

## Original Article

# Glial Scar Stiffness Affects NSCs Differentiated Direction via Piezo1 Post-Stroke

Shengju Wu<sup>1#</sup>, Yuanyue Song<sup>2#</sup>, Yangqianbo Yao<sup>1</sup>, Ze Liu<sup>1</sup>, Yaohui Tang<sup>1</sup>, Wanlu Li<sup>1</sup>, Guo-yuan Yang<sup>1</sup>, Zhijun Zhang<sup>1\*</sup>

<sup>1</sup>Shanghai Jiao Tong Affiliated Sixth People's Hospital, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200030, China. <sup>2</sup>Department of Geriatric Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, 200040, China.

[Received March 26, 2026; Revised July 2, 2026; Accepted July 5, 2026]

**ABSTRACT:** Following ischemic stroke, the progressive formation and mechanical stiffening of the glial scar pose a critical microenvironmental barrier to neural regeneration. However, how this dynamic stiffness influences the fate of neural stem cells (NSCs) remains unclear. To address this, this study aimed to elucidate the mechanotransduction mechanism regulating NSCs differentiation post-stroke. Specifically, thirty male C57BL/6J mice underwent distal middle cerebral artery occlusion to establish a focal cerebral ischemia model. We combined *in vivo* transplantation of NSCs into distal middle cerebral artery occlusion mouse stroke models and *in vitro* culture on polyacrylamide hydrogels mimicking scar stiffness. Furthermore, transcriptomic analysis and functional validation through Piezo1 knockdown were employed. Mechanistic insights were explored by examining calcium influx and the Notch signaling pathway. We found that increased substrate stiffness potently inhibited neuronal differentiation and neurite outgrowth. Importantly, Piezo1 was identified as a key mechanosensitive regulator and its knockdown severely diminished both the neuronal differentiated direction and the NSCs proliferation. Mechanistically, Piezo1-mediated calcium influx modulated Notch signaling, forming a stiffness-sensing axis that coordinates the differentiation gene network. Overall, these findings identify aberrant mechanosensing as an important contributing factor of neurogenesis and highlight Piezo1 as a potential therapeutic target for enhancing neural repair.

**Keywords:** ischemic stroke, glial scar stiffness, Piezo1, neural stem cell, differentiation capacity

## INTRODUCTION

Stroke is characterized by high global rates of mortality and long-term disability [1]. Although intravenous tPA thrombolysis and endovascular thrombectomy are established effective treatments within the 24 h therapeutic window, only approximately 10% of patients are eligible for and benefit from these interventions [2]. Developing effective therapies for the subacute and chronic phases after stroke is needed. Stem cell therapy has emerged as a promising strategy, attracting considerable research attention. Extensive experimental evidence indicates that stem cell transplantation promotes neurological recovery post-stroke, primarily through paracrine-mediated neurogenesis and angiogenesis. However, few evidence supports that functional recovery

is achieved via direct differentiation of transplanted stem cells into mature, integrated neurons. The underlying reason for this phenomenon is not clear.

Neural stem cells are considered promising candidates for post-stroke therapy due to their intrinsic potential for neuronal differentiation [3]. However, following transplantation after stroke, the majority of NSCs differentiate into astrocytes rather than neurons [4]. This observation has prompted us to investigate the key factors that direct NSC lineage fate under these conditions. The microenvironment encompasses not only a variety of biochemical signaling molecules (e.g., inflammatory factors) but also physical properties such as stiffness and surface topography, which profoundly impact stem cell fate decisions and behavioral manifestations [5, 6]. Stem cells perceive these

**\*Correspondence should be addressed to:** Dr. Zhijun Zhang, Med-X Research Institute and School of Biomedical Engineering Shanghai Jiao Tong University, Shanghai 200030, China. Email: [zhangzj@sjtu.edu.cn](mailto:zhangzj@sjtu.edu.cn). <sup>#</sup>These authors contributed equally to this work.

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mechanical cues from the surrounding matrix to regulate their own fate and guide developmental and regenerative processes [7-9]. Studies have shown that variations in substrate stiffness within the 1-10 kPa range can significantly influence the differentiation fate of adult neural stem cells, determining whether they commit to neuronal, astrocytic, or oligodendrocytic lineages [10, 11]. Further *in vitro* experiments indicate that different neural cell types exhibit distinct preferences for substrate stiffness: neurons tend to prefer softer hydrogel surfaces (elastic modulus  $E < 1.0$  kPa), whereas astrocytes show a preference for stiffer substrates ( $E = 9$  kPa) [12, 13]. Overall, neurons, glial cells, and neural stem cells all exhibit differential favor responses to micro-environmental mechanics. Glial scar formation drives a progressive increase in ipsilateral stiffness after 24 h post-stroke [14], implying a potential regulatory role for scar stiffness in neural stem cell lineage choice.

Stem cells perceive external mechanical signals through integrin-mediated cell-matrix adhesion and cell-cell junctions and convert them into biochemical instructions that regulate cell fate [15, 16]. Mechanosensitive ion channels located in the plasma membrane such as Piezo, TRP, and TACAN, can sensitively respond to changes in the physical properties of the extracellular matrix (e.g., increased stiffness) or to mechanical stimuli [17-20]. These signals are subsequently converted into ion fluxes (e.g., calcium influx) or intracellular signaling events, initiating downstream responses [17, 21]. Studies have shown that the mechanosensitive channel Piezo1 highly expressed in human neural stem cells, is regulated by cellular traction forces [22]. Piezo1 can trigger transient calcium signaling following increased substrate stiffness [23]. The calcium signal not only promotes the nuclear translocation of the mechanoresponsive transcriptional coactivator YAP but also directly influences lineage commitment [17]. All this evidence intrigues us to explore whether Piezo1-mediated calcium influx directs NSCs lineage choice during stiffness changes induced by glial scar formation after stroke, and its underlying mechanism.

This study indicated that Piezo1 serves both as a core sensor enabling neural stem cells to perceive microenvironment mechanical changes and as a key regulator of their differentiation fate under the increased stiffness of glial scar after ischemic stroke.

## MATERIALS AND METHODS

### Animal model

The animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, Shanghai, China (permission number:

Bioethics 2023024). A total of 80 (22 - 25 g, 8 weeks) C57BL/6J mice (Vital River Laboratories, Beijing, China) were used. The distal middle cerebral artery occlusion (distal MCAO) model that we have established before [24]. All the animals were housed in microisolator cages under a 12 h light/dark cycle (7:00 a.m. to 7:00 p.m.) with free access to food and water. The housing environment was maintained at 18–22°C and 50%–60% relative humidity.

### Atomic force microscopy

The procedure for determining the Young's modulus of *ex vivo* brain slices using atomic force microscopy (AFM, Fast Scan Bio, Bruker) comprised three main stages: sample preparation, instrument operation, and data processing. AFM probes were first functionalized by attaching silica colloidal microspheres to the tip with a UV-curable adhesive. Immediately after extraction, mouse brains were perfused and rinsed with ice cold PBS, sectioned into 1 cm thick coronal slices using a brain matrix, and maintained on ice. Each slice was affixed to the center of an adhesive-coated culture dish, mounted on a metal disk, and stabilized on the stage by vacuum suction. All procedures were completed within 5 min under ice cold conditions. To facilitate localization of the injury site, small notches were introduced into the adhesive beneath the glial scar region prior to measurement. During AFM testing, the Nanoscope and stage controllers were activated, and contact mode was used with the laser precisely aligned on the cantilever. Sterile PBS was applied to both the probe and the tissue surface to preserve a hydrated environment. Scanning parameters were adjusted in real time to obtain reliable force–distance curves. The acquired force curves were processed using NanoScope Analysis (version 1.80) software. Baseline correction was applied, and Young's modulus values were calculated from the fitted force-indentation curves. Only data yielding a correlation coefficient ( $R^2$ ) approaching 1 were considered valid for subsequent analysis.

### Isolation and culture of primary neural stem cells

The isolation and culture of primary NSCs were performed as follows. Pregnant C57BL/6 mice (Vital River Laboratories, Beijing, CN) at embryonic day 16 (E16) were euthanized, and embryonic brains were carefully harvested. The cerebral cortices were rapidly dissected on ice, rinsed with ice cold PBS, and enzymatically dissociated with 0.25% trypsin (Meilunbio, MA0233-1, CN) at 37 °C for 10 min. Enzymatic digestion was terminated by adding Dulbecco's Modified Eagle Medium (DMEM, HyClone, Logan, UT) containing 10%

fetal bovine serum (FBS, Gibco, 26010074, NM). The tissue was then gently triturated to obtain a single-cell suspension, which was passed through a 40  $\mu\text{m}$  cell strainer (Falcon, USA) and centrifuged. The resulting pellet was resuspended in NSCs proliferation medium supplemented with B27, 20 ng/mL epidermal growth factor (EGF, MCE, HY-P7067, USA) and 20 ng/mL basic fibroblast growth factor (bFGF, MCE, HY-P5321, USA). Cells were seeded and cultured at 37 °C to allow neurosphere formation. Based on neurosphere growth and morphology, passaging was performed using accutase (Sigma, A6964, USA) when necessary to maintain cell viability and proliferation.

