Stem Cell Reports, Volume *8*

Supplemental Information

HuCNS-SC Human NSCs Fail to Differentiate, Form Ectopic Clusters, and Provide No Cognitive Benefits in a Transgenic Model of Alzheimer's Disease

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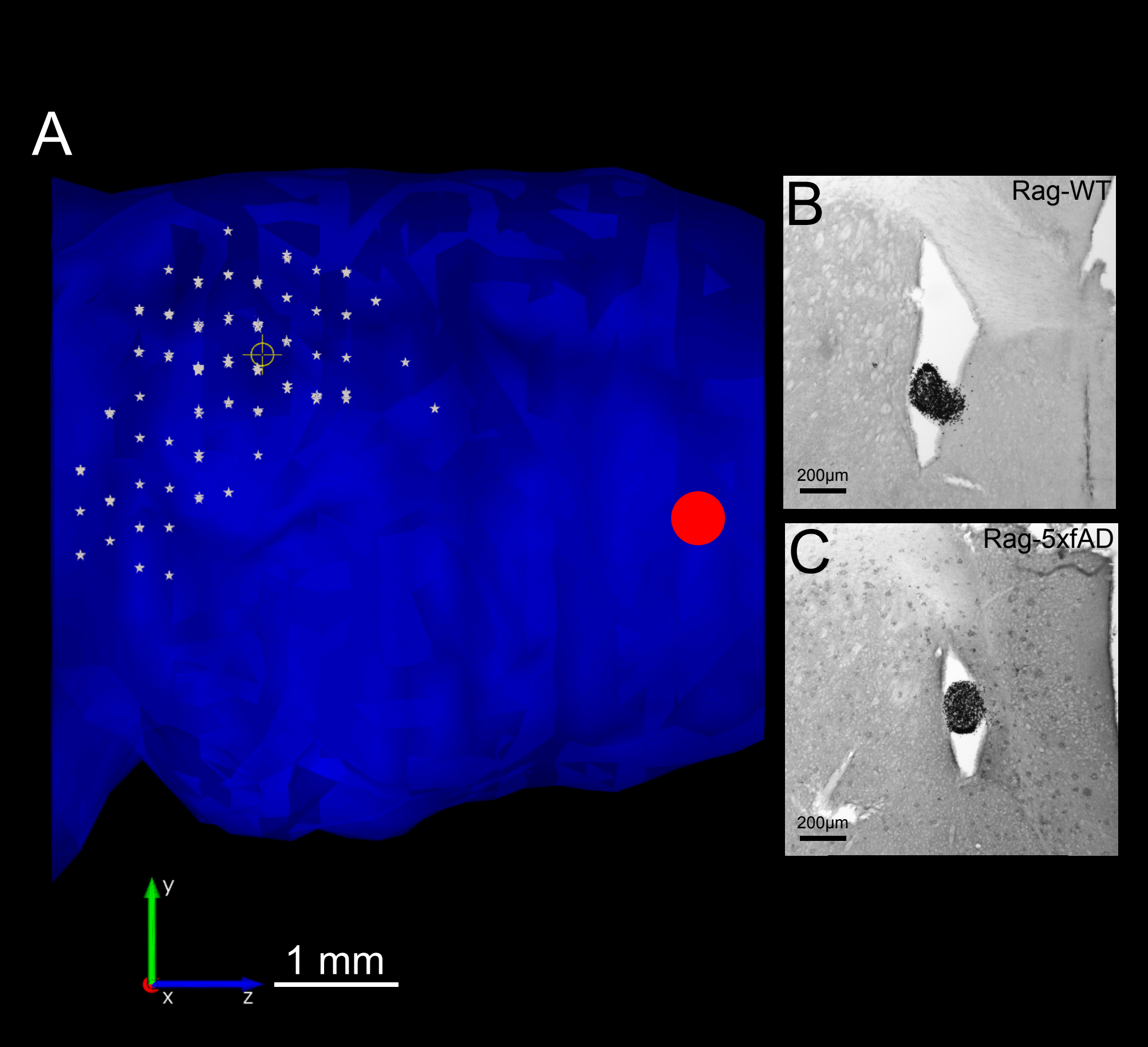
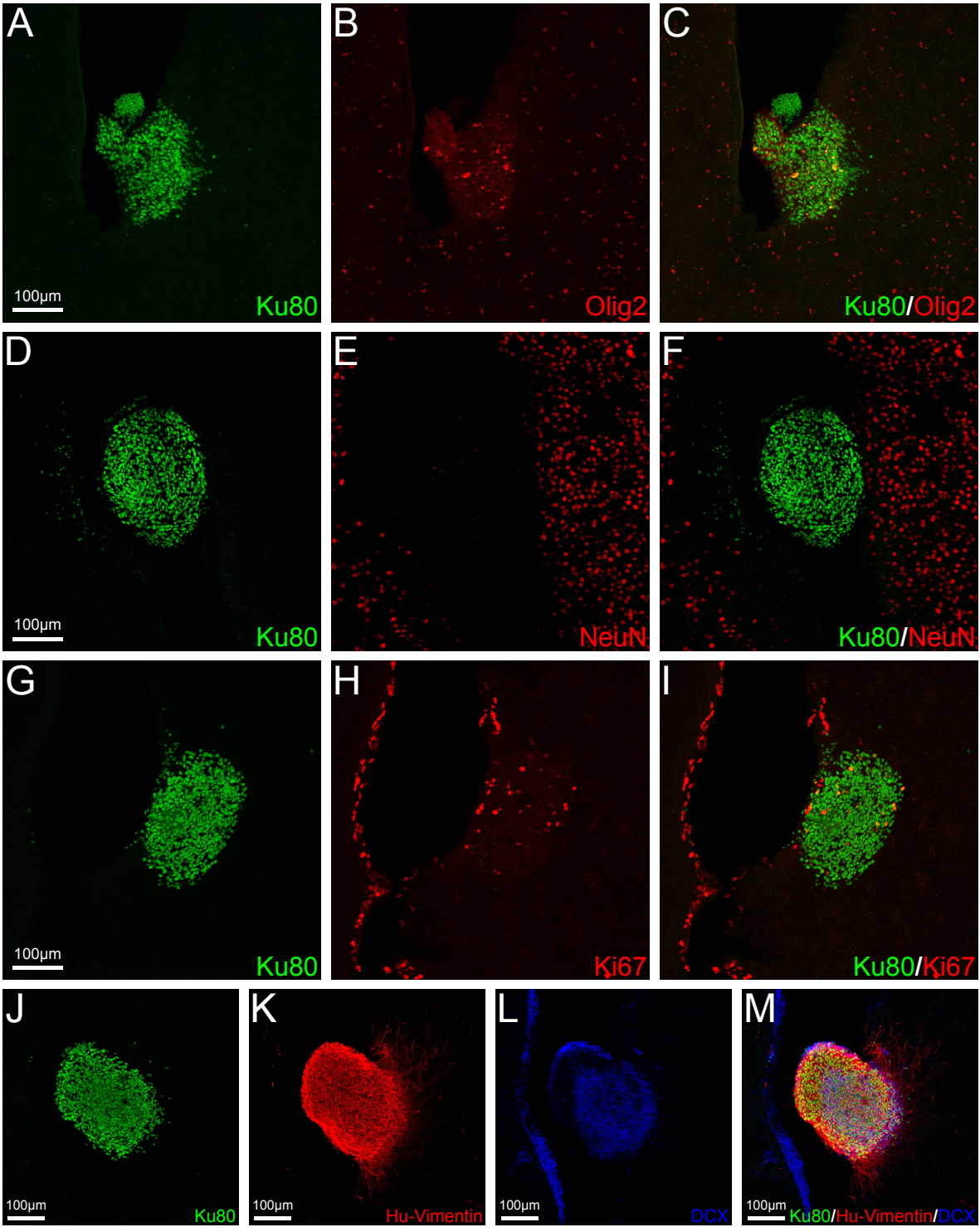

