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Supplemental Information

Subventricular zone adult mouse neural stem cells require insulin receptor for self-renewal

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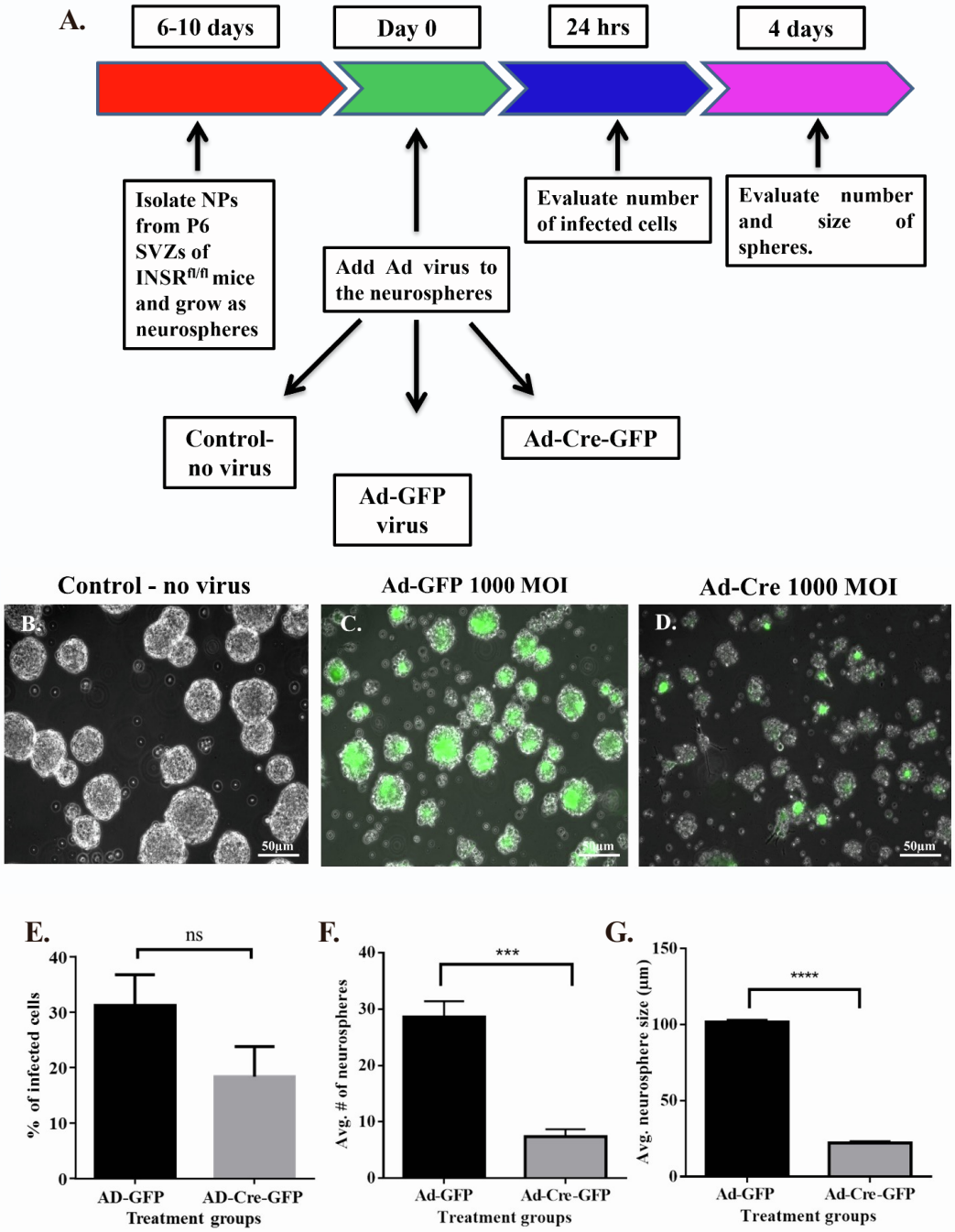

Figure S1. *Insr* knockdown decreases neurosphere size and number of neural progenitors, Related to Figure 1.

Neurospheres generated from *Insr*$^{fl/fl}$ mice were dissociated into single cells and then infected with Ad-GFP, Ad-Cre or no-virus (control). Cells were grown under neurosphere-producing conditions. Images were captured 96 hr after infection. **A.** Schematic of experimental paradigm. **B.** Control spheres produced by uninfected cells (no virus). **C.** Spheres produced by cells infected with Ad-GFP virus. **D.** Spheres produced by cells infected with Ad-Cre-GFP virus. Neurospheres infected with 1000 MOI Ad-GFP, Ad-Cre-GFP virus and control were counted and measured using the ImageJ software. **E.** Infection efficiency. **F.** Average number of neurospheres. **G.** Average size of neurospheres. ****p<0.0001,*** p=0.001 by unpaired t test.