### Distal middle cerebral artery occlusion model

The distal middle cerebral artery occlusion (distal MCAO) model was established as follows. The animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, Shanghai, China (permission number: Bioethics 2023024). Mice were anesthetized in an induction chamber with 3%-5% isoflurane until the loss of the righting reflex (approximately 2-3 min), after which anesthesia was maintained at 1.5% isoflurane via a facemask. The animal was positioned with the head secured and the body aligned parallel to the operator. Following hair removal, the scalp was disinfected with 75% ethanol, and a midline incision was made between the left orbit and ear canal to expose the skull. The temporal muscle was gently dissected along the skull margin to visualize the middle cerebral artery (MCA). The skull above the MCA was thinned uniformly using a round burr drill until it could be carefully lifted with fine forceps without damaging the vessel. The MCA was gently elevated with a bent needle tip from a 1 mL syringe and occluded at three points using bipolar coagulation until blood flow ceased and clot formation was visible in the proximal segment, ensuring complete occlusion while minimizing collateral tissue damage. Successful induction of ischemia was confirmed that cerebral blood flow in the left MCA territory was reduced to 10%-20% of that in the contralateral hemisphere. The incision was sutured, the wound disinfected with iodophor, and the animals were allowed to recover on a heating pad before being returned to their home cages.

### NSCs brain cortical transplantation

To examine the differentiation characteristics of NSCs during distinct stages of glial scar formation following ischemic stroke, the primary green fluorescent protein-expressing NSCs (NSC-GFP) was isolated from the cerebral cortex of GFP transgenic neonatal mice. The cells

were cultured *in vitro* to generate stable, free-floating neurospheres, which were passaged once prior to transplantation. Mature neurospheres (100-200  $\mu\text{m}$  in diameter) were dissociated into single-cell suspensions, adjusted to a concentration of  $1 \times 10^5$  cells/ $\mu\text{L}$ , and stereotactically injected into the peri-infarct glial scar region (2  $\mu\text{L}$  per animal; total of  $2 \times 10^5$  cells). Transplantations were performed in mice subjected to distal MCAO at different post-stroke intervals - day 7 and day 14, as well as in a sham control group. During the procedure, mice were anesthetized with isoflurane and positioned in a stereotaxic frame. Following scalp disinfection and skull exposure, a small craniotomy was made at the target coordinates (AP: +1.0 mm; ML: -3.5 mm). A Hamilton microsyringe (33 G) attached to a microinjection pump delivered the cell suspension at a rate of 0.2  $\mu\text{L}/\text{min}$  to the target depth (DV: +1.0 mm). The needle was left in place for 1 min before and 5 min after injection to minimize reflux. After the injection, the incision was sutured, topical antibiotics were applied, and animals were allowed to recover on a heating pad. All mice were sacrificed 14 days post-transplantation for subsequent histological and molecular analyses.

### Preparation of polyacrylamide hydrogels

To mimic the post-stroke mechanical microenvironment of the glial scar *in vitro* and assess its influence on NSCs differentiation, polyacrylamide (PA) hydrogels with tunable stiffness (0.5, 1.0, 10, and 20 kPa) were fabricated by adjusting the acrylamide (Arc, Sangon, 79-06-1, CN) to N, N-methylene-bis-acrylamide (Bis, Sangon, 110-26-9, CN) ratio. The preparation procedure was as follows. Silanized glass coverslips were ultrasonically cleaned in 75% ethanol, rinsed with deionized water, dried, and treated with Gel Slick solution to facilitate hydrogel detachment. A precursor solution containing 40% (w/v) acrylamide, 2% (w/v) bis-acrylamide, 1 mol 4-hydroxyethylpiperazine-1-ethanesulfonic acid (HEPES, Sigma, 7365-45-9, MO) buffer, and deionized water was prepared in defined proportions, and polymerization was initiated by adding 10% (w/v) ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED, 0.004 g/mL, Thermo Fisher Scientific, 17919, USA). A 200  $\mu\text{L}$  aliquot of the precursor mixture was pipetted onto the treated surface and immediately covered with a cooled, silanized coverslip. After polymerization at room temperature for 10 min, the hydrogel was gently separated, placed gel-side up in a culture plate, and equilibrated with 50 mmol HEPES buffer. The hydrogel surface was then functionalized with sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, 0.2 mg/mL, ProteoChem, 102568-43-4, CN) dissolved in HEPES buffer containing DMSO, followed

by UV exposure for 15 min to induce crosslinking. After washing to remove unbound reagent, the activated hydrogels were coated with 0.1 mg/mL collagen I (Corning Incorporated, 354236, USA) solution and sterilized overnight under UV light at 4 °C.

### NSCs in vitro differentiation induction

To investigate how glial scar stiffness regulates the differentiation potential of NSCs, P1-generation NSCs in a uniform physiological state were seeded onto polyacrylamide hydrogels at a density of  $5 \times 10^4$  cells per well. On the first day after seeding, NSCs were cultured in proliferation medium to facilitate cell adhesion and maintain their undifferentiated state. After 24 h, the medium was replaced with differentiation medium (Neurobasal supplemented with 2% B27) to initiate differentiation. The differentiation medium was refreshed every 2-3 days, and the cells were maintained for a total of 14 days. At the end of the culture period, cells were fixed with 4% paraformaldehyde (PFA, sigma, MO) and subjected to immunofluorescence staining to detect the neuronal marker Tuj-1 and the astrocytic marker GFAP, allowing quantitative assessment of the effect of substrate stiffness on NSCs differentiation toward neuronal or astrocytic lineages.

### CCK-8 assay

To assess the effect of hydrogel stiffness on the proliferation of NSCs, a Cell Counting Kit-8 (CCK-8, Meilunbio, MA0218-2, CN) assay was performed. NSCs were seeded onto polyacrylamide hydrogels with different Young's moduli (0.5, 1, 10, and 20 kPa) at three replicates per group and cultured in proliferation medium for 24 h to allow cell adhesion. The medium was then replaced with fresh medium containing 10% CCK-8 reagent, followed by incubation for 2 h in the dark. Absorbance at 450 nm was measured using a microplate reader to determine cell viability. Differences in absorbance among groups were used to evaluate the influence of substrate stiffness on NSCs proliferation.

### BrdU staining to assess NSCs proliferation

To evaluate the proliferative activity of NSCs on polyacrylamide hydrogels with different stiffnesses, BrdU (Beyotime, ST1056, CN) staining was performed. NSCs were seeded onto hydrogels with graded Young's moduli (0.5, 1, 10, and 20 kPa) and cultured until they reached approximately 70% confluence. Cells were then incubated with BrdU at a final concentration of 10  $\mu$ M for 6 h. After labeling, cells were washed with pre-chilled PBS, fixed with 4% PFA, and permeabilized with 0.3%

Triton X-100 (Sigma, St Louis, MO). For DNA denaturation, cells were sequentially treated with 1 mol/L HCl (Sinopharm, 10011018, CN) for 10 min at room temperature, 2 mol/L HCl for 10 min at room temperature, and 2 mol/L HCl for 20 min at 37 °C, with PBS washing between each step. The acidic environment was neutralized using 0.1 mol/L borate buffer (pH 8.0, Sinopharm, 10004818, CN) for 12 min at room temperature, followed by blocking with 10% FBS for 1 h. Cells were then incubated with an anti-BrdU primary antibody (mouse anti mouse, sc-56255, Santa Cruz, BOL) overnight at 4 °C, followed by a fluorescent secondary antibody at room temperature in the dark. Finally, nuclei were counterstained with DAPI, and images were acquired using a confocal microscope. The proportion of BrdU<sup>+</sup>Nestin<sup>+</sup> cells was quantified to assess the effect of substrate stiffness on NSCs proliferation.

### Quantitative analysis of NSCs differentiation

Neurite Length: Tuj-1<sup>+</sup> immunofluorescence images were imported into ImageJ and converted to 8-bit grayscale. Thresholds were adjusted to accurately capture continuous neurite structures, followed by skeletonization. The Analyze Skeleton plugin was used to quantify total branch length and average neurite length per neuron. Branch Number: The NeuronJ plugin was used to manually trace primary neurites (emanating directly from the soma) and their subsequent branches. The numbers of primary and secondary branches per neuron were recorded. Alternatively, Sholl analysis was employed to automatically quantify neurite intersections with concentric circles. Complexity Analysis: Sholl analysis was performed by drawing concentric circles centered on the neuronal soma. The number of intersections between neurites and each circle was counted to generate a Sholl profile. Key parameters extracted included the maximum number of intersections and the critical radius.

### Transcriptomic analysis of NSCs

To identify differentially expressed genes in NSCs cultured on substrates of different stiffnesses, bulk RNA sequencing (Bulk RNA-Seq) was performed. Total RNA was extracted using TRIzol reagent, and RNA integrity was evaluated with an Agilent 2100 Bioanalyzer. Sequencing libraries were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA), and library construction and transcriptome sequencing were carried out by OE Biotech Co., Ltd. (Shanghai, China). For data analysis, three or four independent biological replicates were used for whole-transcriptome analysis. Differential gene expressions were determined using the DESeq (2012) R package, employing the

estimateSizeFactors and nbinomTest functions. Genes with an FDR-adjusted  $p$ -value  $< 0.05$  (Benjamini-Hochberg method) and  $|\log_2(\text{fold change})| > 1$  were considered significantly differentially expressed. Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the clusterProfiler R package. Significantly enriched GO terms and KEGG pathways were identified using a hypergeometric test with an FDR-adjusted  $p$ -value  $< 0.05$ . The enriched GO terms and KEGG pathways were ranked according to enrichment scores, and representative results were visualized graphically.

