protected from light. The membrane of each insert was cut off by a razor blade. The membrane was placed into one drop of PermaFluor Aqueous Mounting Medium (Epredia, Cat#TA-030-FM) on the uncoated glass slides (Fisherbrand Superfrost Plus Microscope Slides, Fisher Scientific, Cat#1255015), with the side that was facing the migration cues upwards, covered by coverslip, and dried at room temperature in the dark. Cell migration was quantified by counting the migrated cells on each insert filter as described in the “image quantification” section below.

Intracerebroventricular (ICV) injection

Recombinant *Escherichia coli*-derived mouse galectin-9 protein (R&D Systems, Cat#3535-GA) was dissolved in sterile PBS and desalted by Amicon Ultra Centrifugal Filter, 30 kDa MWCO (MilliporeSigma, Cat#UFC503024) at 14,000g. The PBS used for injection into the control mouse brains was centrifuged in parallel with the galectin-9 solution in the same type of filter. The concentration of galectin-9 solution for injection was measured by DC Protein Assay Kit II (Bio-Rad, Cat#5000112). Prior to stereotaxic surgery, 8-week-old male C57BL/6J mice received a subcutaneous dose of



1.2 mg/kg slow-release buprenorphine (Chiron Compounding Pharmacy Inc.) for analgesia. Animals were anesthetized with 2–3% isoflurane and received 1 mL of warmed sterile saline subcutaneously in the flank and 8 mg/kg bupivacaine-HCl (Center de distribution de médicaments vétérinaires, Cat#ORK008504) subcutaneously in the skin overlying the skull prior to being secured in the stereotaxic frame. After removing fur from the scalp and disinfecting the surgical site, a midline incision was made using a #10 scalpel blade and the skin was retracted to expose the skull. A dental drill was used to create a burr hole at 0.5 mm anterior and 1.0 mm lateral to bregma. 3 μ L of galectin-9 (0.1 mg/mL in PBS) was injected into the right lateral ventricle at a depth of 2.3 mm from the skull surface at a rate of 0.1 μ L/min using a 10 μ L Hamilton syringe (10 μ L Microliter #701 Syringe, Hamilton Company, Cat#80330) fitted with a 29G needle (29 Gauge Small Hub Removable Needle Hamilton Company, Cat#7803-06). The control mice received PBS injections. Mouse brains were collected as described in the “[brain tissue preparation and sectioning](#)” section in 1- and 5-day after injection for the first cohort and 1-day post-injection for the second cohort.

Brain tissue preparation and sectioning

Sections were prepared as described previously ([Ouzikov et al., 2024](#)). Mice were anesthetized with 2–3% isoflurane inhalation and then perfused transcardially with PBS followed by 4% PFA. Mouse brains were dissected out, post-fixed for 24–48 h in 4% PFA and then cryoprotected in 30% sucrose in PBS until the brains sank to the bottom of the Falcon tube. Tissues were then embedded with Optimal Cutting Temperature (O.C.T.) compound (Fisher Healthcare Tissue-Plus O.C.T. Compound, Fisher Scientific, Cat#23730571). Brain sections were cut 18- μ m thick in the coronal plane using a Thermo Fisher Scientific Eprelia HM525 NX cryostat (Fisher Scientific, Cat#956640EC70) at -23°C . Sections were collected on glass slides (Fisherbrand Superfrost Plus Microscope Slides, Fisher Scientific, Cat#1255015) coated with gelatin and stored frozen until use.

Antibodies

The primary antibodies used for immunohistochemistry (IHC) in this study included: goat anti-mouse galectin-9 (R&D Systems, Cat#AF3535, RRID: AB_2137240) reconstituted at 0.2 mg/mL in sterile PBS and diluted at 1:30; rabbit anti-doublecortin (DCX) (Cell Signaling Technology, Cat#4604, RRID: AB_561007) at 1:500 dilution; rabbit anti-Iba1/AIF-1 (Cell Signaling Technology, Cat#17198, RRID: AB_2820254) at 1:100 dilution; mouse anti-FOXJ1 (eBioscience, Cat#14-9965-80, RRID: AB_1548836) at 1:200 dilution; biotin anti-mouse CD366 (Tim-3) (BioLegend, Cat#119720, RRID: AB_2571936) at 1:150 dilution; rabbit anti-TLR4 (Proteintech, Cat#19811-1-AP, RRID: AB_10638446) at 1:200 dilution; rabbit anti-SOX2 (Cell Signaling Technology, Cat#3728S, RRID: AB_2194037) at 1:250 dilution; goat anti-SOX2 (R&D Systems, Cat#AF2018, RRID: AB_355110) reconstituted at 1 mg/mL in sterile PBS and diluted at 1:100; mouse anti-GFAP (Cell Signaling Technology, Cat#3670S, RRID: AB_561049) at 1:500 dilution; chicken anti-GFAP (MilliporeSigma, Sigma-Aldrich, Cat#AB5541, RRID: AB_177521) at 1:500 dilution; mouse anti-Ki-67 (BD Biosciences, Cat#550609, RRID: AB_393778) at 1:100 dilution.