A
B
Rag-WT
200µm
C
Rag-5xfAD
200µm
y
x
z
1 mm


A
100μm
Ku80
B
Olig2
C
Ku80/Olig2
D
100μm
Ku80
E
NeuN
F
Ku80/NeuN
G
100μm
Ku80
H
Ki67
I
Ku80/Ki67
J
100μm
Ku80
K
100μm
Hu-Vimentin
L
100μm
DCX
M
100μm
Ku80/Hu-Vimentin/DCX

Supplemental Figure Legends:

Figure S1: 3D Reconstruction of the location of ventricular cell clusters and intraparenchymal HuCNS-SC engraftment (Related to Figure 3). (A) Side view of Supplemental Movie 1. 3D surface reconstruction of mouse half brain and migration span of HuCNS-SCs based on sections utilized for unbiased stereological analysis. White stars represent individual cells counted in randomly sampled counting frames during unbiased stereological analysis. Yellow bullseye represents location of injection site as verified during stereological analysis. Red sphere represents approximate anterior/posterior localization of ectopic cells clusters found in 5 of 18 animals. **(B-C)** Representative light level immunolabeling of Ku80+ HuCNS-SC within the anterior portion of the lateral ventricle in both Rag-WT **(B)** and Rag-5xfAD **(C)** animals. **(A)** Scale Bar = 1mm, **(B-C)** 200μm.

Figure S2: A small percentage of ventricular-localized HuCNS-SC express Ki67 or Olig2, but not NeuN (Related to Figure 3). (A-C) Unlike engrafted cells within the hippocampus, which are nearly 100% positive for immature oligodendrocyte marker Olig2, only a small number of HuCNS-SC within the ventricles co-label with Olig2. **(D-F)** HuCNS-SC ventricular clusters also do not express the mature neuronal marker NeuN. **(G-I)** Clusters exhibit low levels (3.7%) of Ki67 reactivity. **(J-M)** Representative images demonstrating the co-labeling of glial and neuronal markers within the ventricular clusters. (**J-K)** Are the same images shown in **D-F of Figure 3** demonstrating here the co-expression of the glial marker vimentin and **(L-M)** the neuronal marker doublecortin (DCX). Scale Bar =100μm.

Movie S1: 3D Reconstruction of HuCNS-SC engraftment in a Rag-WT mouse (Related to Figure 1). 3D surface reconstruction of mouse half brain and migration span of HuCNS-SC based on sections utilized for unbiased stereological analysis of HuCNS-SC engraftment. White stars represent individual cells counted in randomly sampled counting frames during unbiased stereological analysis. Yellow bullseye represents location of injection site.

Movie S2: 3D Reconstruction of ventricular HuCNS-SC cells infiltrating the adjacent striatum (Related to Figure 3). 3D surface reconstruction of confocal z stack shown in Panel L of Figure 3. Ku80+ cells (red) can be seen in an ectopic cluster within the lateral ventricle. Ventricular-localized HuCNS-SC cells are positive for S100β and the host ventricle wall also highly expresses S100β, clearly delineating its boundaries. As the image rotates, a clear example of disruption of the ventricle wall (lack of S100β) is accompanied by infiltration of HuCNS-SCs beyond the ventricular boundary and into the surrounding parenchyma.

Supplemental Experimental Procedures:

Mice

Briefly, 5xfAD mice (MMRRC Strain: 034848-JAX), express two co-integrated and co-inherited mutant human transgenes (*APP* and *PSEN1*) under control of the murine Thy1.2 promoter (Oakley et al., 2006; Jawhar et al., 2012; Eimer and Vassar, 2013). Purebred C57Bl6 5xfAD were backcrossed with Rag2/il2rγ double knockout mice (Taconic #4111) (Cao et al., 1995), followed by repeated littermate crosses from each generation, to create mice that are heterozygous for the *APP*/*PSEN1*transgenes and lack both copies of the *RAG2* and *IL2RG* genes. The resulting mice, termed Rag-5xfAD, exhibit characteristic AD plaque pathology, microgliosis, and astrocytosis**,** but lack T-cells, B-cells, and NK-cells that normally mediate xenotransplant rejection (Marsh et al., 2016). Immune-incompetent sex- and age-matched littermate controls hereafter referred to as Rag-WT were also used. Mice were group housed (2-4 mice/cage) on a 12h/12h light/dark cycle with access to food and water *ad libitum*. All animal procedures were performed in strict accordance to the National Institutes of Health and University of California animal care and use guidelines.