### PCR detection of mechanosensitive channel genes

NSCs samples were collected, washed with PBS, and lysed in TRIzol reagent (Thermo Fisher Scientific, 15596026CN, USA). The lysates were stored at  $-80^\circ\text{C}$  until further processing. RNA extraction was performed under strict RNase free conditions. After chloroform

(Sinopharm, 10006818, CN) phase separation and centrifugation, the aqueous phase was collected, and total RNA was precipitated with isopropanol (Sinopharm, 80109218, CN), washed with 75% ethanol, and dissolved in RNase-free water. RNA concentration and purity ( $A_{260}/A_{280} \approx 1.8-2.0$ ) were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). High-quality RNA was treated with DNase and reverse-transcribed into cDNA using the Hifair® II SuperMix Plus kit (Yeasen Biotechnology, 11123ES10, CN). For quantitative PCR, gene-specific primers (Table 1) synthesized by Suzhou Hongxun Biotechnology were used with SYBR Green Master Mix (High Rox, Yeasen Biotechnology, 11203ES03, CN) as the detection reagent. Reactions were performed in 384 well plates with three technical replicates per sample. After light-protected loading, centrifugation, and sealing, amplification was conducted using a two-step cycling protocol. Relative expression levels of mechanosensitive genes were calculated using the  $2^{-\Delta\Delta\text{CT}}$  method.

**Table 1.** Primer Sequences.

| Gene   | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|--------|--------------------------|--------------------------|
| Piezo1 | TTCCTGCTGTACCAGTACCT     | AGGTACAGCCACTTGATGAG     |
| Piezo2 | CACCTGGCTACAACTGCTCA     | CCCGATGTCAGGTACAAACA     |
| Trpc4  | GCTGGAGGAGAAGACACTGG     | GACCTGTCGATGTGCTGAGA     |
| Trpc6  | CAGGCCAGATTGATAAGGA      | CCAGCTTTGGCTCTAACGAC     |
| Trpm2  | TGGATCATGAGTGTGCAGGT     | ACAGACAATGCCTGGATCG      |
| Trpm6  | GACGTTTCATAGTGGACCTCTC   | GTTGATCAGCATGTCTCTGTTT   |
| Trpm7  | CCTCATGAAGACCATTTCCTAA   | ACAACGTGAACCTTCCTCACAG   |
| Trpa1  | GCAGGTGGAACCTTCATACCAACT | CACCTTTCGTAAGTACCAGAGTGG |
| Trpv2  | TGATGAAGGCTGTGCTGAAC     | CACCACAGGCTCCTCTTCTC     |
| Trpv4  | ACAACACCCGAGAGAACACC     | TGAACTTGCGAGACAGATGC     |
| Notch1 | GATGGCCCTCAATGGGTACAAG   | TCGTTGTTGTTGATGTCACAGT   |
| Notch2 | ATGCCGGGTTTCAAAGGTGT     | ATGTCGATCTGGCACACTGG     |
| DLL1   | CCCATCCGATTCCTTCG        | GGTTTTCTGTTGCGAGGTCATC   |
| DLL3   | TCGTCATGCTGCCCCCTCC      | TGCTCTCTCCAGGTTTCAATG    |
| DLL4   | CTTTACCGGCTCTAACTGTGAG   | GGCTTGGACCTCTGTTTCAG     |
| JAG2   | TCGTCATTCCCTTTCAGTTTCG   | GTCATTGTCCCAGTCCCAG      |
| Hes5   | GGTCCCGACGCATCTTCT       | GCTCGCTAATCGCCTCCA       |
| GAPDH  | GGTGTGAACCATGAGAAGTATGA  | GAGTCCTCCACGATACCAAAG    |

### Western blot testing

To examine the expression patterns of Piezo proteins in NSCs cultured on hydrogels of varying stiffness, Western blot analysis was performed on samples from the 0.5, 1.0, 10, and 20 kPa groups. Total cellular proteins were extracted and quantified using the BCA assay (MeilunBio, MA0082, CN), followed by denaturation. Equal amounts of protein were separated by SDS-PAGE and transferred onto methanol-activated PVDF membranes. After blocking, membranes were incubated with primary antibodies overnight at  $4^\circ\text{C}$  and

subsequently with HRP-conjugated secondary antibodies for 1 h at room temperature. The antibodies were used as follows: Piezo1 (1:500, Thermo Fisher Scientific, 28511-1-AP), Piezo2 (1:500, Proteintech, 26205-1-AP), GFAP (1:1 000, HUABIO, ET1601-23), Tuj-1 (1:1 000, Proteintech, 66375-1) Notch2 (1:1 000, Wanlebio, WL02409), DLL1 (1:1 000, Wanlebio, WL00968), DLL4 (1:1 000, Wanlebio, WL02214) and  $\beta$ -actin (1:3 000, Proteintech, 66009-1-AP). Protein bands were visualized using an ECL chemiluminescence detection system, and band intensities were quantified by grayscale analysis in ImageJ.

### siRNA silencing of the *Piezo1* in NSCs

For siRNA-mediated gene silencing, specific siRNA sequences targeting the gene of interest were designed and synthesized by OBIO Technology (Shanghai, CN). Two to three effective siRNA sequences were selected to minimize off-target effects, along with a non-targeting negative control. Primary NSCs were seeded in 24-well plates at a density of  $2 \times 10^5$  cells per well and transfected upon reaching 50-70% confluence after two days of stabilization. During transfection, siRNA and Lipofectamine™ 3000 (Thermo Fisher, L3000001, Scientific USA) were separately diluted in Opti-MEM™ reduced-serum medium (Meilunbio, Dalian, China), mixed, and incubated at room temperature for 20 min to form siRNA lipid complexes. The complexes were then added dropwise to the cells. After 4-6 h, the transfection medium was replaced with complete growth medium, and cells were cultured for an additional 24-72 h. Knockdown efficiency was assessed at the mRNA level using qPCR. The siRNA target sequences were as follows (5'-3'): *Piezo1*-1: CACCGGCATCTACGTCAAATA, *Piezo1*-2: ACCAAGAAATACAACCATCTA.

### Calcium imaging assay of NSCs

To examine the role of *Piezo1* channels in calcium influx in primary NSCs, calcium imaging was performed using the Fluo-4 AM fluorescent probe (Thermo Fisher Scientific, F14217, USA). Primary NSCs were seeded in 35 mm confocal dishes and cultured to 50-70% confluence before imaging. Cells were incubated with 1  $\mu$ M Fluo-4 AM for 30 min, washed three times with pre-warmed PBS to remove excess dye, and equilibrated in phenol red-free complete medium for 20 min. Time-lapse imaging was conducted using a confocal microscope with 488 nm excitation, capturing images every 3 s over a 5 min period. Fluorescence intensity was analyzed in regions of interest (ROIs) using ImageJ, and calcium influx was calculated as  $\Delta F/F_0$ , where  $F_0$  represents the baseline fluorescence intensity averaged over the first 10 s and  $\Delta F$  denotes the change in fluorescence. To assess the regulatory function of *Piezo1* channels in calcium signaling, cells were treated with 5  $\mu$ M Yoda1 (agonist) during imaging.

### STRING protein interaction analysis

Based on the list of differential expressed genes obtained from transcriptome sequencing, a protein-protein interaction (PPI) network was constructed using the STRING database (<https://string-db.org/>). The target protein list was uploaded in the “Multiple Proteins” mode, with the species specified. Key parameters were set as

follows: minimum interaction score  $\geq 0.4$  (medium confidence) and “hide disconnected nodes” enabled. The resulting network integrated both physical and functional associations among the target proteins.

### Calcium depletion treatment in NSCs

When neural stem cells (NSCs) reached approximately 80% confluence, calcium depletion was performed. The cells were resuspended in calcium-free medium containing 0.1  $\mu$ M BAPTA-AM (MW = 764.68, C34H40N2O18, purity > 99.68%, MCE, USA. Dissolved in DMSO, with a final DMSO concentration below 0.1%) and incubated at 37 °C in the dark for 48 h to thoroughly chelate intracellular free  $\text{Ca}^{2+}$ . Following incubation, the cells were washed three times with D-PBS to completely remove any uninternalized BAPTA-AM. The treated cells were then ready for subsequent analyses, including cell viability assays, calcium influx imaging, and quantification of mRNA expression levels of relevant genes.