The secondary antibodies used for IHC in this study included: Alexa Fluor 488-conjugated donkey anti-chicken secondary antibody (Jackson ImmunoResearch Laboratories, Cat#703-545-155, RRID: AB_2340375) at 1:500 dilution; Alexa Fluor 647-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Cat#711-605-152, RRID: AB_2492288) at 1:500 dilution; Cy3-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Cat#715-165-150, RRID: AB_2340813) at 1:500 dilution; Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, Cat#705-545-147, RRID: AB_2336933) at 1:500 dilution; Cy3 Streptavidin (Jackson ImmunoResearch Laboratories, Cat#016-160-084, RRID: AB_2337244) at 1:1000 dilution.

Immunohistochemistry (IHC)

Frozen tissue sections were dried for 30 min at 37°C and then rehydrated. Sections were then washed three times with washing buffer (0.1% Triton X-100 in PBS) for 5 min each time. Subsequently, sections were blocked and permeabilized in the blocking solution of 3% donkey serum (Jackson ImmunoResearch Laboratories, Cat#017-000-121, RRID: AB_2337258, reconstituted in Milli-Q water at 60.0 mg/mL) and 0.3% Triton X-100 in PBS for 1 h. Then sections were incubated in primary antibodies listed in the “[antibodies](#)” section diluted in the blocking solution overnight at 4°C in a humidified chamber. On the following day, sections were washed three times in washing buffer for 5 min per wash, then incubated in corresponding secondary antibodies listed in the “[antibodies](#)” section diluted in PBS for 2 h at room temperature in the dark. Afterward, sections were washed three times in the washing buffer for 5 min per wash. Finally, sections were counterstained for 5 min at room temperature in 0.5 μ g/mL Hoechst 33258 (Sigma-Aldrich, Cat#B2883),



washed with PBS for 5 min, and then mounted with PermaFluor Aqueous Mounting Medium (Eprelia, Cat#TA-030-FM) and dried overnight at room temperature.

For the immunostaining of neurospheres, neurospheres were washed with PBS and then fixed with 4% paraformaldehyde (PFA) in PBS for 20 min. Then neurospheres were washed with washing buffer (0.1% Triton X-100 in PBS) for 5 min and collected by centrifugation at 110g for 5 min. Neurospheres were resuspended and incubated in primary antibodies listed in the “[antibodies](#)” section diluted in the blocking solution overnight at 4°C. On the following day, neurospheres were collected by centrifugation at 110g for 5 min, washed once in washing buffer for 5 min, collected by centrifugation at 110g for 5 min, and then resuspended and incubated in corresponding secondary antibodies listed in the “[antibodies](#)” section diluted in PBS for 2 h at room temperature in the dark. Afterward, neurospheres were collected by centrifugation at 110g for 5 min, washed once in the washing buffer for 5 min. Finally, neurospheres were counterstained for 5 min at room temperature in 0.5 µg/mL Hoechst 33258 (Sigma-Aldrich, Cat#B2883), washed with PBS for 5 min, and then mounted with PermaFluor Aqueous Mounting Medium (Eprelia, Cat#TA-030-FM) and dried overnight at room temperature.

Image acquisition

The bright field images of neurosphere were obtained using a Zeiss Apotome 2 live cell system with AxioCam 506 monochrome camera and 10× objective at the University of Toronto Temerty Faculty of Medicine Microscope Imaging Laboratory. Each entire well of the 96-well plate was imaged for subsequent quantification. Immunofluorescence images were collected using a Zeiss Axio Observer 7 equipped with LSM 800 scan head under 40× objective at the Collaborative Advanced Microscopy Laboratories of Dentistry (CAMiLoD) at the University of Toronto Faculty of Dentistry. Images were acquired using Z-stacks at 1 µm interval in ZEN Microscopy Software (version 2.6, ZEISS). All images were acquired with Z-stack sizes ranging between 10 and 22 slices. Images shown were produced using the orthogonal projection feature implemented in ZEN Microscopy Software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image quantification

Image quantifications were all done using Fiji (ImageJ2, version 2.16.0/1.54p) ([Schindelin et al., 2012](#)).

The quantification of the percentage of galectin-9 staining area ([Figure 2C](#)) was done by automatic particle counting in Fiji. The ROI of the ipsilateral stroke infarct regions and the corresponding contralateral regions were manually selected. All images were processed by an identical process written in a Macros implemented in Fiji of steps as follows. The image was converted to 8-bit. The thresholds were set to (25, 255). The percentage area was counted by “Analyze Particles” function implemented in Fiji. To obtain a percentage area value for each individual brain, five anatomically matched 18-µm thin coronal sections containing the ipsilateral and contralateral region were analyzed, totaled and averaged.