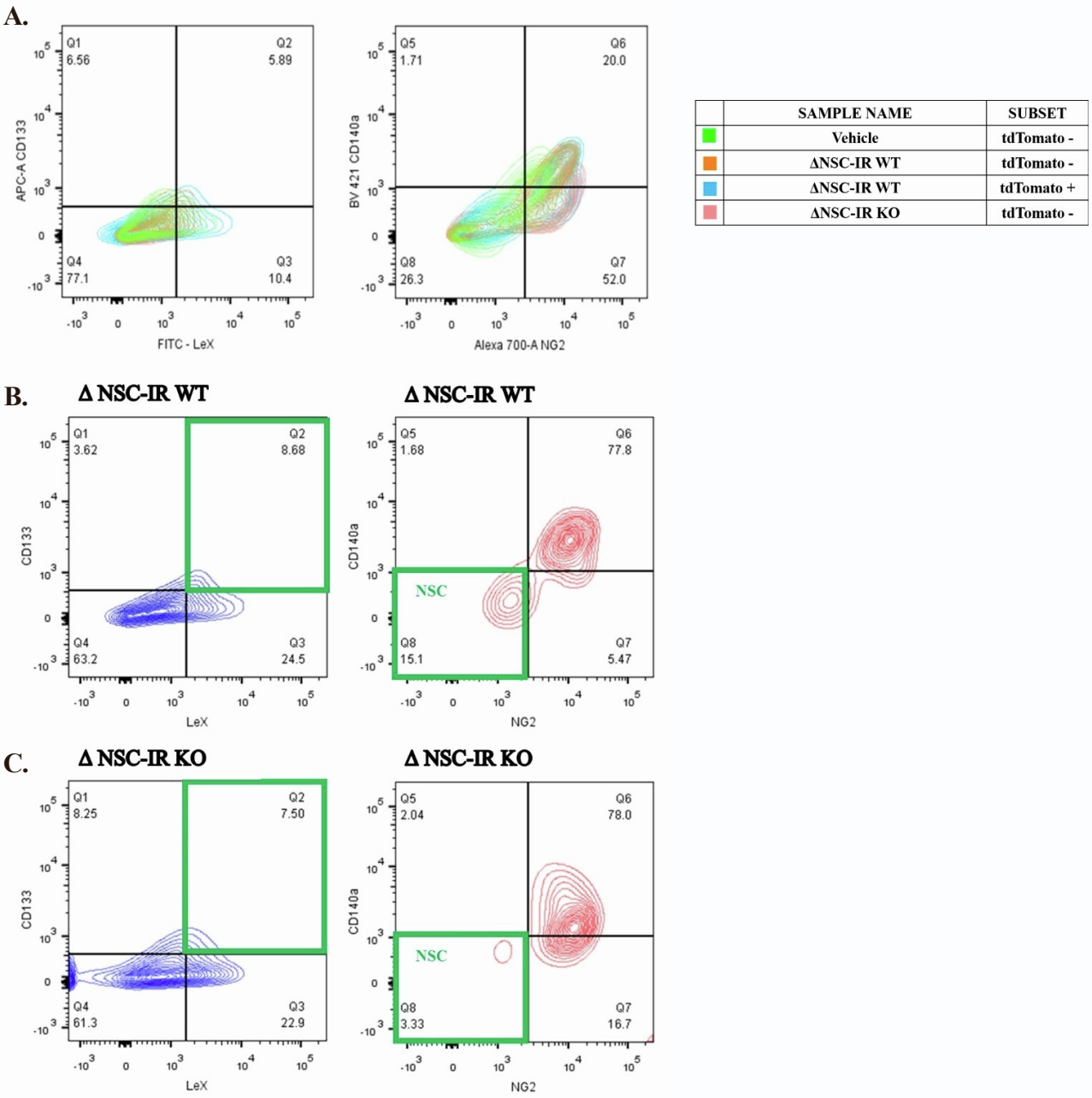

Figure S2. *Insr* deletion decreases NSC population, Related to Figure 1.

Neurospheres generated from ΔNSC-IR WT and ΔNSC-IRKO mice were treated with 0.5 μM of 4-OH tamoxifen or vehicle for 24 hour followed by dissociation for flow cytometry. **A**. Control groups showing similar CD133, LeX, NG2 and CD140a expression. **B.** NSC numbers in ΔNSC-IR WT (control) induced with tamoxifen **C.** NSC numbers in ΔNSC-IRKO induced with tamoxifen.