### DAPT treatment

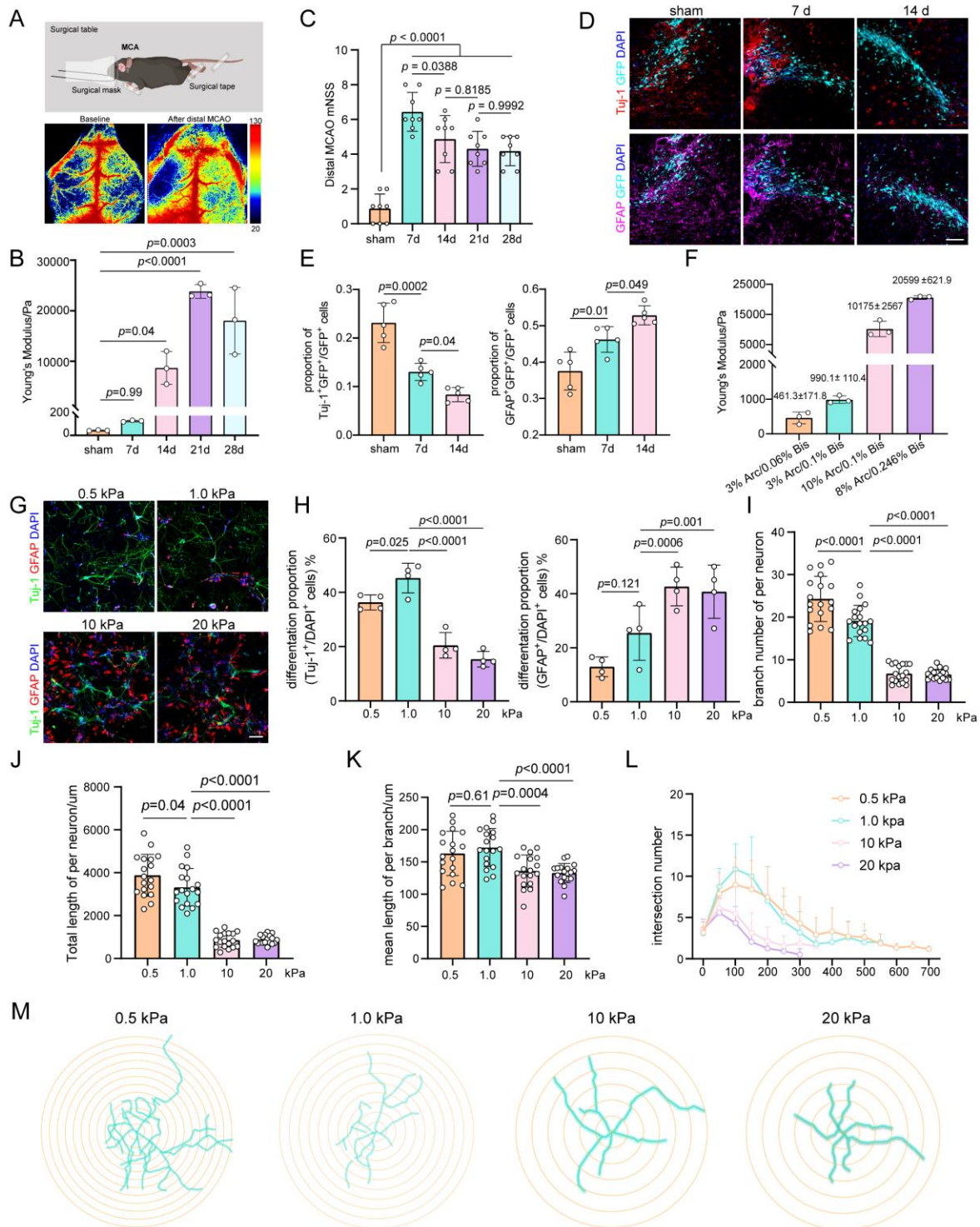
Neural stem cells (NSCs) were cultured in proliferation medium. For Notch inhibition, cells were treated with 1  $\mu$ M DAPT (a  $\gamma$ -secretase inhibitor, HY-13027, MCE, USA) or an equivalent volume of DMSO (vehicle control) for 24 h. Fresh DAPT was added with each medium change. After treatment, cells were harvested for differentiation assays, proliferation analysis, or immunofluorescence staining.

### Statistical Analysis

All experiments were performed with biological replicates as indicated in the figure legends. The number of biological replicates was chosen based on preliminary power analysis to ensure adequate statistical power. Specifically, we used G-Power software with an effect size of (e.g., 0.4), an  $\alpha$  level of 0.05, and a power ( $1-\beta$ ) of 0.8 to determine the sample size. For animal experiments, mice were randomly assigned to treatment groups using a random number generator. For *in vitro* experiments, cell culture plates were randomly allocated to different stiffness groups. Data collection and analysis were performed blinded to group allocation. The outcome measurements (e.g., immunofluorescence staining, neurite length quantification, RNA sequencing) were conducted by an investigator unaware of the experimental conditions. The measurements from multiple cells, slices, or fields derived from the same biological replicate were averaged prior to analysis and not considered as independent replicates. All data in this study are presented

as mean  $\pm$  standard deviation (SD). All data were normally distributed (Shapiro-Wilk test,  $p > 0.05$ ) and showed homogeneity of variances (Brown-Forsythe test,  $p > 0.05$ ). Therefore, an unpaired two-tailed Student's *t*-test was used for comparisons between two groups, and one-way ANOVA followed by Dunnett's or Tukey's post

hoc test was used for multiple group comparisons. Specific *p* values indicating statistical significance are shown in the corresponding figures. All analyses were conducted using GraphPad Prism 9 and Microsoft Excel 2019.



**Figure 1. Reduced neuronal differentiation of NSCs on stiffer glial scar substrates.** (A) Schematic of the distal MCAO model and representative images of mice cerebral blood flow measured by Laser speckle contrast imaging (LSCI) at the baseline and 10 min after distal MCAO. (B) Young's modulus of glial scars measured by atomic force microscopy (AFM) in sham-operated mice and at 7, 14, 21 and 28 days post-stroke (N = 3 mice). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (C) The mNSS (modified Neurological Severity Score) assessments at 7, 14, 21, and 28 days after distal MCAO modeling (N = 8 mice). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (D) Representative immunofluorescence images of graft-derived GFP<sup>+</sup> cells (cyan) co-labeled with the neuronal marker Tuj-1 (red) or the astrocytic marker GFAP (purple) in sham and ischemic glial scars region at 7 and 14 days after NSCs transplantation. White arrows indicate colocalization. Scale bar = 50  $\mu$ m. (E) Quantification of the percentage of GFP<sup>+</sup>Tuj-1<sup>+</sup> and GFP<sup>+</sup>GFAP<sup>+</sup> cells among total GFP<sup>+</sup> grafted NSCs (N = 5 mice per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (F) Young's modulus of polyacrylamide hydrogels fabricated with varying acrylamide (Acr) and bis-acrylamide (Bis) ratios, as measured by AFM. (G) Immunostaining of Tuj-1<sup>+</sup> (green) and GFAP<sup>+</sup> (red) cells after NSCs differentiation on polyacrylamide gels with stiffnesses of 0.5, 1.0, 10, and 20 kPa. Scale bar = 100  $\mu$ m. (H) Quantitative analysis of Tuj-1<sup>+</sup> and GFAP<sup>+</sup> cell proportions following NSCs differentiation on stiffness-variant hydrogels (N = 4 slices per group). One-way ANOVA with Dunnett's test was used for statistical evaluation. (I-L) Morphometric analysis of neuronal complexity after NSCs differentiation on hydrogels of varying stiffness (0.5-20 kPa), including branch number per neuron (I), total neurite length (J), mean branch length (K), and intersection number (L) (N = 18 cells per group). Statistical comparisons were performed using Kruskal-Wallis followed by Dunn's test. (M) Schematic illustrating the method for quantifying neuronal intersection number with radial increment  $\Delta d = 50$   $\mu$ m.

