Prenatal maternal stress promotes neural stem cell proliferation in the ependymal-subventricular zone of adult male offspring

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Abstract

Perturbations during critical time windows in the normal series of development can lead to adverse functional consequences that manifest later in life. Here, we report that prenatal stress (PNS) during the last week of gestation (E14-delivery) dramatically increased the number of proliferating neural stem/progenitor cells (NSC/NPCs) in the ependymal-subventricular zone (E-SVZ) of male mouse offspring. PNS did not cause significant cell death or a deficit in neuroblast migration to the olfactory bulb (OB). Olfactory behavioral tests showed that while prenatally stressed male mice displayed normal olfactory function in differentiating nonsocial odors, these mice showed impairment in discriminating different social smells. Bulk and single nucleus transcriptomic analyses combined with rescue assays using mitogen-activated protein kinases (MAPK) phosphorylation inhibitors revealed that PNS exposure during the critical period of neurogenesis promoted NSC/NPC proliferation in adult male offspring by sustaining MAPK3/1 activity. Compared to prenatally stressed males, their female littermates showed less change in the number of proliferating cells in the E-SVZ.

Introduction

The prenatal period represents a critical time window open to neural plasticity changes and is most vulnerable to the influence of external disturbances. In humans and model animals, environmental disturbances during the critical period have been shown to cause neural morphological, neurobiological, and behavioral deficits[1-11].

Since the evidence of cell mitosis in the SVZ of adult rats was published over 100 years ago[12], it has now been widely accepted that adult neurogenesis occurs in mammals[13-17]. Numerous studies have reported that the ependymal-subventricular zone (E-SVZ) and the dentate gyrus (DG) are the main brain regions that harbor NSC/NPCs to give rise to neurons and glial cells throughout life[14, 15, 18-22]. Mechanistically, EGF and VEGF are two key growth factors to activate dormant/quiescent neural stem cell and neural progenitor cell proliferation in the E-SVZ of the lateral and/or the 4th ventricles in mice[14, 19]. MAPK (a.k.a. extracellular signal-regulated kinase (ERK)), as a major transducer of growth factors like EGF and VEGF, plays a vital role in mediating their intracellular signaling[23, 24]. MAPK(ERK) has a TxY motif in its activation domain, which requires dual phosphorylation by upstream MAPK kinases (e.g., MAP2K1/2 (MEK1/2)) for catalytic activation. In cancer cells, MAPK3 and 1 (ERK1/2) have been shown to interact with wide-range signaling pathways, such as EGF/FGF/VEGF, WNT, BMP/TGFB, Estrogen, SHH[25-29] to regulate cell proliferation and to facilitate epithelial-mesenchymal transition (EMT)[30, 31].

In the past decade or so, studies reported that prenatal maternal environmental disturbances have profound impact on hippocampal neurogenesis but spare OB neurogenesis in postnatal offspring[32-35]. Although tremendous progress has been made to understand the long-lasting effects of PNS on brain development, little is known about the molecular programs that govern adult NSC/NPC proliferation and neurogenesis in response to the prenatal

stimuli. In the current study, by employing daily mild restraint stress during the last week of gestation, we found that PNS increased the numbers of $BrdU^+$ and $MKI67^+$ proliferating cells in the E-SVZ and the rostral migratory stream (RMS), and PNS also increased $BrdU^+$ cells in the OB. To reveal the underlying molecular program that mediates PNS triggered cell proliferation in adult brain, we carried out bulk and single nucleus transcriptomic analyses. Combined with rescue assays, we showed that PNS exposure at the late stage of embryonic development sustained postnatal phosphorylation levels of MAPK3/1, which in turn promoted NSC/NPC proliferation in the E-SVZ of adult male offspring. By olfactory behavioral tests, we found that prenatally stressed male mice had an intact olfactory function to locate food pellets and discriminate nonsocial odors, whereas they showed impairment in differentiation of different social smells.

Results

PNS triggers cell proliferation in the lateral E-SVZ. To evaluate the effects of the prenatal aversive stimulus on cell proliferation in the E-SVZ, we employed a widely used stress-inducing method, the restraint stress on pregnant dams during the third week of gestation (60min/day, 0800h-0900h)[36]. The third week of gestation in mice roughly corresponds to the second trimester of human gestation, which is considered to be a critical period for the development of psychiatric disorders[37, 38]. To eliminate the postnatal influence from stressed mothers, the prenatally stressed (STR) pups were fostered by unstressed dams at postnatal day 0 (P0). As a control (CTL), pups from unstressed dams also underwent the fostering process. BrdU was administered by intraperitoneal (IP) injection one hour before tissue collection (Fig.1A).

Compared to the CTL, the ratio of proliferating cells to the total number of cells in the lateral E-SVZ ($MKI67^+$ cells/total E-SVZ cells) was dramatically increased in STR male offspring at P60 and P180, whereas similar increases were not detected in neonatal or juvenile mice (P0.5-21) (Fig.1B; Sup.Fig.1). We found that the numbers of $BrdU^+/PROM1^+/GFAP^-$ and $MKI67^+/PROM1^+/GFAP^-$ ependymal cells, and $MKI67^+/DCX^+$, $BrdU^+/DCX^+$, and $MKI67^+/TUBB3^+$ neuroblasts increased dramatically in STR male mice (Figs.1C, D, 2A; Sup.Fig.3A). There were more $BrdU^+$ and $MKI67^+$ migrating neuroblasts in the rostral migratory stream (RMS) of stressed male mice than that of the CTL mice (Fig.2B). We did not observe statistically significant changes in the numbers of $GFAP^+/BrdU^+$ cells in either female or male offspring (Sup. Figs.2D, 3B).

In the female littermates, aforementioned changes were statistically insignificant, suggesting that female offspring were resilient to PNS (Sup.Figs.2, 4A,B). We found the ratio of proliferating cells to the total number of cells in the E-SVZ in CTL female mice were higher than that of their male littermates ($BrdU^+/PROM1^+$ cells: female = 2.1 ± 0.4% vs. male = 0.9 ± 0.2%; $MKI67^+/PROM1^+$ cells: female = 4.1 ± 0.4% vs. male = 2.7 ± 0.3%. mean ± SEM). A similar finding was demonstrated in the subgranular zone of female mice by Tanapat et al[39]. It seems that the influence of PNS exposure is highly sex dependent. Similar findings have been observed in both rats and humans. In rats, PNS significantly increased anxiety- and depression-related behaviors in male offspring,

but not in female offspring[40, 41]. In humans, it has been shown that prenatal disturbances preferentially affect males in a late-onset manner[42, 43].

E-SVZ NSC/NPC progenies migrate to and mature in the OB. To observe adult neurogenesis in the OB of STR offspring, we labeled proliferating cells with two BrdU administrations separated by a 12hr interval. The animals were sacrificed six weeks later (Fig.3A). BrdU labeled cells migrated to the OB and mainly resided in the granule cell layer (GrO) with a few cells in the glomerular layer (Gl) and the external plexiform layer (EPl) in both CTL and STR offspring (8-9-month-old). As expected, the number of BrdU-labeled, newborn RBFOX3$^+$ neurons increased in the OB of stressed mice (Fig.3B, C). We did not observe substantial cell death in the OB of either the CTL or STR mice (Fig.3D).

PNS promotes E-SVZ cell proliferation via regulating MAPK pathway. To reveal the underlying molecular programs of PNS-increased cell proliferation, we carried out transcriptomic analyses. 49 eight-month-old STR male and female mice were sacrificed, the brains were sectioned, and bilateral E-SVZ regions were dissected out, rapidly frozen using liquid nitrogen, and stored at -80°C. Tissues from left and right sides were stored separately: one for bulk RNA sequencing, and one for single nucleus RNA sequencing (snRNAseq) (Fig.4A).