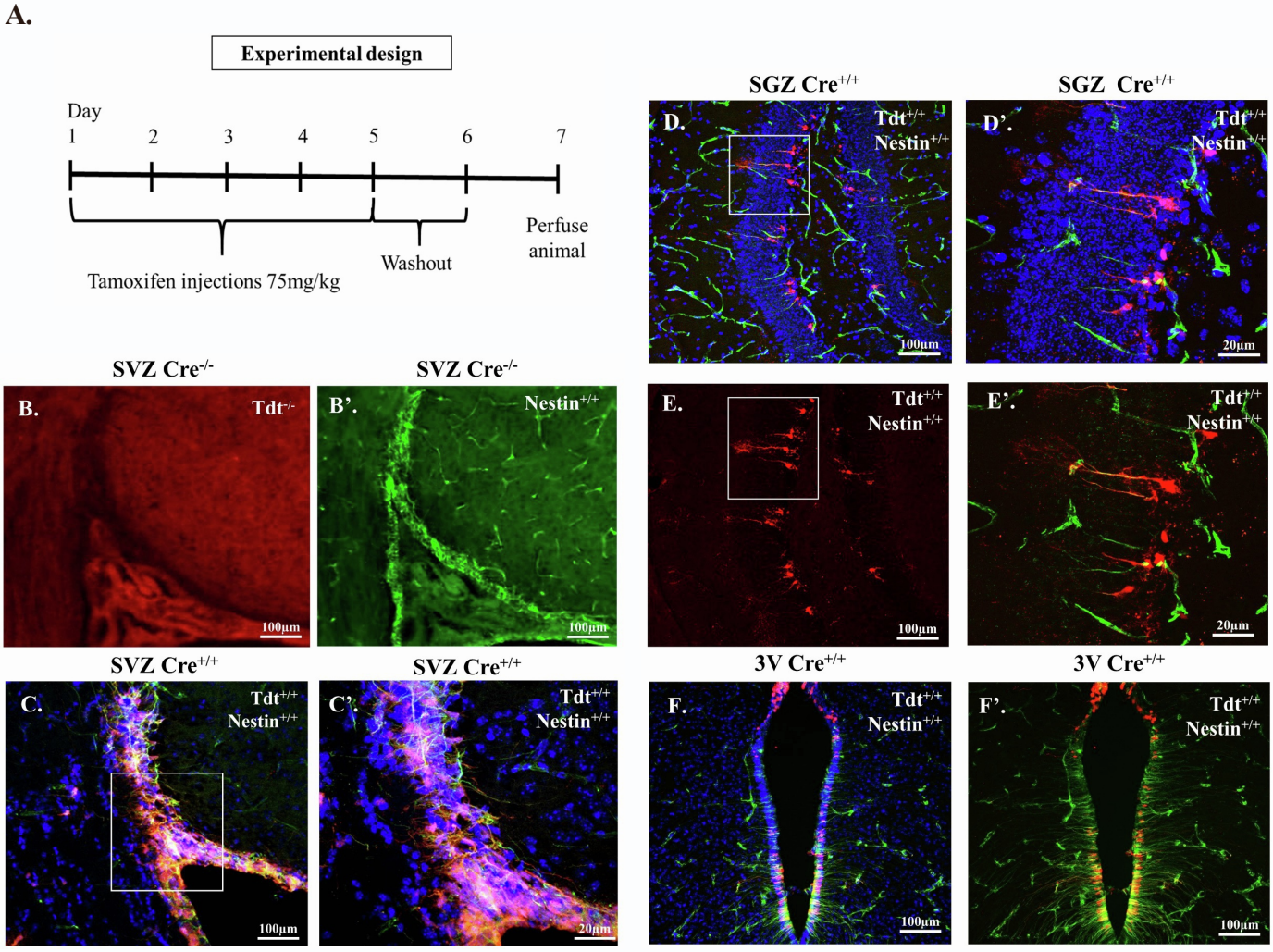

Figure S3. NestinCre promoter induces TdTomato expression in FB SVZ, SGZ and hypothalamus, Related to Figure 2.

A. Schematic of experimental timeline showing administration of tamoxifen and tissue collection. **B.B'.** SVZ of Cre negative mice induced with tamoxifen that are negative for tdTomato expression but stain positive for nestin with nestin antibody. **C.C'.** SVZ of Cre positive mice induced with tamoxifen showing tdTomato expression that colocalizes with nestin positive cells (yellow cells). **D.D'.** SGZ of Cre positive mice induced with tamoxifen showing tdTomato expression that colocalizes with nestin positive cells (yellow cells). **E.E'.** SGZ of Cre positive mice induced with tamoxifen showing tdTomato expression that colocalizes with nestin positive cells (yellow cells) without DAPI. **F.F'.** 3rd ventricle of Cre positive mice induced with tamoxifen showing tdTomato expression that colocalizes with nestin positive cells (yellow cells) with and without DAPI.