## RESULTS

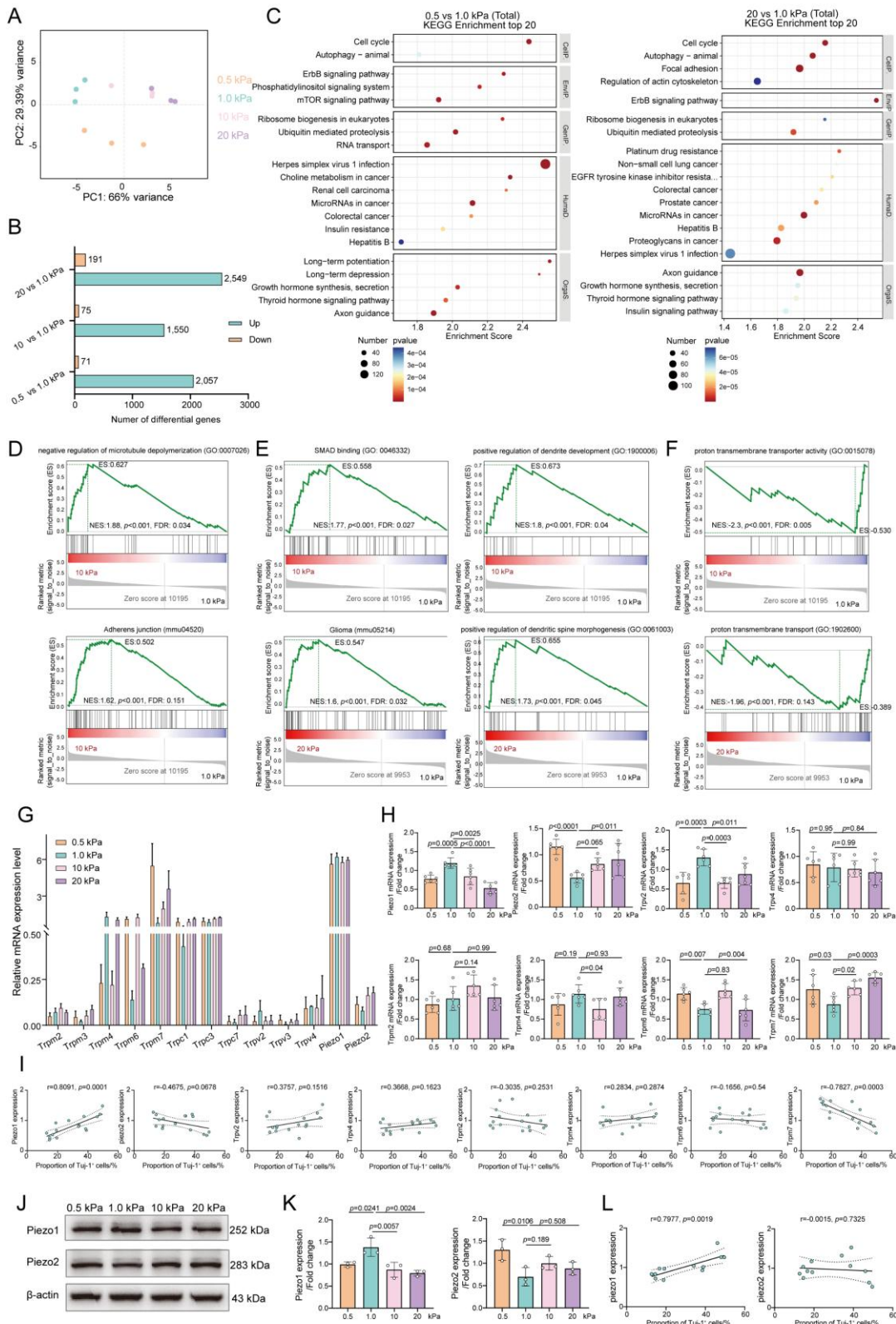
### The increase of glial scar stiffness reduces the neuronal differentiation capacity of neural stem cells

During the progression of ischemic stroke, the physicochemical properties of glial scars undergo dynamic evolution. To precisely characterize the temporal changes in its mechanical stiffness, this study employed atomic force microscopy (AFM) as the reference standard for micro-nanoindentation testing to measure the Young's modulus of the glial scar following distal MCAO (Fig. 1A). Analysis of the absolute Young's modulus demonstrated distinct trends within glial scar regions, comparing the sham group with stroke-affected cohorts at both subacute (7 and 14 days) and recovery (21 and 28 days) stages (Fig. 1B). The Young's modulus showed a pattern of sharp increase from a low baseline to a peak, followed by a slight decrease, which correlated with the progression of glial scar formation. Furthermore, following distal MCAO, the mNSS showed a progressive decline along with glial scar formation, with a significant difference observed only between days 7 and 14 (Fig. 1C). To overcome the limitation posed by the sparse population of endogenous neural stem cells, GFP-NSCs were harvested from GFP-expressing fetal mice. This approach enabled a precise assessment of how glial scar stiffness at distinct time points influences neural differentiation. Following expansion *in vitro* to passage 2, the GFP-NSCs were delivered via stereotaxic injection. The cells were grafted into the sham-operated hemisphere or directly into the glial scar tissue present at 7 and 14 days post ischemic stroke (Fig. 1D). Targeted injections were guided by the following stereotaxic coordinates: AP: +1.0 mm, ML: -3.5 mm, DV: +1.0 mm. The results showed that the differentiated fate of transplanted NSCs correlated with the post-stroke time point. At 14 days

post-stroke, neuronal differentiation (GFP<sup>+</sup>Tuj-1<sup>+</sup>) was significantly suppressed, whereas astroglialogenesis (GFP<sup>+</sup>GFAP<sup>+</sup>) was prominently enhanced (Fig. 1E). However, multiple microenvironmental factors-including inflammation, cytokines, and ECM remodeling-also change over this period, and the *in vivo* data alone cannot attribute the observed lineage shift solely to stiffness.

To directly investigate the role of glial scar stiffness as a biophysical cue directing NSCs differentiation, we engineered polyacrylamide hydrogel substrates with stiffness gradients (0.5, 1, 10, and 20 kPa) to mimic the mechanical evolution of the scar *in vivo* (Fig. 1F). AFM measurements confirmed that collagen I coating did not alter the Young's modulus of polyacrylamide hydrogels (Fig. S1A), and rhodamine-labeled collagen I showed comparable ligand density across different substrate stiffnesses (0.5-20 kPa), ruling out coating-induced mechanical changes or uneven ligand presentation as confounding factors (Fig. S1B-C). Primary NSCs isolated from the fetal mice cortex were seeded onto these substrates and induced to differentiate for 14 days in neurobasal medium. Differentiation outcomes were subsequently quantified by immunofluorescence staining. The lineage choice of NSCs was found to be directed by the changes of substrate stiffness. Softer matrices (0.5 and 1.0 kPa) promoted neuronal differentiation, whereas a pronounced shift toward astrocytic fate occurred on stiffer substrates (10 and 20 kPa) (Fig. 1G-H). Critically, a significant discrepancy in differentiation outcomes was identified specifically between the 1.0 kPa and 10 kPa conditions, indicating the existence of a decisive mechanical threshold that governs NSCs fate commitment. Furthermore, as stiffness increased, we observed a parallel decrease not only in the number of NSCs adopting a neuronal fate but also in subsequent neurite complexity, encompassing branch number, the length of branches and the intersection number (Fig. 1I-M).

These results indicated that substrate stiffness mediated a concerted reduction in both neuronal yield and differentiation.



**Figure 2. Transcriptomics reveals the key role of Piezo1 for mechanical signaling in NSCs differentiation.** (A) Principal component analysis (PCA) of transcriptomes from NSCs cultured on 0.5, 1.0, 10, and 20 kPa substrates. (B) Bar graph displaying the number of differentially expressed genes in 0.5 vs 1.0 kPa, 10 vs 1.0 kPa and 20 vs 1.0 kPa comparisons. (C) Bubble plot of the top 20 enriched KEGG pathways for 10 vs 1.0 kPa and 20 vs 1.0 kPa comparisons (filtered by  $p < 0.05$  and fold change  $> 2$ ). (D–F) Gene set enrichment analysis (GSEA) with the 1.0 kPa condition set as the control. (G) Expression levels of stress-related genes in NSCs RNA-seq under 0.5, 1.0, 10, and 20 kPa culture conditions ( $N = 4$  samples per group). (H) Real-time PCR quantification of NSCs stress-related gene expression across the four levels of substrate stiffnesses ( $N = 6$  samples per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (I) Pearson correlation between the proportion of Tuj-1<sup>+</sup> neurons and the expression levels of stress-related genes under matching substrate stiffness. (J–K) Western blot analysis of Piezo1 and Piezo2 protein levels in NSCs cultured on 0.5–20 kPa substrates ( $N = 3$  samples per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (L) Pearson correlation between neuronal differentiation rate (Tuj-1<sup>+</sup> cells) and Piezo1/Piezo2 protein expression across substrate stiffness conditions.

### Piezo1 identified as a key mechanosensor transducing substrate stiffness to promote neuronal fate

To elucidate the molecular mechanisms of substrate stiffness affecting neuronal differentiation, we conducted transcriptome sequencing of NSCs cultured on hydrogels with stiffness values of 0.5, 1.0, 10, and 20 kPa (Fig. 2A). Using the 1.0 kPa condition as a reference, differential gene expression analysis was performed. Relative to this baseline, the 0.5 kPa, 10 kPa, and 20 kPa groups exhibited 2,057, 1,550, and 2,549 upregulated genes, and 71, 75, and 191 downregulated genes, respectively (Fig. 2B). KEGG pathway enrichment analysis of the differentially expressed genes identified significant enrichment in the 0.5 and 20 kPa groups, relative to the 1.0 kPa control. Key pathways altered included ErbB signaling, focal adhesion, cell cycle, and axon guidance (Fig. 2C). Gene Set Enrichment Analysis (GSEA) further indicated that stiffer substrates (10 and 20 kPa) upregulated gene sets related to the negative regulation of microtubule depolymerization and adherens junction assembly, implicating cytoskeletal stabilization and intercellular adhesion in stiffness-mediated neural polarization (Fig. 2D). Concurrently, pathways for “SMAD binding”, “positive regulation of dendrite development”, “glioma”, and “dendritic spine morphogenesis” were also elevated. These results indicated that high-stiffness conditions activate mechanosensitive programs linked to gliogenic commitment and aberrant neurite remodeling, thereby favoring astrocytic differentiation while restricting the development of functional neuronal circuitry (Figure 2E). Moreover, transcriptomic profiling also showed a marked downregulation of genes involved in proton transmembrane transporter activity within the stiffer (10 and 20 kPa) microenvironments. These data suggested the involvement of mechanosensitive ion channels, including specific proton channels, as key transducers. This transduction mechanism effectively converts the mechanical substrate signal into a biochemical cascade, thereby orchestrating the shift toward astrocytic fate (Fig. 2F).