Weighted gene co-expression network analysis (WGCNA) and gene ontology (GO) enrichment analysis of bulk RNAseq data implied that PNS dysregulated gene expression networks related to the regulation of three biological functions: transferase activity (*Dusps1, 6, 5, Rgs, Spry2*; the purple module), cellular response to morphogens (*Shisa2, Ccn1, Spry2, Bambi, Smad7*; the purple module), and gliogenesis (*Mag, Trf, Myrf, Plp1, Gjc2*; the magenta module) in adult male offspring (Fig.4B, C). Many genes in the purple module are critical components of RAS-RAF-MEK-MAPK(ERK) signaling cascade, which has been shown to be the major downstream of growth factor signaling (e.g., EGF, FGF, VEGF) to promote cell proliferation[14, 18, 44-47]. GO enrichment analysis of the greenyellow and pink modules did not identify any signaling pathways. The major sex differences between males and females were the regulation of the immune effector process and cytokine production (the yellow module) (Fig.4B,C, Sup.Fig.6A)[48].

To further examine PNS elicited changes in the E-SVZ, we carried out snRNAseq. The nuclei isolated from the E-SVZ cells of different litters were pooled, purified, and subjected to droplet-based snRNAseq analysis (Fig.4A). Quality control filtering and integration of CTL and STR datasets were performed using the Seurat package[49] and the SingCellaR package[50, 51], which generated a merged dataset consisting of 7,407 cells and 20,634 genes for further analysis. According to marker gene expression, we annotated the 18 clusters to 10 different types of cells: 1) *Foxj1*$^+$ ependymal cell (EpC, cluster 6); 2) *Prom1*$^+$ endothelial-like cell (ElC, cluster 17); 3) dorsal neural progenitor cell (NPCd, cluster 14); 4) neuroblasts (NB, clusters 7 and 11); 5) *Drd1*$^+$ spiny projection neurons (D1 SPN, clusters 2, 3, 10, 12); 6) *Drd2*$^+$ SPN (D2 SPN, clusters 1, 8, 13); 7) ventral neural progenitor cell (NPCv, cluster 16); 8) astrocytes (AC, clusters 4, 5); 9) oligodendrocytes (ODC, cluster 9) and

oligodendrocyte precursor cells (OPC, cluster 18); 10) microglia (MG, cluster 15) (Fig.4D,Sup.Fig.6C). Cluster 11 NBs are dividing cells expressing *Mki67* and *Top2a*, while cluster 7 NBs are postmitotic cells. Cluster 14 NPCs express *Egfr*, *Mki67*, and *Btg2*, but not the NB markers *Dcx* and *Tubb3*. Astrocytes are separated into *Aqp4*$^{-}$ (cluster 4) and *Aqp4*$^{+}$ (cluster 5) cells. We detected a significant number of D1 and D2 SPNs that reside close to the E-SVZ (Sup.Fig.6B,C). Compared to the CTL, there were no obvious cell type changes in STR samples (Sup. Fig.5C).

To determine potential intrinsic programs that control NSC/NPC proliferation, we reanalyzed proliferating neural lineage cells. These cells express at least one of the cell cycle markers, *Mki67*, *Mcm2*, *Top2a*, and *Pcna* (UMI>0). We identified eight types of cells: EpC (cluster 7), ElC (cluster 8), NPC (cluster 3), NB (clusters 4 and 6), AC (cluster 5), D1 SPN (cluster 1), D2 SPN (cluster 2), and 'eccentric' eSPN (cluster 9) (Fig.4E, Sup.Fig.6C)[52, 53]. Cell cycle position estimation analysis (estimating a continuous cell-cycle pseudotime by principal component analysis of cell-cycle genes) showed that most cells in cluster 3 (NPC), cluster 4 (NB), and some cells in cluster 8 (ELC) were actively dividing cells, while cells of other clusters were predicted to be in the G1-phase (Sup.Fig.6D, cell cycle score)[54]. Cells in cluster 3 (NPC, *Egfr*$^{+}$, *GFAP*$^{-}$*)* were likely to be the previously described activated NSC (aNSC) and/or transit-amplifying cells (TACs, C cells)[55]. Cluster 5 astrocytes expressed *Gfap* and *Pdgfrb*, but not *Egfr* and *Prom1*, suggesting an astroglial lineage (Sup.Fig.6B). It remains to be determined why a small subset of spiny projection neurons (D1, D2, and eSPN) expresses low levels of cell cycle markers. It seems that in addition to olfactory bulb neurons, this region could produce new striatal neurons[56]. Another possibility is that cells in or close to the E-SVZ could sense some environmental cues, and thus express cell cycle markers.

We next employed the bigSCale2 algorithm to construct gene regulatory networks for the proliferating cells[57] (Fig. 4F). Compared to the CTL network, the STR network showed a lower edge to node density (CTL = 8.17, STR = 7.63) and a higher modularity (CTL = 0.41, STR = 0.54), suggesting weaker intramodular connectivity but higher gene expression heterogeneity in the STR network. The analysis implicated two major network modules that regulate cell cycle progression and neural maturation (Fig.4F). GO analysis of differentially expressed hub genes showed that PNS promoted expression of genes that regulated cell cycle progression (e.g., *Atad2*, *Top2a*, *Brd8*, *Atad5*, *Smchd1*, *Ncapg2*, *Pola1*, *Ezh2*) and neuronal differentiation (e.g., *Sox2*, *Sox5*, *Nfib*, Zmiz1, *Zeb2*), while it downregulated expression levels of genes modulating synapse organization and membrane potential (e.g., *Slc8a2*, *Kcnd2*, *Grm5*, *Nfia*, *Ntrk3*, *Slit3*) (Fig.4F,G). More specifically, PNS enhanced the expression of bromodomain containing genes *Atad2* (ATPase family AAA domain containing 2) and *Brd8* (bromodomain containing 8) that have been shown to be involved in ESR1 (estrogen receptor alpha) mediated transcriptional regulation of cell proliferation in cancer cell lines[58-60]. PNS increased expression levels of Wnt pathway modulators *Ezh2*, *Tcf4*, *Ephb2*, and *Sox2*, which play important roles in sustaining cell stemness and promoting cell proliferations[61-65]. The Wnt and the estrogen signaling pathways are tightly associated with

MAPK3/1 signaling to stimulate tumorigensis[26, 66, 67]. Moreover, MAP4K4 (serine/threonine kinase mitogen-activated protein 4 kinase 4) has been identified as a positive regulator of MAPK(ERK) signaling in cancer cells[68]. In addition, previous studies by our group and others have shown that MAPK3/1 (ERK1/2) are essential in maintaining proliferation of adult NSC/NPCs[68-70]. Considering the above transcriptomic analysis, we hypothesized that PNS promoted cell proliferation via the activation of the MAPK(ERK) signaling in the E-SVZ NSC/NPCs of adult male offspring.

PNS regulates MAPK3/1 phosphorylation. To test this hypothesis, we first employed immunostaining approach to examine phosphorylation levels of MAPK3/1 in the E-SVZ of both CTL and STR mice. The antibody was developed to recognize phosphorylated MAPK3/1 at threonine 202/185 and tyrosine 204/187 loci specifically (ThermoFisher 44-680G). We found that immunostaining of phosphorylated MAPK (p-MAPK) showed a punctate pattern (Sup.Fig.8A). This pattern was affirmed by using another p-MAPK3/1 specific antibody from a different vendor (Cell Signaling, 4370) and an antibody against unphosphorylated MAPK(ERK) (Cell Signaling, 4695) (Sup.Fig.7A,B). Furthermore, when treating the section with lambda protein phosphatase (Lambda PP) before primary antibody incubation, the immunoreactivity of the p-MAPK3/1 antibody was completely abolished, suggesting the high specificity of the antibody in recognizing p-MAPK (Sup.Fig.7C).