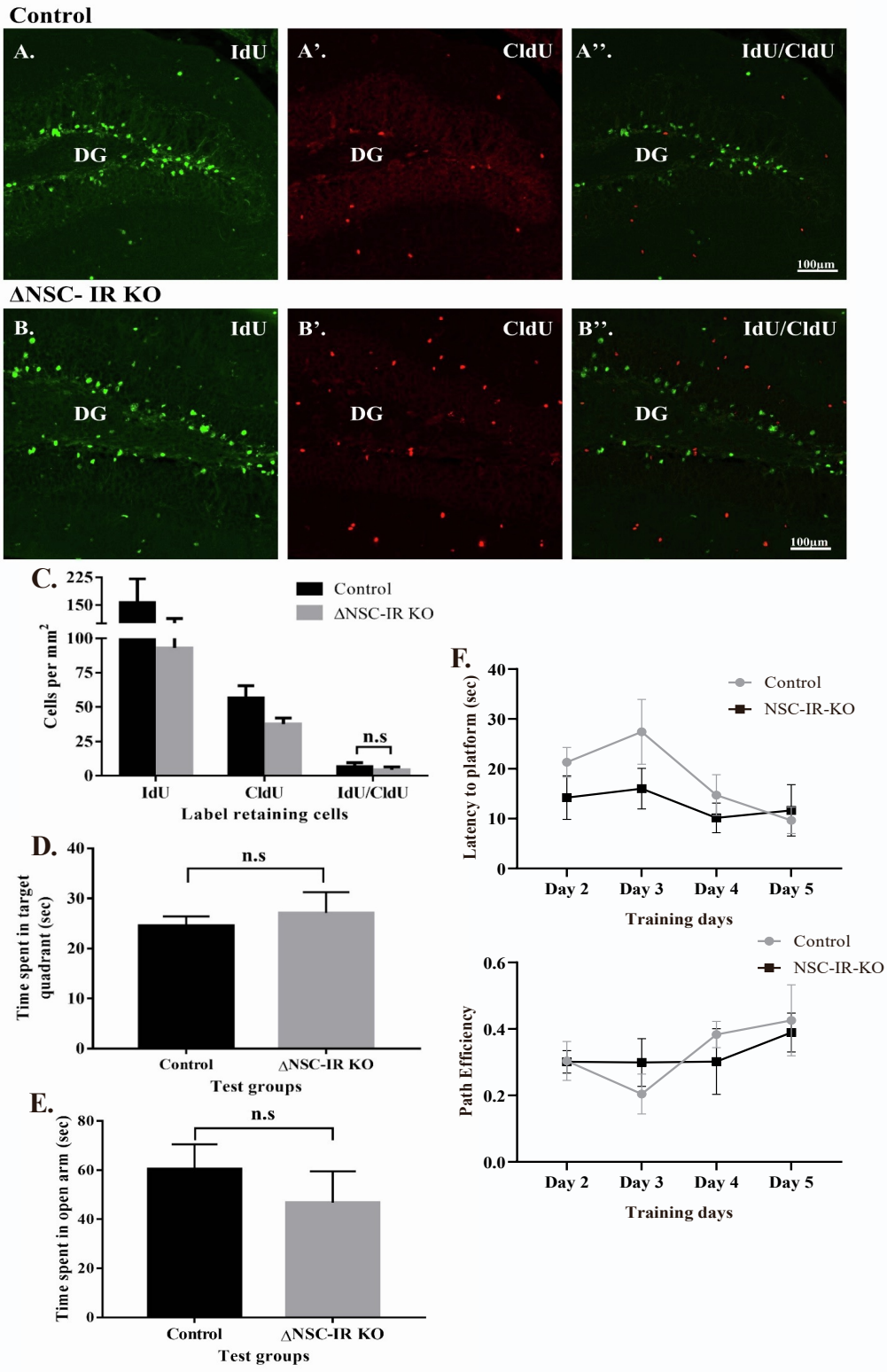

Figure S4. Hippocampal neurogenesis and hippocampal dependent behaviors are unchanged in ΔNSC-IRKO mice, Related to Figure 3.

A. Representative images of IdU and CldU labeling in control SGZ. **B.** Representative images of IdU and CldU labeling in ΔNSC-IRKO SGZ. **C.** Number of IdU, CldU single and double positive cells (unpaired t-test, n.s). **D.** Time spent in the target quadrant during the probe test of the Morris water maze (n=10 control, n=12 ΔNSC-IRKO). **E.** Time spent in the open arms of the elevated plus maze (n=10 control, n=12 ΔNSC-IRKO). **F.** Latency to find platform and path efficiency to find platform (n=10 control, n=12 ΔNSC-IRKO). Mice were analyzed 9 weeks after inducing *Insr* deletion for label retention (n =4 control, n=5 ΔNSC-IRKO).