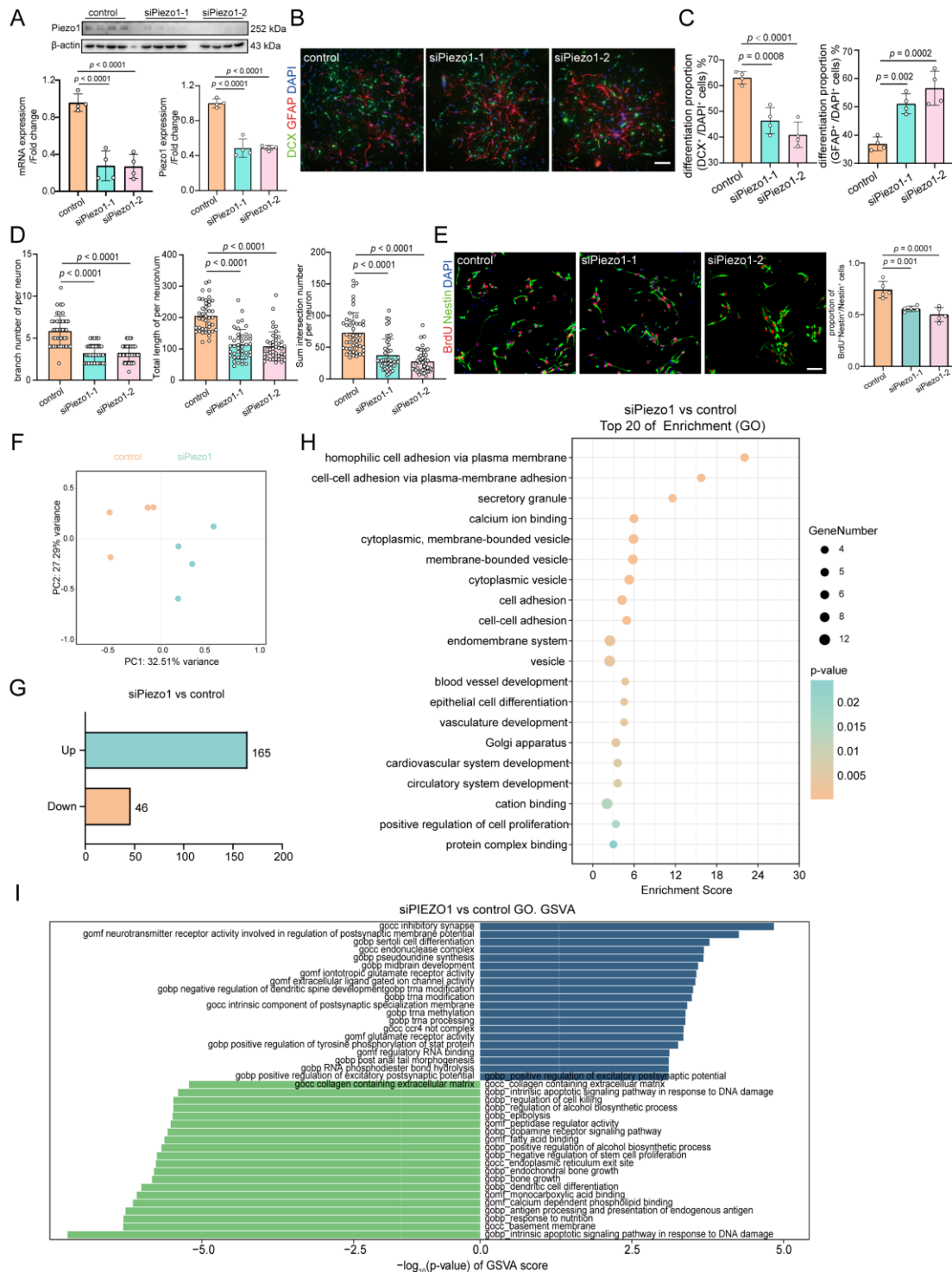
Given the role of mechanosensitive ion channels in substrate stiffness perception, we systematically quantified their gene expression in NSCs. The expression profile related to neuronal differentiation rates (as Tuj-1<sup>+</sup> cells) was subsequently evaluated under matching stiffness conditions (Fig. 2G–I). By assessing the correlation between mechanosensitive channel expression and neuronal differentiation, Piezo1 was identified the most prominent and strong positive correlation with the Tuj-1<sup>+</sup> neuron proportion ( $r = 0.8091$ ,  $p = 0.0001$ ). Conversely, Trpm7 levels correlated negatively ( $r = -0.7827$ ,  $p = 0.0003$ ), whereas Piezo2 exhibited a non-significant inverse trend ( $r = -0.4675$ ,  $p = 0.0678$ ). Simultaneously, we examined the correlation between protein expression levels of Piezo1/Piezo2 and the proportion of Tuj-1<sup>+</sup> neuron differentiation under different stiffness conditions. Piezo1 similarly exhibited a strong positive correlation ( $r = 0.7977$ ,  $p = 0.0019$ ), whereas Piezo2 showed a weaker negative correlation ( $r = -0.0015$ ,  $p = 0.7325$ ) (Fig. 2J–L). These results indicated Piezo1 as a principal mechanosensor in stiffness-dependent neurogenesis.

### Piezo1 is required for stiffness-dependent neurogenesis

To establish the functional contribution of Piezo1 to mechanosensing in NSCs, we performed siRNA-mediated knockdown of Piezo1 at mRNA and protein levels (Fig. 3A). Cell viability assays (CCK-8 and PI staining) confirmed that Piezo1 knockdown did not alter NSC survival (Fig. S2). Following neuronal induction, immunofluorescence analysis revealed that Piezo1 knockdown significantly decreased the proportion of DCX<sup>+</sup> neurons while concomitantly elevating the proportion of GFAP<sup>+</sup> astrocytes compared to control conditions (Fig. 3B–C). Moreover, Piezo1 knockdown impaired neuronal outgrowth and complexity, resulting in significant reductions in branch number, total length, and synaptic density (Fig. 3D). These findings demonstrate that Piezo1 is essential for directing neuronal lineage commitment and supporting subsequent neuronal

morphogenesis and differentiation. Moreover, we also assessed whether Piezo1 regulates NSCs proliferation. Under proliferation-promoting conditions, Piezo1 knockdown significantly reduced the proportion of BrdU<sup>+</sup>Nestin<sup>+</sup> cells, demonstrating its requirement for maintaining the proliferation state (Fig. 3E). Notably,

Piezo1 knockdown markedly suppressed neuronal differentiation and proliferation of NSCs, and these effects were effectively reversed by treatment with the Piezo1 agonist Yoda1, which restored both differentiation and proliferative capacity (Fig. S3).



**Figure 3. Loss of Piezo1 impairs mechanosensitive neurogenesis.** (A) Validation of Piezo1 knockdown efficiency via siRNA interference at mRNA and protein levels (N = 4 samples per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (B-C) Representative immunofluorescence images and quantitative analysis showing the proportion of DCX<sup>+</sup> (green) and GFAP<sup>+</sup> (red) cells following NSCs differentiation under control and siPiezo1 conditions (siPiezo1-1 and siPiezo1-2, N = 4 samples per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (D) Morphometric analysis of total neurite length, branch number, and intersection number per neuron after NSCs differentiation in control and siPiezo1 groups (siPiezo1-1 and siPiezo1-2) (N = 42 cells per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (E) Immunostaining of BrdU<sup>+</sup> (red) and Nestin<sup>+</sup> (green) for NSCs under control and siPiezo1 conditions (scale bar = 100  $\mu$ m), and quantification of the BrdU<sup>+</sup>Nestin<sup>+</sup>/Nestin<sup>+</sup> cell ratio (N = 5 samples per group). Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (F) Principal component analysis (PCA) of transcriptomes from control and siPiezo1-treated NSCs. (G) Bar graph summarizing the number of differentially expressed genes between control and siPiezo1 conditions. (H) GO functional enrichment analysis of the top 30 differentially expressed genes between siPiezo1 and control groups (filter criteria:  $p < 0.05$ , fold change  $> 2$ ). (I) GSVA scores for significant GO terms in siPiezo1 versus control comparisons ( $p < 0.05$ , fold change  $> 2$ ). All data are presented as mean  $\pm$  SD.

To delineate the mechanistic basis of Piezo1 function, we interrogated the transcriptomic consequences of its knockdown (Fig. 3F). Sequencing identified 165 upregulated and 46 downregulated genes in Piezo1-deficient NSCs compared to controls (Fig. 3G). GO enrichment analysis demonstrated significant involvement of these differentially expressed genes in biological processes including cell adhesion, calcium ion binding, positive regulation of cell proliferation, and differentiation (Fig. 3H). Subsequent Gene Set Variation Analysis (GSVA) further revealed that Piezo1 depletion specifically downregulated gene sets related to neuronal differentiation, such as those governing inhibitory synapse function and postsynaptic membrane activity (Fig. 3I). These transcriptomic findings further revealed Piezo1 as a central regulator of gene networks coordinating neurogenesis and synaptic length.