Since both phosphorylated and unphosphorylated MAK3/1 staining displayed punctate patterns, we reasoned that the number of puncta correlates with the status of phosphorylation. By employing this quantitative methodology, we next examined the phosphorylation of MAPK3/1 in the E-SVZ at different postnatal developmental time points (P0.5-180). Concomitant with the cell proliferation finding (Fig.1B, Sup.Fig.1), we found phosphorylation level of MAPK3/1 was considerably upregulated in the E-SVZ of stressed adult offspring (P60, P180) but not at earlier stages (Sup.Fig.8A,B).

To definitively verify the role of the MAPK3/1 pathway in regulating cell proliferation in stressed male mice, we carried out rescue assays by treating the STR and CTL animals with a selective MAPK3/1 inhibitor, LY3214996, and an inhibitor of upstream protein kinases MAP2K1/2 (MEK1/2), GSK1120212. We found that administration of these inhibitors effectively reduced the numbers of p-MAPK3/1 puncta and proliferating cells in the E-SVZ of both CTL and STR mice (Fig.5). Taken together, the rescue assays suggested that MAPK(ERK) signaling played a critical role in promoting NSC/NPC proliferation in STR adult male mice.

Discussion

Psychosocial, cultural, and environmental stressors experienced during gestation can be detrimental to pregnancy as well as maternal and fetal health. Recent studies have suggested that prenatal stress can shape neural circuitry, neural cell fate, and behavioral development in offspring, which may set the stage for postnatal disease vulnerability[7, 9-11]. In the current study, we showed that prenatal restraint stress sustained MAPK3/1 phosphorylation levels, thereby increasing the numbers of adult-stage $BrdU^+/PROM1^+$ and $DCX^+/TUBB3^+$

NSC/NPCs in the lateral E-SVZ and BrdU$^+$/RBFOX3$^+$ newborn neurons in the OB of male offspring. This finding is in line with a previous study that showed 'mild' prenatal stress (30min/day, E15-17) enhanced neurogenesis in the DG of neonatal rat brains[71]. Nevertheless, other studies showed that PNS inhibited neurogenesis in the DG under severe stress protocols[32-35], suggesting that different stress levels ranging from mild to severe can have different negative influences on the neural development of offspring.

Interestingly, snRNAseq data analysis suggested that PNS upregulated the expression level of *Zeb2* (zinc finger E-box binding homeobox 2) and its downstream target *Sox6*[72] (Fig.4F). ZEB2 is one of the essential transcription factors that induces epithelial-mesenchymal transition (EMT) during pluripotent stem cell differentiation, embryonic development, wound healing, and tumorigenesis[73-75]. Studies by Miquelajauregui et al. and Deryckere et al. found that ZEB2 plays a critical role in controlling neurogenesis in prenatal and postnatal brains, repsectively[72, 76]. Moreover, Wnt and MAPK(ERK) pathways have been shown to induce *Zeb2* expression and promote EMT in cancer cells[73, 74]. MAPK(ERK) is a potent signaling transducer of EGF and VEGF, which are essential for promoting dormant/quiescent neural stem cell activation and neural progenitor cell proliferation[14, 19, 24, 45, 69, 70, 77]. Therefore, it is possible that PNS could activate dormant or quiescent neural stem cell proliferation via promoting MAPK(ERK)-mediated EMT in the germinal zones of adult brain.

To examine the possible association between PNS and basic olfactory function, we carried out the buried food test and the habituation/dishabituation test. Our initial study showed that STR male mice (5-8 months old) had a largely intact olfactory function to locate food pellets and discriminate nonsocial odors, while these animals showed impairment in differentiation of different social odors and habituation of social odor 2 (STR Social 1-3 median=2.98 sec to Social 2-1 median=2.58sec; CTL Social 1-3 median=1.34sec to Social 2-1 median=4.66sec) (Sup.Fig.5B-D). Calbindin 2 (CALB2, a.k.a. Calretinin) staining showed a disorganized glomerular layer (Gl) and a slightly increased number of CALB2$^+$ cells in the external plexiform layer (EPl) and the granule cell layer (GrO) of the OB in stressed male mice (Sup.Fig.5A). Glomeruli and granular cells have been shown to play essential roles in the regulation of olfactory habituation behavior[78, 79]. Moreover, scRNAseq analysis showed that PNS attenuated expression of genes modulating synapse organization and membrane potential (Fig.4G). For example, PNS upregulated the expression of glutamate metabotropic Receptor 5 (*Grm5*, a.k.a. mGluR5), which has been shown to regulates granule cell-mediated inhibition and odor discrimination[80, 81]. Taken together, immunostaining and snRNAseq analyses showed that environmental perturbation during the peak of prenatal neurogenesis elicited great changes in neuronal cytoarchitecture and gene expression in the OB of postnatal offspring. These changes may directly contribute to the behavioral deficit in discriminating different social odors[82-85].

Materials and Methods

Experimental Animals. Mice were kept and fed in standard conditions on a 12hr light/dark cycle. Experimental

procedures on animals were performed in accordance with the guidelines of UCLA Institutional Animal Care and Use Committee and UCLA Animal Research Committee, and the Regional Committee for Medical Research Ethics of Tongji University. The inbred strain C57BL/6NCrlVr mice were used in this study. Pregnancy was timed by daily monitoring for vaginal plugs.

Restraint stress and tissue collection. To induce stress between gestational days 14 and delivery (~E18.5), the pregnant dam was placed individually in a plastic restrainer fitted closely to body size for 60 min/day (session at 0800h). Control pregnant females were left undisturbed in their home cages. After delivery, the prenatally stressed (STR) and the CTL pups were fostered by unstressed, same strain mothers at P0. All pups were weaned at P21. For tissue collection, the animals were sacrificed, and brains were removed for immunostaining or RNA sequencing.

Buried food and olfactory habituation/dishabituation tests.
The tests were described by Yang and Crawley[86]. Details are given in SI Appendix.

Bromodeoxyuridine (BrdU) administration. The BrdU injection was described in our previous study[49]. Details are given in SI Appendix.

Immunohistochemistry. The primary antibodies used in this study were BrdU (Abcam ab6326, 1:100; ab1893 1:250), MKI67 (Abcam ab15580, 1:200), GFAP (ThermoFisher PA1-10004, 1:500), TUBB3 (R&D MAB1195, 1:500), CALB2 (Synaptic Systems 214111, 1:200), DCX (Santa Cruz sc-8066, 1:400), RBFOX3 (Abcam ab177487, 1:500), p-MAPK3/1(ThermoFisher 44-680G, 1:100), p-MAPK3/1 (Cell Signaling 4370, 1:200), and MAPK3/1 (Cell Signaling 4695, 1:250). Fluorophore-conjugated secondary antibodies were purchased from ThermoFisher Scientific and Jackson ImmunoResearch.

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay. TUNEL assay was carried out following manufacturer's instructions (*In Situ* Cell Death Detection Kit, Roche-11684795910).

Tissue processing for BrdU immunostaining. Details are given in SI Appendix.

Transcriptomic analysis. The brains of 7- or 8-month-old STR and CTL offspring were sectioned at 300μm thickness in artificial cerebrospinal fluid supplied with actinomysin D (3μM, Sigma-Aldrich). The lateral walls of ependymal SVZ (E-SVZ) were dissected, and snap frozen in liquid nitrogen, and stored in air-tight tubes in -80°C freezer.