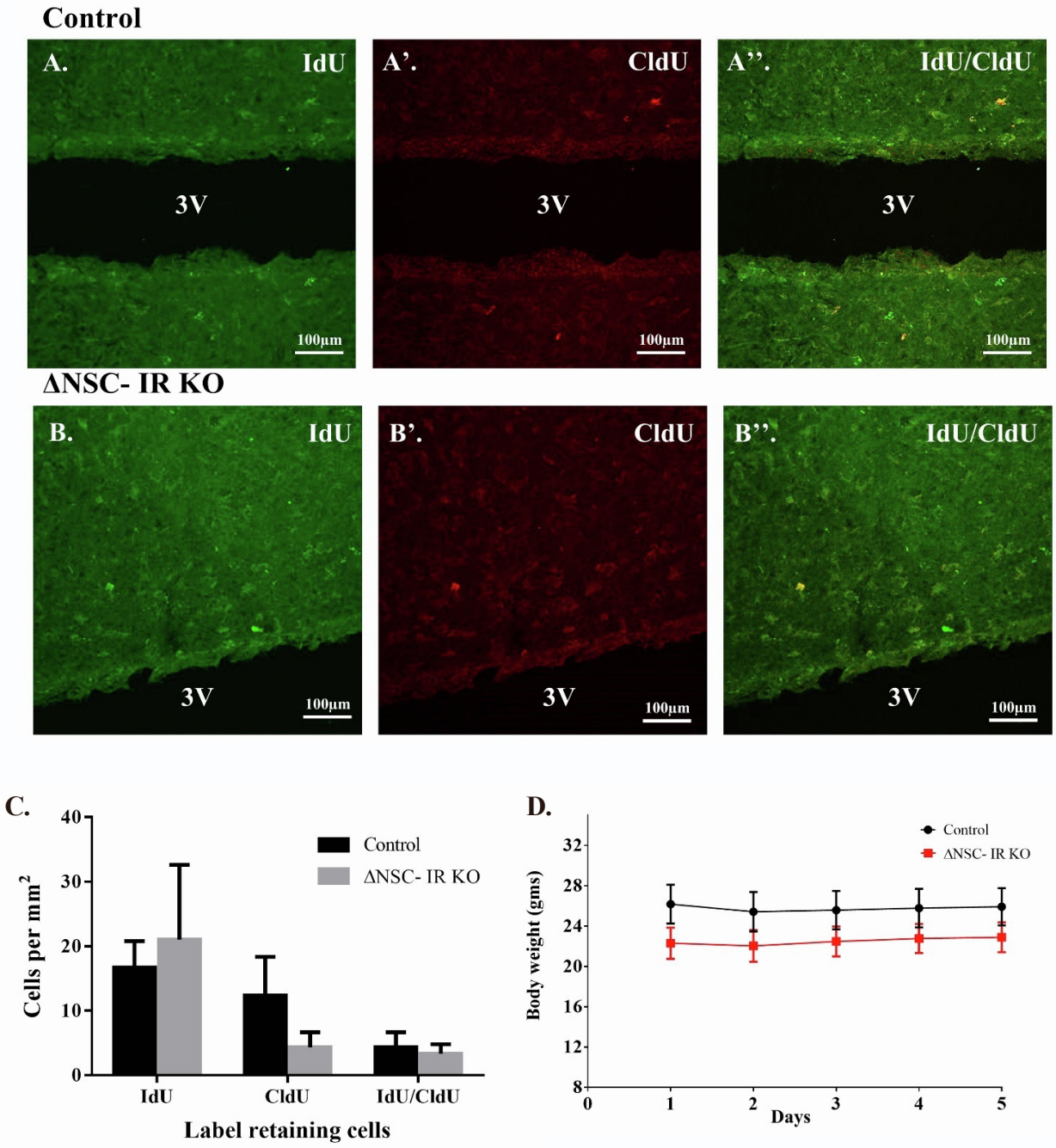

Figure S5. α-tanycytes in the 3rd ventricle do not require INSR signaling for self-renewal, Related to Figure 3.

A-A''. Representative images of IdU and CldU labeling in control 3rd ventricle (3V). **B-B''.** Representative images of IdU and CldU labeling in ΔNSC-IRKO 3rd ventricle (3V). **C.** Number of IdU, CldU single and double positive cells in the median eminence (n=3 per genotype). **D.** Weight of control and ΔNSC-IRKO mice over the 5 day injection period (n=10 control, n=12 ΔNSC-IRKO).