The diameter of the neurospheres ([Figure 3C](#)) was quantified by “Measure” function implemented in Fiji and then converted to µm according to the scale bar obtained in ZEN Microscopy Software. The number of neurospheres ([Figure 3D](#)) was quantified by manual counting for each well of a 96-well plate. Neurospheres cultured from the combined tissue from the V-SVZ of two mouse brains were pooled together as one biological replicate (defined as one *N*, i.e., one data point in the figure). For the quantification of neurosphere diameter and count for galectin-9, three independent batches of cells that had ten biological replicates (*N* = 10, i.e., 20 mice) in total were analyzed, where the first batch of cells had four biological replicates (*N* = 4, i.e., 8 mice), the second batch of cells had two biological replicates (*N* = 2, i.e., 4 mice), and the third batch of cells had four biological replicates (*N* = 4, 8 mice). Technical triplicates were quantified for each biological replicate as described in the “[cell proliferation assay](#)” section. For the quantification of percentage of proliferating (Ki-67+) ([Figure 3E](#)) and apoptotic (CC3+) ([Figure 3F](#)) cells in neurospheres, neurospheres cultured from the combined tissue from the V-SVZ of two mouse brains were pooled together as one biological replicate (defined as one *N*, i.e., one data point in the figure). Neurospheres of four biological replicates (*N* = 4, i.e., 8 mice) in total were analyzed. 10–12 neurospheres were imaged for each biological replicate and the Ki-67+, CC3+ cells, and nuclei were manually counted, respectively.

For quantification of the images from the Boyden chamber assay ([Figure S1](#)), the nuclei staining images of the insert filter were obtained under 10× objective. A region of interest (ROI) with an identical size of 3360 × 3468 pixels covering most part in the center of the image was selected for all images in Fiji. The number of pixels in the ROI were calculated and converted to mm² according to the scale bar obtained in ZEN Microscopy Software. All migrated cells represented by the stained nuclei inside the ROI were counted. The migrated cells were presented by dividing the numbers of cells in the ROI of the insert filter on the side facing the migration cues by the area of the ROI. The cells dissociated from the secondary



spheres cultured from the combination of V-SVZ tissue of two mouse brains were used as one biological replicate (defined as one N) for each group in one cohort of experiment. Two independent batches of cells that had seven biological replicates ($N = 7$, i.e., 14 mice) in total were analyzed, where the first batch of cells had four biological replicates ($N = 4$, i.e., 8 mice) and the second batch of cells had three biological replicates ($N = 3$, i.e., 6 mice). Each biological replicate was seeded with two technical replicates in the Boyden chamber.

For quantification of the percentage of proliferating NSCs (Figures 5B and 6D) and doublecortin positive (DCX+) cells (Figure 6E) in the V-SVZ, only visibly immunostained cells along the lateral wall (LW), closest to the ventricle (periventricular area) were counted. The anatomically matched sections were quantified for the groups of control and treatment. To obtain a cell count value for each individual brain (N represented one animal), the positive cells counted from five anatomically matched 18- μm thin coronal sections containing the V-SVZ LW region were totaled and averaged. The percentage of proliferating (Ki-67+) NSCs was represented by dividing the number of Ki-67+SOX2+GFAP+ cells by the number of SOX2+GFAP+ cells. The percentage of doublecortin positive (DCX+) cells was represented by dividing the number of DCX+ cells by the number of nuclei.

For quantification of the density of the NSCs in the V-SVZ (Figure 6F), the V-SVZ at the LW was manually selected in Fiji. The number of pixels in the selected area were quantified by Fiji and converted to μm^2 according to the scale bar obtained in ZEN Microscopy Software. Cells inside the selected V-SVZ area were counted. SOX2+GFAP+ cells represented NSCs. Then the density of NSCs in the V-SVZ was calculated by dividing the number of NSCs by the area of the selected V-SVZ region. To obtain a density of cell value for each individual brain (N represented one animal), the double positive cells counted from three anatomically matched 18- μm thin coronal sections containing the V-SVZ LW region were totaled and averaged.

The quantification of the density of the puncta of TIM-3 staining (Figure 6G) was done by automatic particle counting in Fiji. A ROI with a size of 1500×2000 pixels covering most part of the image field obtained by a Zeiss Axio Observer 7 equipped with LSM 800 scan head under $40\times$ objective was selected for analysis for all images of anatomically matched 18- μm thin coronal sections containing the V-SVZ LW region. The number of pixels in the ROI were calculated and converted to μm^2 according to the scale bar obtained in ZEN Microscopy Software. All images were processed by an identical process written in a Macros implemented in Fiji of steps as follows. The image was converted to 8-bit. The thresholds were set to (140, 255). The number of puncta was counted by “Analyze Particles” function implemented in Fiji. The density of the puncta of TIM-3 staining was calculated by dividing the number of puncta by the area of the ROI. To obtain a density of puncta value for each individual brain (N represented one animal), three anatomically matched 18- μm thin coronal sections containing the V-SVZ LW region were analyzed, totaled and averaged.

Statistics

All statistical analyses and graphical representations were done in GraphPad Prism software (version 10.5.0 for macOS). For Figures 3B, a Friedman test followed by Dunn’s multiple comparisons test were conducted. For Figures 2C, 3C, and 3D a paired t test was used. For Figures 3E, 3F, 5B, and 6D–6G, the unpaired t test was used. For Figure S1, a Friedman test was conducted.