Bulk RNAseq. Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen 74104). 100ng of qualified RNA was subjected to VAHTS™ mRNA-seq V3 Library Prep Kit for Illumina® (Vazyme NR611) for library preparation, followed by quality assessment using Agilent Bioanalyzer 2100, and sequencing on Illumina NovaSeq 6,000. The mRNA sequencing reads were mapped to the reference Mouse reference genome GRCm38 using HISAT2 (version 2.2.1) and StringTie (version 2.1.4), and the mapped reads were assigned using featureCounts function of R package Rsubread (version 2.0.1). The counts were normalized using the DESeq2 R package (version 1.30.0)[87]. Pseudogenes (gene names with the prefix "Gm") were filtered out, and top 10,000 highly variable genes were kept for weighted gene co-expression network analysis (WGCNA) using the R package WGCNA (version 1.69)[88]. Specifically, a soft-power of 12 was chosen to construct a topological overlap matrix from the gene correlation network. Modules were determined by the Dynamic Hybrid Cut algorithm using a "deepSplit" parameter of 2. Highly correlated modules (Pearson correlation of module eigengene > 0.9) were merged as one module using a "mergeCutHeight" parameter of 0.25. Gene ontology (GO) and KEGG pathway enrichment analyses of genes in each identified module were performed using the R package clusterProfiler (version 4.4.4)[89] set at default parameters.

Single nucleus RNAseq (snRNAseq). Single nuclei were extracted from pooled frozen lateral walls of ependymal SVZ (E-SVZ) from CTL and STR mice using a previously described ultracentrifugation method[90] with some modifications. The single nuclei RNA sequencing library construction was performed using the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (10× Genomics) according to the user guide. The quality assessement of the libraries was conducted using Agilent Bioanalyzer 2100, and the sequencing was performed on a Illumina Novaseq 6000. The alignment, filtering, barcode assignment, and UMI counting of the sequenced reads were processed using the Cell Ranger pipeline (version 5.0.1, 10xGenomics). Quality control (QC) of cells and genes was performed using R package Seurat (version 4.1.1). Doublets were removed using R package scDblFinder (version 1.10.0). Additional downstream workflow (including further QC, sample integration, highly variable gene selection, dimensional reduction, clustering, cluster marker gene identification, and visualization) was processed via SingCellaR R package (version 1.2.1)[50, 51]. According to expression of the marker genes, along with well-established celltype specific gene sets, the 18 clusters were assigned to 11 major celltypes: dorsal neural progenitor cells (NPCd), ventral neural progenitor cells (NPCv), neuroblasts (NB), astrocytes (AC), ependymal cells (EpC), endothelia-like cells (ElC), *Drd1*$^+$ SPN (D1), *Drd2*$^+$ SPN (D2), oligodendrocyte progenitor cells (OPC), oligodendrocytes (ODC), and microglia (MG).

Cells (except for clusters without neuronal lineage potential, OPC, ODC and MG) expressing at least one of the four cell cycle related genes (*Mki67*, *Top2a*, *Mcm2*, and *Pcna*) were isolated and re-clustered via SingCellaR

workflow. The potentially dividing cells were clustered into 9 clusters, and annotated as neural progenitor cells (NPC), AC, NB, EpC, ElC, D1, D2, "eccentric" spiny projection neuron (eSPN), respectively. The cell-cycle position of each potentially dividing cell was estimated using the tricycle R package (version 1.4.0)[54]. The putative gene-to-gene correlated network of the potential dividing cells was inferred using the bigSCale2 algorithm (version 2.0)[57]. The network centrality PageRank (PR) was chosen to represent gene essentiality. Gene communities in each network were identified, and those communities consisting of over 5 genes were considered as main communities. Hub genes with maximal biological relevance in each main community were defined as the intersection of three gene sets: 1) top 500 genes with the biggest change in PR absolute value between STR and CTL; 2) genes with over 0.75 quantile of PR value; 3) DEGs between STR and CTL determined using SingCellaR package (version 1.2.1)[50, 51]. GO enrichment analysis of the hub genes was performed using R package clusterProfiler (version 4.4.4)[89] set at default parameters. Details are gaven in SI Appendix.

Rescue assay. A MAPK3/1 inhibitor LY3214996 (Selleck, 16 mg/kg) and a MAP2K1/2 inhibitor GSK1120212 (Selleck, 0.35 mg/kg) and the vehicle solution were daily intraperitoneally (IP) injected to 9- to 10-month-old prenatally stressed male offspring for 27 days (except for the 11th and 22nd days), respectively. Details are given in SI Appendix.

Lambda Protein Phosphatase (Lambda PP) reaction. The assay was carried out following manufacturer's instructions (NEB, P0753S). Details are given in SI Appendix.

Imaging and Statistical Analysis. At least three mice of each gender from different litters at different postnatal stages were randomly selected for immunostaining and quantification analysis. Stained sections were imaged with Zeiss LSM800 confocal microscope as tiled or single images. Images obtained were processed and quantified with Imaris software and ImageJ. Statistical analyses of the data were carried out with R-package, GraphPad Prism, and SAS (Statistical Analysis Software). Mann-Whitney test was used to assess statistical significance between independent experimental groups. All reported significant levels represent two-tailed values. Details are given in SI Appendix.

Figure legends

Fig. 1. PNS promoted PROM1^{+} cell proliferation in the lateral E-SVZ of prenatally stressed male offspring. (A) The pregnant dams were stressed in a plastic restrainer from E14 until delivery. Samples were collected at postnatal different time points. To label proliferating cells, BrdU was IP injected 1hr before sacrifice. **(B)** PNS significantly increased E-SVZ NSC proliferation in prenatally stressed adult offspring (P60 and P180), but not neonatal and juvenile mice (P0.5-21). CPu, caudate putamen; LV, lateral ventricle; Sept, septum. Scale bar, 30μm.

(C) MKI67 (green color), PROM1 (red color), and GFAP (white color) immunostaining. The enlarged images at the bottom showed MKI67$^+$/PROMl$^+$ ependymal cells are GFAP negative (MKI67$^+$ cells: CTL = 24.5 ± 1.6%, n = 6; STR = 31.8 ± 2.8%, n=6. P = 0.026. MKI67$^+$/PROM1$^+$ cells: CTL = 2.7 ± 0.3%, n = 13; STR= 5.5 ± 0.8%, n = 10. P= 0.0002). The box plot showed the minimum, the maximum, the median, the mean (the + symbol), and the first and the third quartiles. Scale bars, 10μm, 2μm. **(D)** BrdU (green color), PROM1 (red color), and GFAP (white color) immunostaining (BrdU$^+$ cells: CTL = 13.3 ± 0.9%, n = 28; STR =21.4 ± 1.4%, n = 26. P<0.0001. BrdU$^+$/PROM1$^+$ cells: CTL = 0.9 ± 0.2%, n = 26; STR = 2.9 ± 0.5%, n = 18. P = 0.0002). The enlarged images on the right showed BrdU/PROMl double positive ependymal cells were GFAP negative. Scale bars, 10μm, 2μm. CTL, control; STR, stressed. Mann-Whitney test. All data were presented as mean ± SEM. *p < 0.05,***p < 0.001, ****p < 0.0001.

Fig. 2. PNS promotes neuroblast proliferation in the E-SVZ and the RMS of male offspring. (A) MKI67/BrdU/DCX immunostaining shows that the ratio of neuroblasts increased dramatically in the lateral E-SVZ (BrdU$^+$/DCX$^+$ cells: CTL = 3.5 ± 0.4%, n = 21; STR = 7.0 ± 0.6%, n = 23. P<0.0001. MKI67$^+$/DCX$^+$ cells: CTL = 11.8 ± 1.3%, n = 21; STR = 17.3 ± 1.3%, n = 23. P=0.0071). Scale bars, 30μm. **(B)** The ratio of proliferating A-cell (BrdU$^+$ or MKI67$^+$) increased in the RMS (MKI67$^+$ cells: CTL = 27.3 ± 1.8%, n = 38; STR = 35.1 ± 1.4%, n = 40. P = 0.0021. BrdU$^+$ cells: CTL = 14.6 ± 1.1%, n = 38; STR = 17.3 ± 0.6%, n = 40. P = 0.021). Scale bars, 100μm. Mann-Whitney test. All data were presented as mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001.