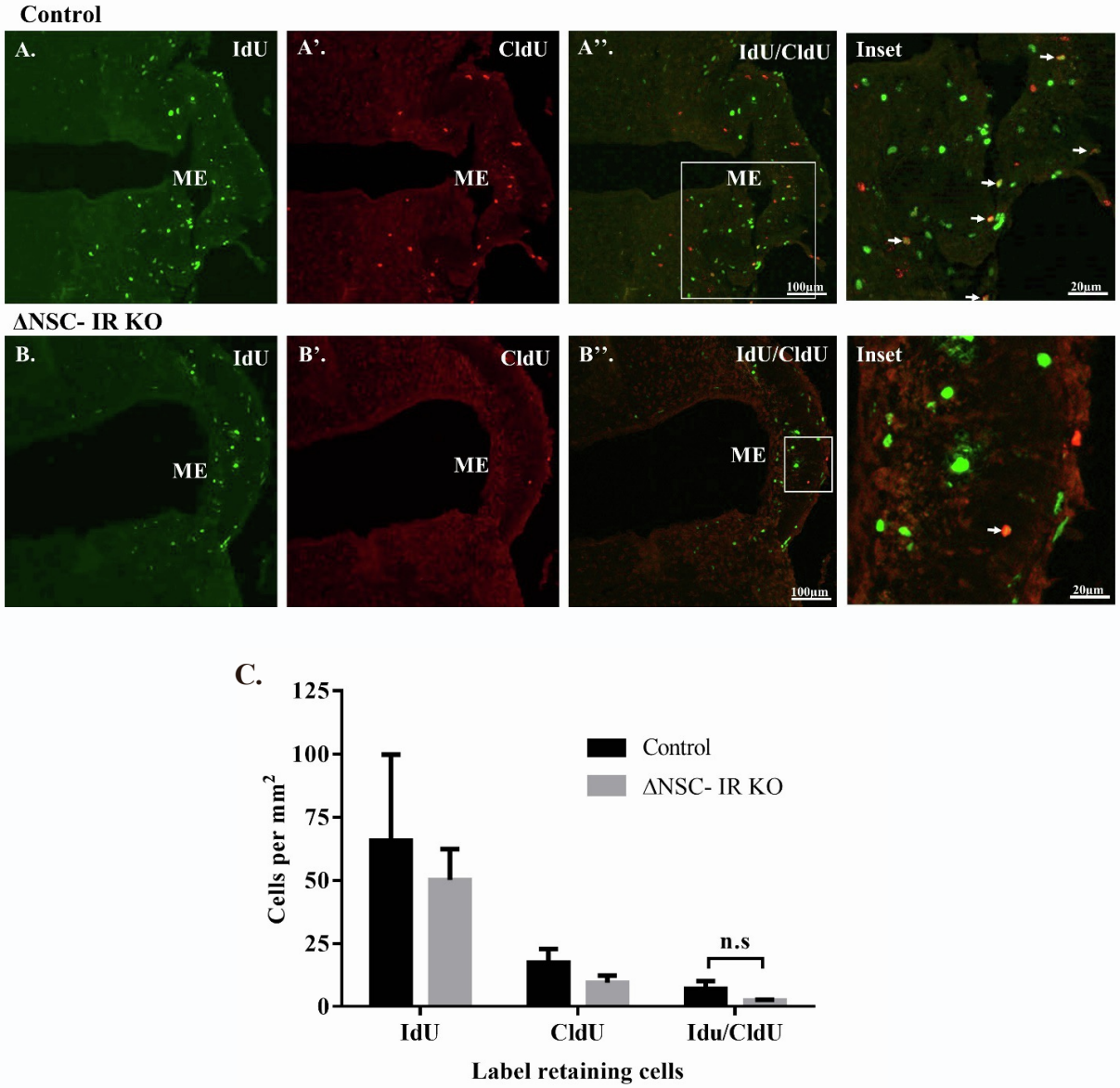

Figure S6. Hypothalamic median eminence may contain an INSR responsive nestin+ sub-population of NSCs, Related to Figure 3.

A-A''. Representative images of IdU and CldU labeling in control median eminence (ME). **B-B''.** Representative images of IdU and CldU labeling in ΔNSC-IRKO ME. White arrows represent $IdU^+/CldU^+$ double positive cells. Scale bars represent 100 µm in panels A and B, and 200 µm for the insets. **C.** Number of IdU, CldU single and double positive cells in the ME (n=3 per genotype).