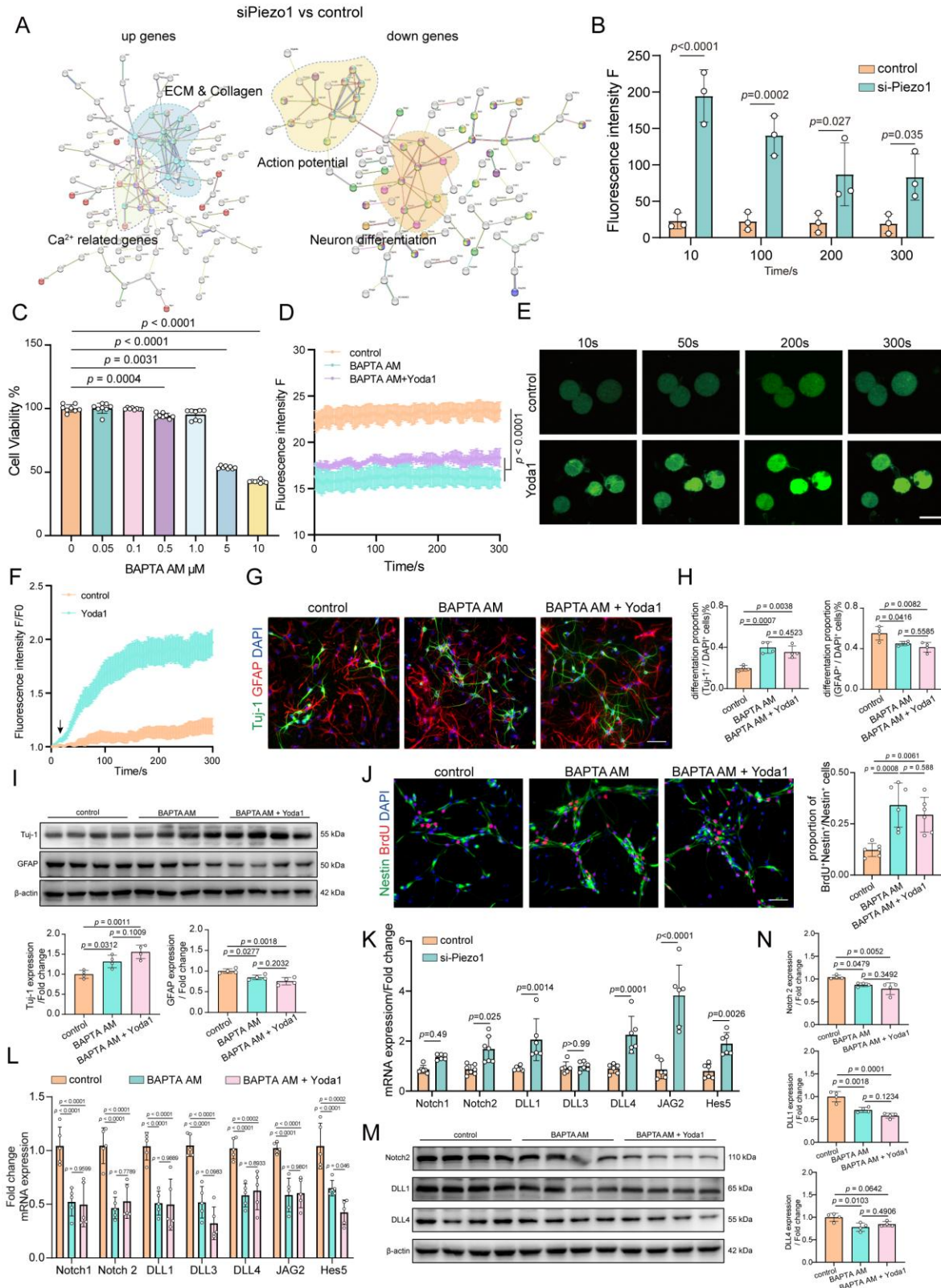
### Piezo1 couples calcium dynamics with Notch signaling to determine NSCs fate specification

To examine the molecular mechanism of Piezo1 regulating NSCs differentiation, we conducted a String-based protein-protein interaction network analysis of transcriptomic data. The results showed that Piezo1 knockdown resulted in upregulation of genes associated with calcium-related processes as well as extracellular matrix (ECM) and collagen organization, whereas downregulated genes were predominantly implicated in action potential propagation and neuronal differentiation (Fig. 4A). Since Piezo1 is a cardinal calcium ion channel, we next to check whether these transcriptional changes were coupled to alterations in calcium dynamics. Unexpectedly, knockdown of Piezo1 led to a significant increase in cytosolic calcium levels, as measured by Fluo-4 AM, rather than the anticipated decrease. This counter-intuitive observation suggests that the net calcium signal reflects complex homeostatic adjustments rather than simply the loss of Piezo1-mediated calcium influx (Fig. 4B). To further elucidate the critical role of calcium

signaling in Piezo1-mediated fate determination of NSCs, we treated the cells with 0.1  $\mu$ M of the calcium chelator BAPTA-AM for 48 h under conditions that did not affect cell viability (Fig. 4C). The results showed that this treatment significantly attenuated calcium influx signals. Moreover, activation of Piezo1 with the agonist Yoda1 failed to reverse this effect (Fig. 4D). However, treatment of neural stem cells alone with the same concentration of Yoda1 does indeed effectively enhance cellular calcium influx signals (Fig. 4E-F). Additionally, effective chelation of intracellular calcium by BAPTA-AM significantly increased the proportion of NSCs differentiating into Tuj-1<sup>+</sup> neurons, while reducing their tendency to differentiate into GFAP<sup>+</sup> astrocytes (Fig. 4G-H). This effect could not be reversed by treatment with the Piezo1 agonist Yoda1. Consistent trends were also observed at the protein level (Fig. 4I). Furthermore, Piezo1 affected the proportion of BrdU<sup>+</sup>Nestin<sup>+</sup>/Nestin<sup>+</sup> neural stem cells by modulating calcium signaling (Fig. 4J). Notably, although BAPTA-AM or Yoda1 treatment did not alter the overall viability of NSCs, it concurrently enhanced proliferation and reduced the dead cell percentage to a certain extent (Fig. S4). Based on these findings, we further evaluated the activity of the canonical Notch signaling pathway-known to influence NSC lineage choice. Piezo1 knockdown significantly elevated the expression of multiple Notch pathway components, including Notch2, DLL1, DLL4, JAG2, and the downstream effector Hes5, implying that Piezo1 normally constrains Notch signaling to preserve neuronal differentiation potential (Fig. 4K). Conversely to Piezo1 knockdown, chelation of calcium signals led to a significant suppression of key Notch pathway components in NSCs, particularly the Notch2, DLL1, and DLL4, evident at both transcriptional and protein levels (Fig. L-N). Mechanistically, Notch inhibition by DAPT (1  $\mu$ M, 24 h) not only enhanced NSC neuronal differentiation and proliferation on its own but also completely rescued the deficits caused by Piezo1 knockdown, demonstrating that Notch activation is

required downstream of Piezo1 to suppress neuronal differentiation and proliferation (Fig. S5). Taken together, our findings suggest that Piezo1 regulates NSC fate

commitment by serving as a key modulator of both calcium signaling and the Notch pathway.



**Figure 4. Piezo1 integrates calcium dynamics and Notch signaling to direct NSCs fate.** (A) Protein interaction network analysis (STRING) of the top 200 differentially expressed genes between control and siPiezo1 groups. (B) Quantification of calcium influx intensity from live-cell imaging in control versus siPiezo1 NSCs (N = 3 samples per group). (C) Bar graph of NSCs viability following treatment with various concentrations of BAPTA-AM, N = 8 samples per group. Data were analyzed by one-way ANOVA with Dunnett's post hoc test. (D) Calcium influx graph in NSCs after treatment in control, 0.1  $\mu$ M BAPTA AM, and 0.1  $\mu$ M BAPTA AM + Yoda1 (5  $\mu$ M) group (N = 3 samples per group). (E) Representative Fluo-4 AM calcium imaging traces of NSCs under different pharmacological conditions: DMSO (control) and Yoda1 (5  $\mu$ M, Piezo1 agonist). Scale bar = 10  $\mu$ m. (F) Quantification data of NSCs under different pharmacological conditions: DMSO (control) and Yoda1 (5  $\mu$ M, Piezo1 agonist). (G) Immunostaining of Tuj-1<sup>+</sup> (green) and GFAP<sup>+</sup> (red) cells after NSCs differentiation at control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups. Scale bar = 50  $\mu$ m. (H) Quantitative analysis of Tuj-1<sup>+</sup> and GFAP<sup>+</sup> cell proportions following NSCs differentiation at control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups, N = 4 samples per group. One-way ANOVA with Tukey's HSD test was used for statistical evaluation. (I) Western blot analysis of Tuj-1 and GFAP protein levels in NSCs cultured at control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups, N = 4 samples per group. Data were analyzed by one-way ANOVA with Tukey's HSD post hoc test. (J) Immunostaining of BrdU<sup>+</sup> (red) and Nestin<sup>+</sup> (green) for NSCs under control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups (scale bar = 50  $\mu$ m), and quantification of the BrdU<sup>+</sup>Nestin<sup>+</sup>/Nestin<sup>+</sup> cell ratio (N = 6 samples per group). Data were analyzed by one-way ANOVA with Tukey's HSD post hoc test. (K) Expression levels of Notch signaling pathway components in control and siPiezo1 NSCs groups (N = 6 samples per group; two-way ANOVA with Dunnett's test). (L) Expression levels of Notch signaling pathway components in control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups, N = 4 samples per group. Data were analyzed by two-way ANOVA with Tukey's HSD post hoc test. (M-N) Western blot analysis of Notch signal pathway proteins levels in NSCs cultured at control, BAPTA AM (0.1  $\mu$ M) and BAPTA AM (0.1  $\mu$ M) + Yoda1 (5  $\mu$ M) groups, N = 4 samples per group. All data are presented as mean  $\pm$  SD, and one-way ANOVA with Tukey's HSD test was used for statistical evaluation.