Fig. 3. PNS disturbs bulbar neurogenesis. **(A)** To determine fates of lateral E-SVZ NSCs in prenatally stressed male mice, NSCs were labeled with two BrdU IP injections. After 6 weeks, the brain samples were collected. **(B)** BrdU immunostaining (white color). The enlarged images on the right show BrdU staining in the GrO (BrdU$^+$ cells/OB section: CTL = 92 ± 8, n = 8. STR = 166 ± 19, n =8 . P = 0.0011. **p < 0.01. mean ± SEM). Mann-Whitney test. Scale bar, 200μm. **(C)** BrdU labeled cells migrated to the OB and matured to become RBFOX3$^+$ neurons. The arrow heads show BrdU/RBFOX3 double positive cells in the GrO. Scale bars, 200μm. **(D)** TUNEL assay shows that PNS does not cause cell apoptosis in the OB. Scale bars, 300μm. GrO, granular cell layer of OB; Mi, mitral cell layer; EPl, external plexiform layer; Gl, glomerular layer.

Fig. 4. Bulk and single nucleus transcriptomic analyses of cells in the lateral E-SVZ of STR and CTL mice. (A) Schematic overview of tissue and single nucleus isolation for bulk RNA sequencing and single nucleus RNA sequencing (snRNAseq). **(B)** Module-trait correlation analysis revealed 3 modules in male offspring were corelated with PNS (Magenta, Greenyellow, Purple). **(C)** GO analysis shows top 7 GO terms enriched in major gene modules (Magenta and Purple), excluding redundant terms. The top 5 enriched genes of each term are listed.

(D) Force-directed graph is used to visualize clusters of the total single nucleus dataset (the left panel). EpC, ependymal cell; ElC, endothelial-like cell; NPCd, dorsal neural progenitor cell; NPCv, ventral neural progenitor cell; NB, neuroblast; D1, dopamine receptor D1, Drd1^{+} spiny projection neuron; D2, Drd2^{+} spiny projection neuron; AC, astrocyte; OPC, oligodendrocyte progenitor cell; OD, oligodendrocyte; MG, microglia. Violin plot (the right panel) shows the top cell-type-specific genes expressed in the clusters defined in the left panel. **(E)** Force-directed graph drawing of the potentially dividing cells dataset (the left panel), and the violin plot (the right panel) of the expression of cell-type marker genes. MCM2, minichromosome maintenance complex component 2; TOP2A, DNA topoisomerase II alpha; PCNA, proliferating cell nuclear antigen; eSPN, 'eccentric' spiny projection neuron. The expression of top cell-type-specific genes of each cluster is showed in the right panel. **(F)** Gene regulatory network analysis of proliferating cells (E). The node size is proportional to its PageRank (PR) centrality. Nodes highlighted in darkorange/cadetblue colors in the network indicate genes in the intersection of three gene sets: 1) top 500 genes with the biggest changes in PR absolute value between STR and CTL; 2) genes with over 0.75 quantile of PR value in each main community (community consisting of over 5 genes) detected in the network; 3) DEGs between STR and CTL. The Nodes with text labels refer to hub genes with maximal biological relevance. **(G)** Bar plots show the GO enrichment results of up- and down- regulated hub genes in (F).

Fig. 5. Inhibition of MAPK activity counteracted PNS triggered E-SVZ NSC/NPC proliferation. (A, B) Administration of MAPK3/1 inhibitor LY3214996 (LY) and MAP2K1/2 inhibitor GSK1120212 (GSK) decreases the total phosphorylated MAPK3/1 puncta in the E-SVZ (CTL = 6.2 ± 0.3, n = 18; CTL/LY = 5.1 ± 0.5, n = 16; CTL/GSK = 5.3 ± 0.2, n = 17; STR = 8.7 ± 0.5, n = 16; STR/LY = 4.2 ± 0.3, n = 16; STR/GSK = 7.2 ± 0.3, n = 15. CTL vs LY, P = 0.0056; CTL vs GSK, P = 0.0032; CTL vs STR, P = 0.0002; STR vs LY, P < 0.0001 ; STR vs GSK, P = 0.0405). Scale bars, 30μm. **(C)** Inhibition of MAPK pathway reduces the number of both PROM1^{+}/MKI67^{+} ependymal cells and TUBB3^{+}/MKI67^{+} neuroblasts. MKI67^{+}cells: CTL = 20.7 ± 0.8%, n = 18; CTL/LY = 17.3 ± 1.0%, n = 15; CTL/GSK = 14.1 ± 1.6%, n = 12; STR = 29.2 ± 1.7%, n = 24; STR/LY = 20.3 ± 0.8%, n = 38; STR/GSK = 22.4 ± 0.9%, n = 38. CTL vs LY, P = 0.0229; CTL vs GSK, P = 0.0008; STR vs LY, P < 0.0001; STR vs GSK, P = 0.0013. PROM1^{+}/MKI67^{+} cells: CTL = 1.8 ± 0.3%, n = 16; CTL/LY = 1.4 ± 0.2%, n = 15; CTL/GSK = 1.4 ± 0.5%, n = 12; STR = 2.9 ± 0.4%, n = 20; STR/LY = 0.8 ± 0.2%, n = 17; STR/GSK = 1.0 ± 0.2%, n = 14. CTL vs LY, P = 0.5263; CTL vs GSK, P = 0.3770; STR vs LY, P < 0.0001; STR vs GSK, P < 0.0001. TUBB3^{+}/MKI67^{+} cells: CTL = 10.6 ± 0.6%, n = 18; CTL/LY = 7.9 ± 0.7%, n = 15; CTL/GSK = 6.0 ± 0.5%, n = 12; STR = 14.3 ± 0.8%, n = 20; STR/LY = 11.7 ± 0.7%, n = 17; STR/GSK = 9.1 ± 1.0%, n = 14. CTL vs LY, P = 0.0091; CTL vs GSK, P < 0.0001; STR vs LY, P < 0.0151; STR vs GSK, P < 0.0010. Scale bars, 30μm. Mann-Whitney test. All data were presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