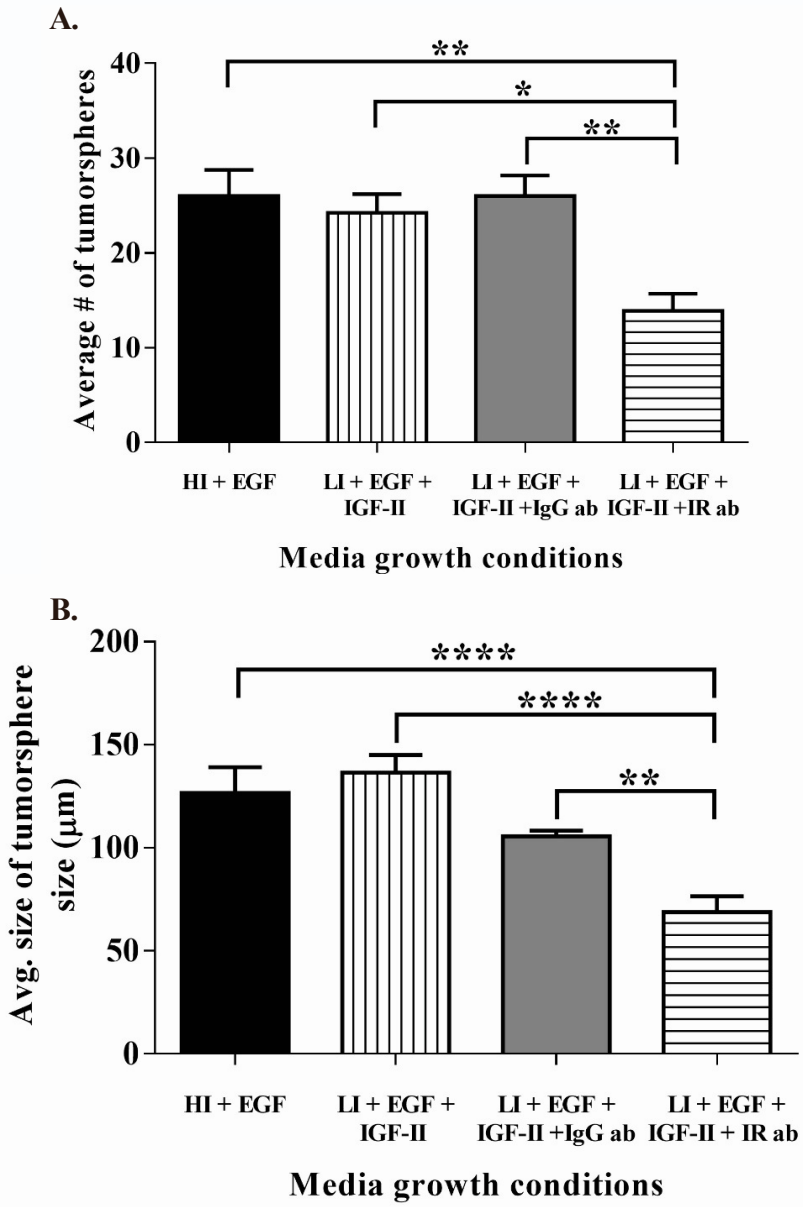

Figure S7. Mesenchymal tumorspheres grown with control antibody, Related to Figure 5.

A. Average number of tumorspheres grown in different media groups; HI+EGF vs LI+EGF+IGF-II+IR ab (**p<0.001), LI+EGF+IGF-II vs LI+EGF+IGF-II+IR ab (* p<0.01), LI+EGF+IGF-II+ IgG ab vs LI+EGF+IGF-II+IR ab (** p<0.001) **B.** Average size of tumorspheres grown in different media groups; HI+EGF vs LI+EGF+IGF-II+IR ab (*****p<0.0001), LI+EGF+IGF-II vs LI+EGF+IGF-II+IR ab (****p<0.0001), LI+EGF+IGF-II+ IgG ab vs LI+EGF+IGF-II+IR ab (**p<0.001). Data are from one experiment performed in triplicate. IR=INSR.

S. Chidambaram et al., 2022 SCR Supplemental Methods

Neurosphere propagation and quantification

Neurospheres were generated by enzymatically dissociating the periventricular region of $IR^{fl/fl}$ pups (P4-5) as described previously. The cells were plated at a density of 2.5 x 10^5 cells/ml in B27 media minus insulin supplemented with 20 ng/ml of recombinant human epidermal growth factor (EGF) (PeproTech) and 4.4 μM of insulin (Sigma) and passaged to secondary spheres (Alagappan et al., 2009). Secondary spheres were counted 5 days after plating which allowed sufficient time for sphere growth. Only regularly shaped spheres that were visibly identifiable as containing tdtomato+ cells were counted. At least 6 random fields per well and 3 wells per experiment were quantified. ImageJ software was used to measure neurosphere diameter, where any sphere that was at least 30 μm in diameter was defined as a neurosphere.

Flow cytometry

Spheres were dissociated and counted as previously described(Buono et al., 2012; Velloso et al., 2022). Briefly, spheres were dissociated by incubation in 0.2 Wünsch unit (WU)/ml of Liberase DH (Roche) and 250 μg of DNase1 (Sigma) in PGM solution (PBS with 1 mM $MgCl_2$ and 0.6% dextrose) at 37°C for 5 min with gentle shaking. An equal volume of PGM was added and the spheres were placed onto a shaker (LabLine) at 225 rpms at 37°C for 15 min. After enzymatic digestion, Liberase DH was quenched with 10 ml of PGB (PBS without Mg^{2+} and Ca^{2+} with 0.6% dextrose and 2 mg/ml fraction V of BSA (Fisher Scientific, BP1600-100) and cells were collected by centrifugation for 5 min at 200 x g. Cells were dissociated by repeated trituration, collected by centrifugation, counted using ViCell (Beckman Coulter, Miami,

FL) and diluted to at least 10^6 cells per 50 µl of PGB. All staining was performed in 96 V-bottom plates using 150 µl/well. For surface marker analysis, cells were incubated in PGB for 25 min with antibodies against Lewis-X (1:20, LeX/CD15 FITC, MMA; BD Bioscience), CD133-APC (1:50,13A4; eBioscience), CD140a (1:400, APA5; BioLegend) and NG2 Chondroitin Sulfate Proteoglycan (1:50, AB5320; Millipore). Cells were washed with PGB by centrifugation at 278 x g. Goat anti-rabbit IgGAlexa Fluor 700 (1:100; Invitrogen) was used for NG2. Cells were then incubated in LIVE/DEAD fixable Violet (Invitrogen) for 20 min for dead cell exclusion. Cells were washed with PGB by centrifugation at 278 x g. They were fixed with 1% ultrapure formaldehyde (50000; Polysciences, Inc) for 20 min, collected by centrifugation for 9 min at 609 x g, resuspended in PBS w/o Mg^{2+} and Ca^{2+} and stored at 4°C for next day analysis. All sample data were collected on the BD LSR II (BD Biosciences Immunocytometry Systems). Matching isotype controls were used for all antibodies and gates were set based on these isotype controls. Post-acquisition analysis was performed using FlowJoX (Tree Star Inc, Ashland, OR). Among the live cells only tdtomato positive cells were analyzed.