## DISCUSSION

Our study first transplanted NSCs into post-stroke glial scar regions with varying stiffness and demonstrated that the increased scar stiffness during disease process impedes the neuronal differentiation of exogenous NSCs while promoting their differentiation toward astrocytes. Subsequently, we employed polyacrylamide hydrogels to simulate the stiffness variation of glial scars and seeded primarily NSCs on the gels for induced differentiation. The results indicated that a softer substrate not only enhanced the proportion of NSCs differentiating into neurons but also increased the branching complexity of the differentiated neurons. Further transcriptomic analysis revealed that glial scar stiffness regulates NSC differentiation through the mechanosensitive ion channel Piezo1. It was validated that loss of Piezo1 significantly reduced both the neuronal differentiation and proliferative capacity of NSCs, together with a marked decrease in differentiation complexity, underscoring the pivotal role of Piezo1. Given the substantial size of the Piezo1 gene coding sequence, which presents technical challenges for constructing stable overexpression plasmids, this study employed RNA interference technology to achieve its knockdown, thereby effectively validating its function. Finally, we also found that Piezo1 may modulate the classical differentiation pathway Notch signaling via calcium homeostasis to mediate its function in regulating NSC differentiation.

Although transplanting NSCs into the post-stroke glial scar region demonstrate the significant impact of substrate stiffness on their differentiation potential, we

cannot exclude the potential confounding effects of differences in cytokine profiles and inflammation-related signaling pathways in the microenvironment at 7 and 14 days after stroke. During the acute stage following stroke, the release of IL-1 $\alpha$  [25], IL-1 $\beta$  [26], and TNF- $\alpha$  from activated astrocytes and microglia [27], coupled with the upregulated expression of ICAM-1 and VCAM-1 adhesion molecules on damaged endothelial cells, acts to inhibit the process of neurogenesis after injury [28]. And peripheral immune cells (neutrophils, monocytes, macrophages, and lymphocytes) infiltrate the brain parenchyma and contribute to secondary injury by releasing substantial amounts of reactive oxygen species (ROS) and matrix metalloproteinases (MMPs). The secreted inflammatory factors can exacerbate BBB disruption, thereby compromising the migration and survival of transplanted neural stem cells [29, 30]. As the condition progresses into the subacute and recovery phases, the inflammatory response in the peri-injured area gradually subsides, and reparative programs are initiated. During this period, the levels of pro-inflammatory cytokines decline, whereas anti-inflammatory mediators such as IL-10 and TGF- $\beta$  increase [31, 32]. This shift in the microenvironment promotes neuronal differentiation while suppressing the proliferation of NSCs [33]. Together, these findings indicate that the state of the inflammatory response at different time points after stroke can also influence the differentiation potential of exogenous NSCs. Therefore, in this study, we employed polyacrylamide hydrogels to simulate the stiffness variations of glial scars to avoid other influence factor in vivo, allowing a more direct investigation of the effect of

stiffness on NSC lineage differentiation in an *in vitro* system.

The elasticity and stiffness of the extracellular matrix critically modulate the proliferation and differentiation capacities of NSCs [7, 34]. Although integrin mediated mechanosensing process involved in NSC differentiation direction [34-36], our results supported NSCs also respond to mechanical changes via the mechanosensitive ion channel Piezo1 on the membrane. Activation of Piezo1 in astrocytes triggers the release of  $\text{Ca}^{2+}$  and ATP, which has been shown to modulate neurogenesis in the adult hippocampus [7, 14]. In NSCs, activation of Piezo1 triggers  $\text{Ca}^{2+}$  influx, which subsequently initiates downstream signaling cascades such as the BMP2/SMAD and YAP/TAZ pathways, reported to promote a neuronal phenotype [18]. Moreover, depletion of Piezo1 significantly downregulates key genes in the cholesterol biosynthesis pathway and impairs the normal proliferative and differentiation capacities of NSCs [37]. Contrary to expectation, Piezo1 knockdown elevated cytosolic  $\text{Ca}^{2+}$ , indicating compensatory or indirect effects on  $\text{Ca}^{2+}$  homeostasis rather than a simple loss of Piezo1-mediated influx. Thus, we propose that Piezo1 regulates Notch signaling by modulating global  $\text{Ca}^{2+}$  homeostasis, including but not limited to direct  $\text{Ca}^{2+}$  entry. Lineage selection between neuronal and astrocytic differentiation is classically regulated by the Notch signaling pathway [38]. Notably, the extracellular domains of Notch receptors and their ligands (JAG, DLL) contain calcium-binding EGF-like repeats, enabling them to sense fluctuations in extracellular  $\text{Ca}^{2+}$  concentration. This sensing modulates ligand–receptor interactions and ultimately influences lineage commitment in NSCs [39, 40]. This evidence supported our study that a plausible mechanistic link whereby Piezo1-mediated  $\text{Ca}^{2+}$  influx may affect NSC differentiation by modulating Notch signaling activity. However, the specific molecular details of this crosstalk require further experimental investigation.

Several limitations of this study should be acknowledged. First, although the polyacrylamide hydrogel system allowed precise control of substrate stiffness, it represents a two-dimensional (2D) culture environment that does not fully recapitulate the complex three-dimensional (3D) architecture and biochemical composition of the glial scar *in vivo*. Therefore, future studies employing 3D scaffolds or *ex vivo* tissue models are needed to confirm the translational relevance of our findings. Second, the involvement of Piezo1 in regulating NSC fate is primarily supported by our *in vitro* experiments; the *in vivo* data presented remain largely correlative. Moreover, embryonic NSCs may respond differently to stiffness and Notch cues than adult NSCs, and this should be validated in future studies using adult-

derived or human iPSC-derived cells. Consequently, while our *in vitro* results suggest a causal role for Piezo1 in stiffness-dependent differentiation, direct *in vivo* validation, for instance, using conditional knockout or pharmacological inhibition of Piezo1 in animal models of stroke [22], is required to avoid overinterpretation and to establish the therapeutic potential of targeting Piezo1.

In summary, this study provides insights into the potential mechanism of substrate stiffness regulating NSC differentiation via the mechanosensitive ion channel Piezo1. We demonstrate that Piezo1 activation coordinates the gene networks governing neurogenesis and synaptic length by modulating cell adhesion, calcium signaling, and the expression of Notch pathway genes critical for proliferation and differentiation. Our findings suggest several potential strategies to improve NSC-based therapy for stroke. First, an early time window with low glial scar stiffness may be preferable for transplantation over the acute edema or chronic recovery phase. Second, modulating Piezo1 expression or using Piezo1 antagonists prior to transplantation could enhance neuronal differentiation and therapeutic efficacy. Third, briefly culturing NSCs on a soft matrix mimicking normal brain tissue or early-stage scar stiffness may promote neuronal differentiation and increase neuronal yield. Finally, combining small-molecule drugs (e.g., calcium channel modulators or Notch inhibitors) with stem cell transplantation might mimic a soft-matrix molecular environment, thereby facilitating neuronal differentiation even on stiff scar tissue and overcoming physical barriers. These translational implications warrant further investigation in clinical settings. Our findings uncover a novel mechanosensory mechanism underlying impaired neural regeneration, providing a fresh perspective on the microenvironmental regulation of NSC fate after stroke.

## Acknowledgements

Thank others for any contributions.

## Funding

This study was supported by grants from National Natural Science Foundation of China (82571494 [ZZ], 82271320 [ZZ], 82371307 [YT], 32301146 [LW]), the National Key R&D Program of China (2022YFA1603600 [ZZ]).

## Author contributions

SW designed the projects, conducted the experiments, analyzed the data, and wrote the manuscript. SY and YYQ assisted in the experiments and data analysis. Additionally, YT and WL helped to revise the manuscript. GY, and ZZ revised and edited the manuscript. All authors

have seen and approved the final version of the manuscript being submitted.

### Data availability

The raw RNA-seq reads generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) and are publicly available under accession numbers SRR39357122, SRR39357121, SRR39357120, SRR39357119, SRR39357118, SRR39357117, SRR39357116, SRR39357115, SRR39357114, SRR39357113, SRR39357112, and SRR39357111.

### Competing interests

The authors declare that they have no conflict of interest.

### Supplementary Materials

The Supplementary data can be found online at: [www.aginganddisease.org/EN/10.14336/AD.2026.0347](http://www.aginganddisease.org/EN/10.14336/AD.2026.0347).

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