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Figure 1

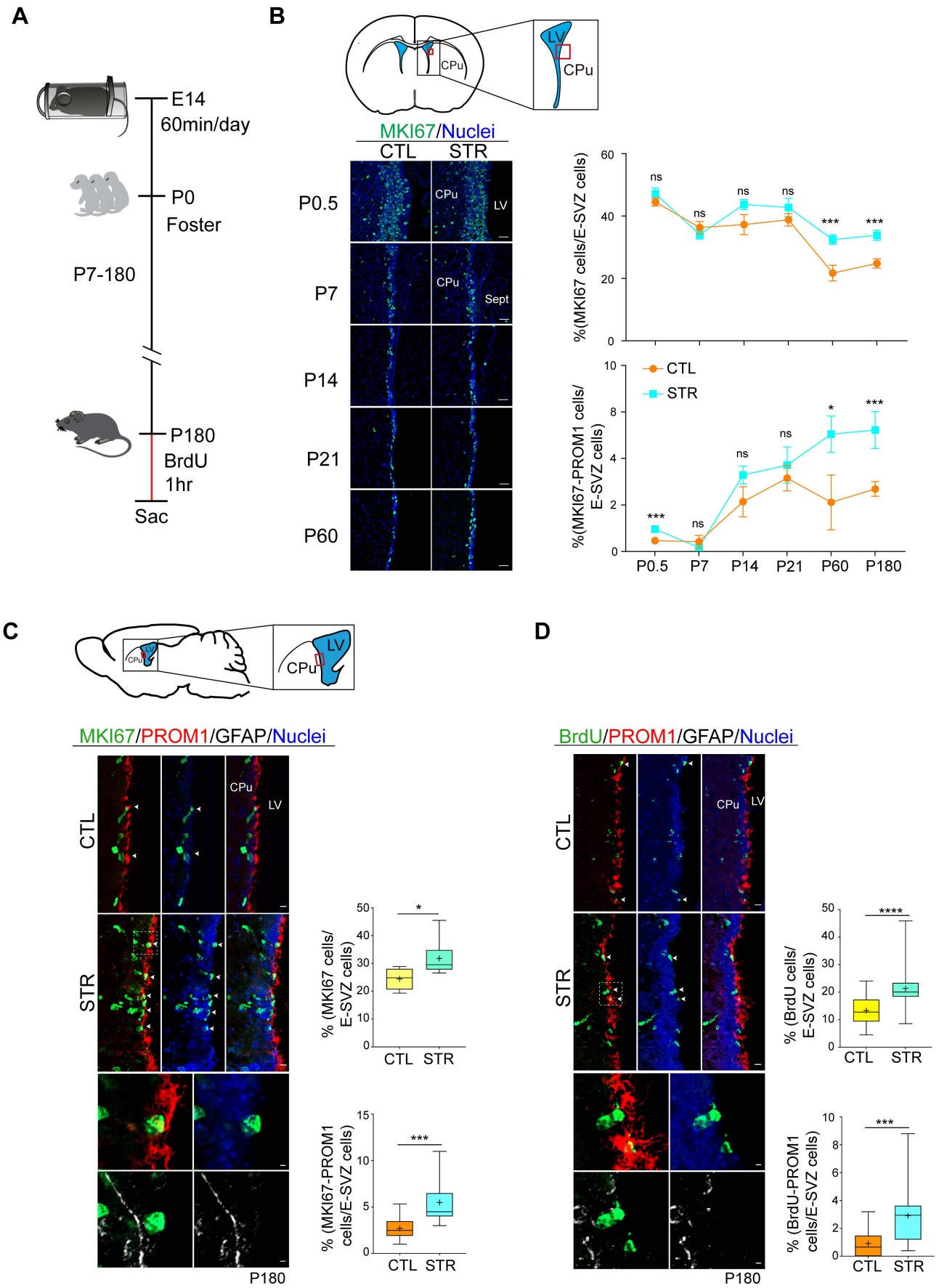
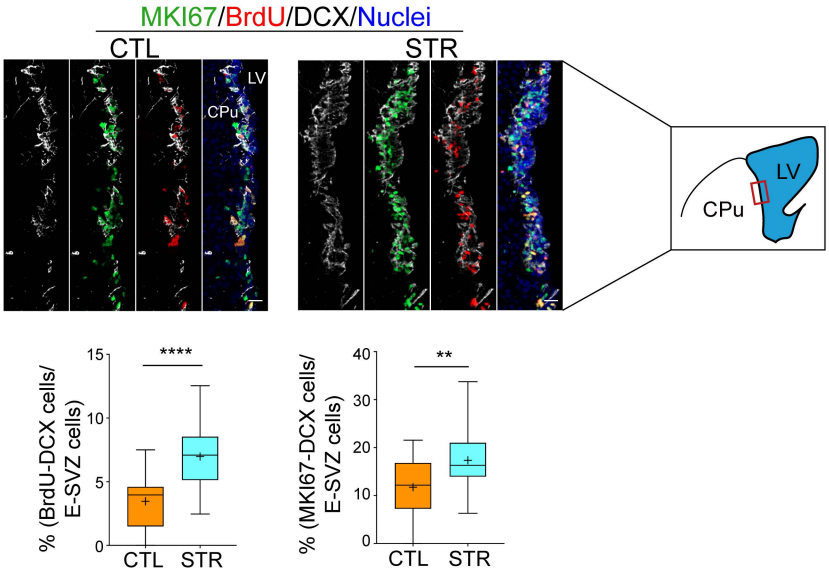

A
E14
60min/day
P0
Foster
P7-180
P180
BrdU
1hr
Sac
B
LV
CPu
MKI67/Nuclei
CTL
STR
P0.5
P7
P14
P21
P60
Sept
%(MKI67 cells/E-SVZ cells)
%(MKI67-PROM1 cells/ E-SVZ cells)
CTL
STR
ns
***
*
P0.5
P7
P14
P21
P60
P180
C
MKI67/PROM1/GFAP/Nuclei
CTL
STR
% (MKI67 cells/ E-SVZ cells)
% (MKI67-PROM1 cells/E-SVZ cells)
P180
D
BrdU/PROM1/GFAP/Nuclei
% (BrdU cells/ E-SVZ cells)
% (BrdU-PROM1 cells/E-SVZ cells)
****

Figure 2

A


MKI67/BrdU/DCX/Nuclei
CTL
STR
LV
CPu
LV
CPu
% (BrdU-DCX cells/ E-SVZ cells)
****
CTL
STR
% (MKI67-DCX cells/ E-SVZ cells)
**
CTL
STR

B

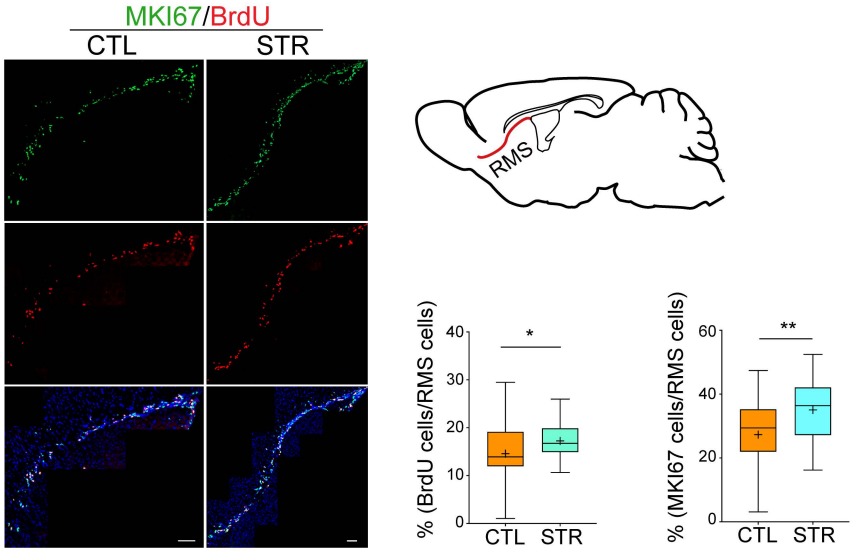

MKI67/BrdU
CTL
STR
RMS
% (BrdU cells/RMS cells)
*
CTL
STR
% (MKI67 cells/RMS cells)
**
CTL
STR