Thymidine analogs and Dual Thymidine Analog Detection and Stereology

IdU (Sigma I7125) was provided in the drinking water at 1 mg/mL in 1% sucrose. CldU (Sigma C-6891) was dissolved in 0.9% saline and administered intraperitoneally at 42.5 mg/kg. (Ziegler et al., 2019). IdU and CldU detection was performed as described by Tuttle et. al. 2010 (Tuttle et al., 2010) with the following modifications: cryosections of forebrain SVZ and hippocampus (30 µm) were washed with 0.3% of triton-X-100 for 30 min at RT. Antigen retrieval was performed using Sodium citrate for 20 min at 96^0C in a steamer. Tissue was blocked using 10% donkey serum for one hour at RT. IdU (1:100) was added to tissue overnight

at 4^0C. The following day slides were washed with low salt TBST solution (36mM Tris, 50mM NaCl, 0.5% tween-20; pH 8.0) for 20 min at 37^0C at 225 rpm. CldU antibody (1:100) was added overnight at 4^0C. IdU was used with dylight 488, CldU with dylight 647 and DAPI at 1:5000 in PBS for 15 min. Slides were mounted in fluorogel (Electron Microscopy Sciences 17985). An Olympus BX51 microscope was used to measure immunofluorescence of mounted slides of FB SVZ, SGZ and HT SVZ. Stereoinvestigator software was used to quantify the number of IdU^+, $CldU^+$ and $IdU^{+/}CldU^+$ double positive cells.

Morris Water Maze

Hidden Platform: The set up and testing for the water maze was as previously described (Ziegler et al., 2019) (see also Supplementary Methods). Mice were tested in the Morris Water Maze for 5 consecutive days. Day 1 consisted of 1 pre-training session performed with the platform visible. For the pre-training session, mice were placed on the platform for 10 seconds, allowed to swim for 20 seconds, and then placed on the platform for another 10 seconds; each mouse received 4 trials. On days 2-5, mice received 2 training sessions per day. For each training trial, mice were placed in the maze and allowed 60 seconds to find the hidden platform, the latency to find the platform was measured for each trial. Each training session consisted of 6 trials with the mouse placed in a different quadrant of the maze each time, omitting placement in the TQ. On day 6, mice received a probe trial: the platform was removed, and mice were placed in the maze and allowed to explore for 60 seconds; the time spent in the TQ was measured.

Elevated Plus Maze

Briefly, the animal was placed on the EPM apparatus facing the open arm. The time was recorded, and the animal was tracked using a video camera placed on top of the EPM. The animal was tested on the EPM for a total of 5 mins. After this the animal was removed and placed back into the cage. AnyMaze software was used to track the total amount of time spent in the open arms, indicating less anxiety.

Expression analysis of RNAseq data

RNA was obtained from cultures of stem cells isolated from two glioblastoma tumors (code-named GB2, WCR8) and from one normal human neural stem cell culture (HNSC). Next-generation RNA-sequencing was performed in the Epigenomics Core Facility of the Albert Einstein College of Medicine, NY, using an Illumina HiSeq2500 machine. Detailed protocols for library preparation can be found at http://wasp.einstein.yu.edu/index.php/Protocol:directional_WholeTranscript_seq. Sequence reads were aligned to the human genome (hg19 build) using *gsnap* Genomic locations of genes and exons, as defined in Refseq, were extracted from the *refGene.txt* file (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/refGene.txt.gz). Read summarization at the gene level was done for all the genes in Refseq using the bam alignment files and in-house scripts, taking only reads with mapping quality of 20 or greater. The number of raw reads mapping to a gene was standardized to reads per kilobase per million reads (RPKM). The number of RPKM for the insulin receptor (INSR) and for the insulin-like growth factor 1 receptor (IGF1R) was extracted and the ratio of INSR to IGF1R was obtained for each sample.

References

Alagappan, D., Lazzarino, D.A., Felling, R.J., Balan, M., Kotenko, S.V., and Levison, S.W. (2009). Brain injury expands the numbers of neural stem cells and progenitors in the SVZ by enhancing their responsiveness to EGF. ASN NEURO 1, e00009

Buono, K.D., Vadlamuri, D., Gan, Q., and Levison, S.W. (2012). Leukemia inhibitory factor is essential for subventricular zone neural stem cell and progenitor homeostasis as revealed by a novel flow cytometric analysis. Dev Neurosci 34, 449-462

Tuttle, A.H., Rankin, M.M., Teta, M., Sartori, D.J., Stein, G.M., Kim, G.J., Virgilio, C., Granger, A., Zhou, D., Long, S.H.*, et al.* (2010). Immunofluorescent detection of two thymidine analogues (CldU and IdU) in primary tissue. Journal of visualized experiments : JoVE

Velloso, F.J., Kumari, E., Buono, K.D., Frondelli, M.J., and Levison, S.W. (2022). Analyzing mouse neural stem cell and progenitor cell proliferation using EdU incorporation and multicolor flow cytometry. STAR Protocols 3, 101065

Ziegler, A.N., Feng, Q., Chidambaram, S., Testai, J.M., Kumari, E., Rothbard, D.E., Constancia, M., Sandovici, I., Cominski, T., Pang, K.*, et al.* (2019). Insulin-like Growth Factor II: An Essential Adult Stem Cell Niche Constituent in Brain and Intestine. Stem Cell Reports 12, 816-830