Figure 3

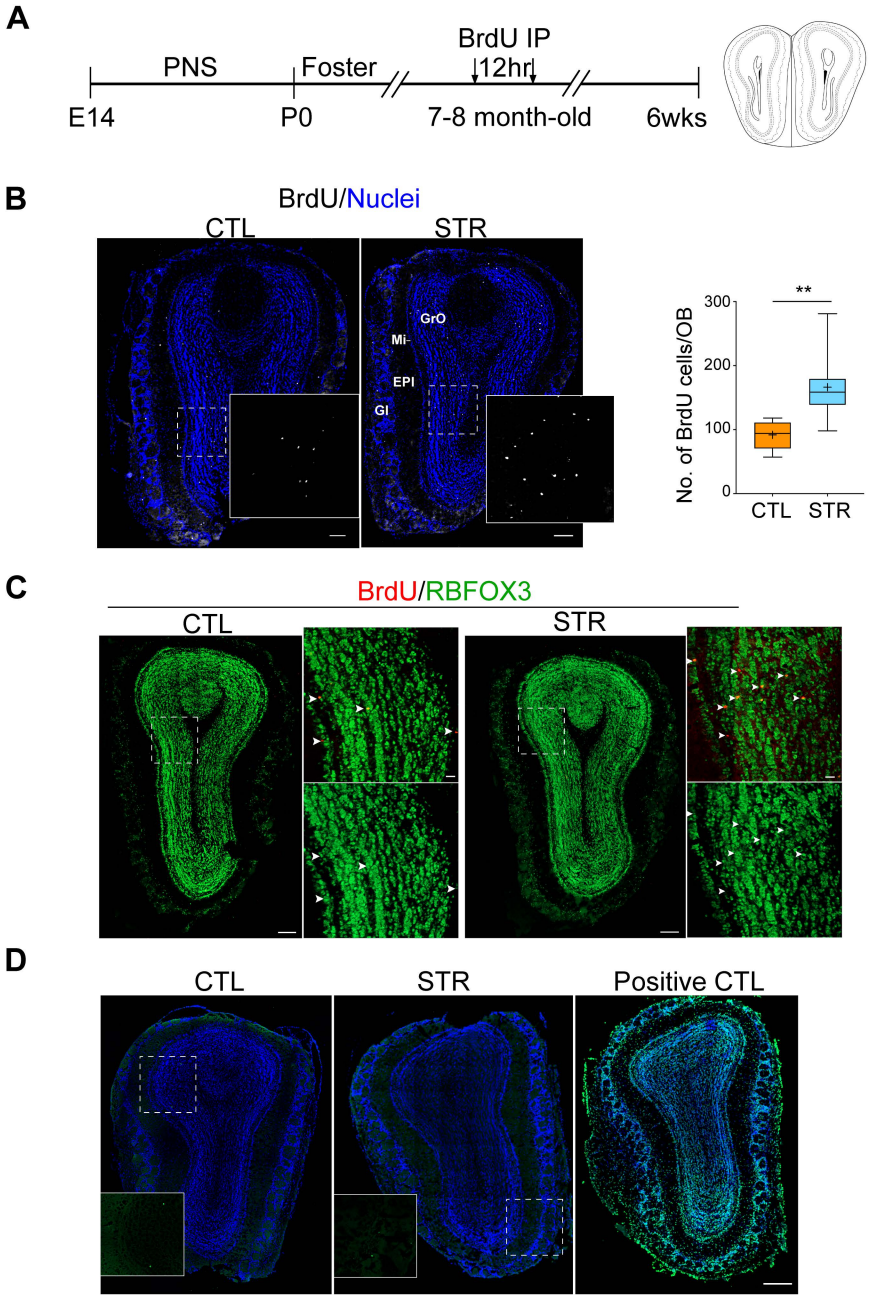

A
PNS
Foster
BrdU IP
12hr
E14
P0
7-8 month-old
6wks
B
BrdU/Nuclei
CTL
STR
GrO
Mi-
EPI
GI
No. of BrdU cells/OB
300
200
100
0
**
CTL
STR
C
BrdU/RBFOX3
CTL
STR
D
CTL
STR
Positive CTL

Figure 4

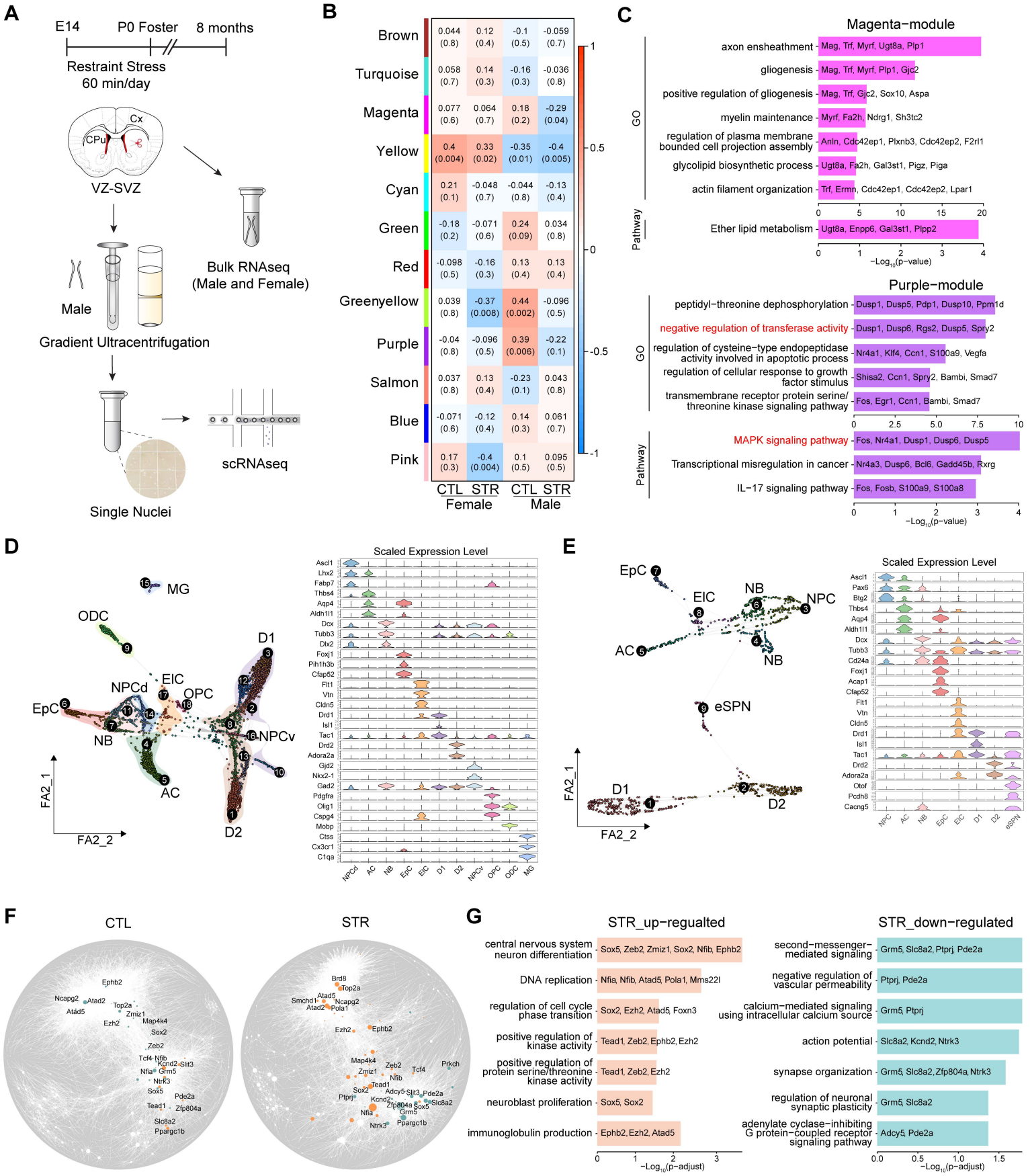
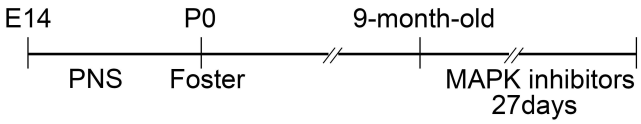
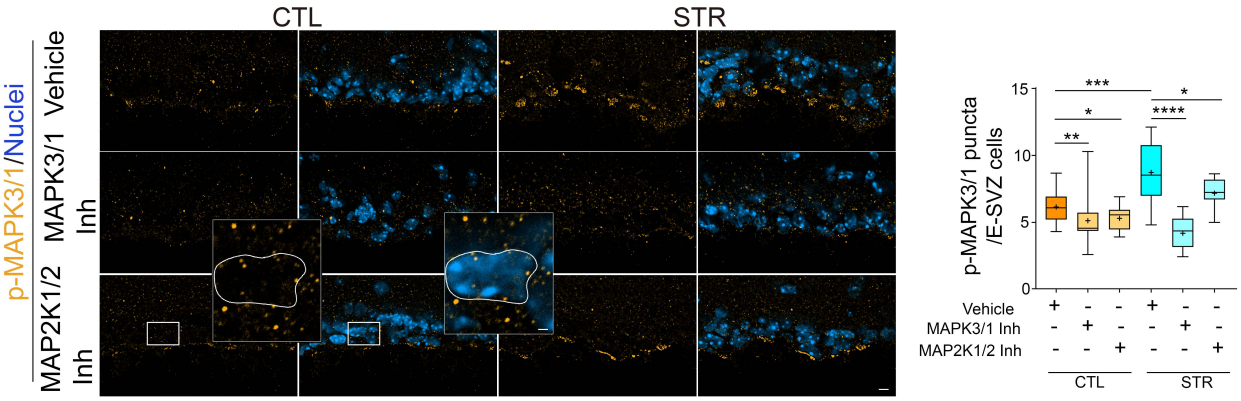

A
E14
P0 Foster
8 months
Restraint Stress
60 min/day
Cx
CPu
VZ-SVZ
Bulk RNAseq
(Male and Female)
Male
Gradient Ultracentrifugation
scRNAseq
Single Nuclei
B
Brown
Turquoise
Magenta
Yellow
Cyan
Green
Red
Greenyellow
Purple
Salmon
Blue
Pink
CTL STR CTL STR
Female Male
C
Magenta−module
GO
Pathway
axon ensheathment
gliogenesis
positive regulation of gliogenesis
myelin maintenance
regulation of plasma membrane bounded cell projection assembly
glycolipid biosynthetic process
actin filament organization
Ether lipid metabolism
Purple−module
peptidyl−threonine dephosphorylation
negative regulation of transferase activity
regulation of cysteine−type endopeptidase activity involved in apoptotic process
regulation of cellular response to growth factor stimulus
transmembrane receptor protein serine/threonine kinase signaling pathway
MAPK signaling pathway
Transcriptional misregulation in cancer
IL−17 signaling pathway
−Log10(p−value)
D
MG
ODC
D1
EIC
NPCd
OPC
EpC
NB
NPCv
AC
D2
FA2_1
FA2_2
Scaled Expression Level
E
EpC
EIC
NB
NPC
AC
NB
eSPN
D1
D2
FA2_1
FA2_2
Scaled Expression Level
F
CTL
STR
G
STR_up−regualted
central nervous system neuron differentiation
DNA replication
regulation of cell cycle phase transition
positive regulation of kinase activity
positive regulation of protein serine/threonine kinase activity
neuroblast proliferation
immunoglobulin production
STR_down−regulated
second−messenger−mediated signaling
negative regulation of vascular permeability
calcium−mediated signaling using intracellular calcium source
action potential
synapse organization
regulation of neuronal synaptic plasticity
adenylate cyclase−inhibiting G protein−coupled receptor signaling pathway
−Log10(p−adjust)

Figure 5

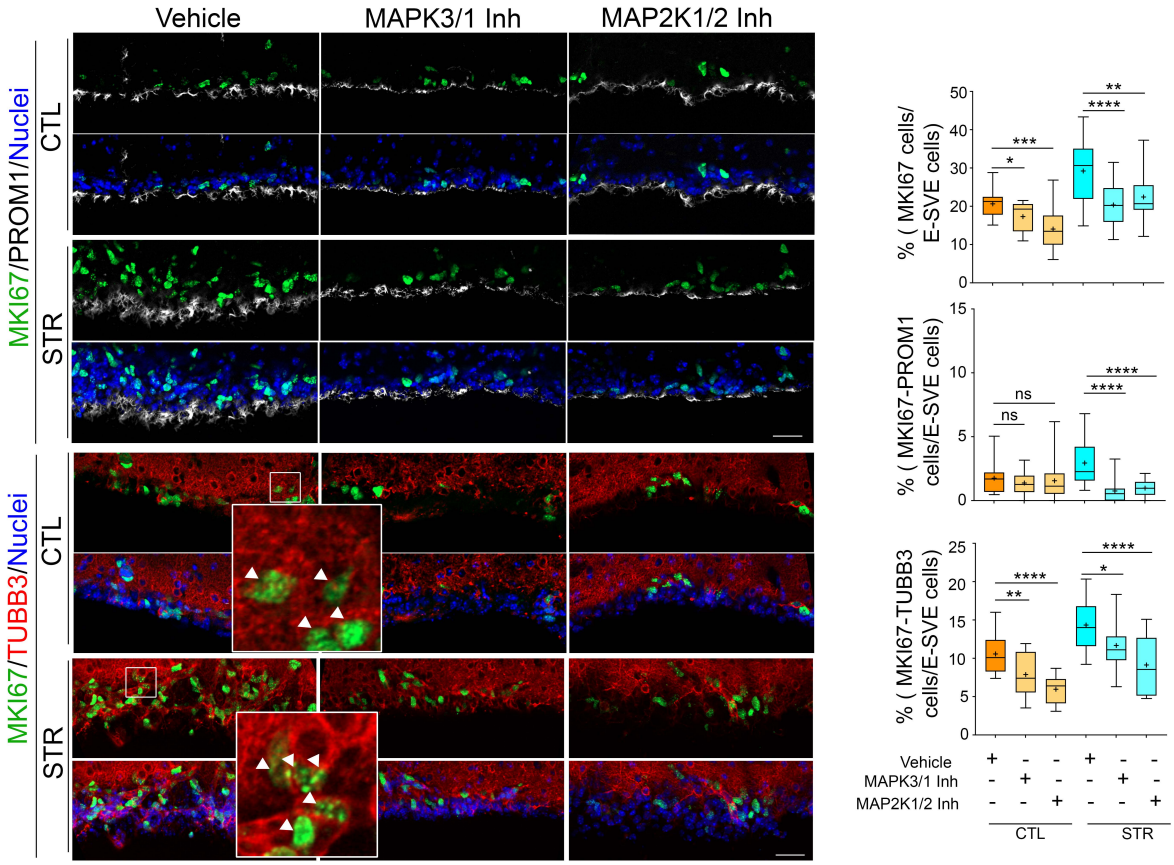
A


E14
P0
9-month-old
PNS
Foster
MAPK inhibitors
27days

B


CTL
STR
p-MAPK3/1/Nuclei
Vehicle
MAPK3/1 Inh
MAP2K1/2 Inh
p-MAPK3/1 puncta /E-SVZ cells
Vehicle
MAPK3/1 Inh
MAP2K1/2 Inh
CTL
STR

C


Vehicle
MAPK3/1 Inh
MAP2K1/2 Inh
MKI67/PROM1/Nuclei
CTL
STR
MKI67/TUBB3/Nuclei
CTL
STR
% (MKI67 cells/ E-SVE cells)
% (MKI67-PROM1 cells/E-SVE cells)
% (MKI67-TUBB3 cells/E-SVE cells)
ns
Vehicle
MAPK3/1 Inh
MAP2K1/2 Inh
CTL
STR