Stereotactic HuCNS-SC Transplantation Surgery

Prior to surgeries, cells were counted and assessed for viability and then resuspended in injection buffer (X-VIVO 15 media (Lonza; Basel, Switzerland) with anti-oxidant supplement, generously provided by Stem Cells Inc.) at a concentration of 50,000 cells/µl. Cell viability measured before surgery was 85.9% and 82.6% on days 1 and 2 respectively. Vehicle and cell surgeries were performed on both Rag-5xfAD and Raw mice on both days of surgery. Surgeries were performed on 2 month old mice using hippocampal coordinates AP: -2.06, ML: ± 1.75, DV: - 1.95 relative to Bregma. Accuracy of injection sites was confirmed by subsequent post-mortem visualization of the needle tract in coronal brain sections of all animals. Cells were bilaterally injected using a 10µl Hamilton microsyringe (30-gauge), in volume of 2µl (100,000 cells total/hemisphere; 200,000 cells/animal) at rate of 1 µl/min. Following the injection the syringe remained in place for a period of 4 minutes before being slowly withdrawn to minimize potential reflux of the cells along the needle track. Surgical incisions were closed with Tissuemend (VetEquip) and mice were allowed to recover on a heating pad before being placed back in to their home cage. Following completion of the surgeries, the remaining cells were again counted and assessed for viability for comparison to pre-surgical counts. Cell viability following completion of surgeries (~3.5 hours post-initial count) was 76.3% and 77.3% on days 1 and 2 respectively (5-10% decreased viability).

Morris Water Maze

The task consists of a pool of opaque white water to obscure the location of hidden escape platform submerged just beneath the water's surface. On all walls surrounding the pool were high-contrast, easily visible spatial cues. Mice were placed in pool at randomly assigned starting positions and allowed to swim freely until they reached the platform or 60 seconds elapsed, which ever came first. If the mouse found the platform they were allowed to remain there for 15 seconds before being removed to a pre-warmed holding cage. If the mouse failed to find the platform, they were gently guided by the experimenter to the platform and remained there for 15 seconds before being returning to the warming cage. Mice were given 4 trials per day and placed at different starting positions for each trial. The order of these starting positions was semi-randomly chosen every day. After 5 days of training, the platform was removed and a 24-hour probe trial was conducted. Mice were placed in the pool at starting position directly opposite from the platform's previous location and allowed to swim freely for 60 seconds and then removed and placed in a warming cage.

Novel Arm Y-Maze

Novel Arm Y-maze examines spatial working memory. Each arm of standard Y-maze was covered in different high contrast patterns. For training, mice were placed in the start arm of the maze with one of the choice arms blocked. Mice were allowed to freely explore the open arms for 7 minutes. The arm blocked during training was controlled for evenly within each treatment group to account for any bias to arm pattern or turning preference. Following completion of the training trial mice were removed and placed back in their home cage for 30 minutes. For the testing trial, mice were returned to the start arm with all arms open and allowed to freely explore for 7 minutes. In between all trials, the maze and walls were cleaned with 70% ethanol and dried to prevent odor cues from affecting performance.

Elevated Plus Maze

A standard mouse elevated plus maze (Stoelting Co.) was used to measure anxiety. The maze consists of four equal length arms which measured 35 x 5 cm and was raised 40cm above the ground. The two closed arms were enclosed with 15cm high walls. Mice were placed in the center of maze facing an open arm and allowed to freely explore the maze for 5 minutes in low light conditions (14 Lux). In between all trials, the arms and walls of the maze were cleaned with 70% ethanol and dried to prevent odor cues from affecting performance.

Tissue Processing

Immediately following perfusion, brains were quickly removed and hemispheres separated. The cerebellum was removed and then the right hemisphere was immediately flash-frozen in dry ice for subsequent biochemical analysis. The left hemisphere was drop-fixed in 4% paraformaldehyde for 48 hours at 4˚C. After 48 hours postfixation, the left hemispheres were transferred to 0.01M PBS and 0.02% NaN_3 for storage until sectioning. Brains were submerged in 30% sucrose for 48 hours prior to sectioning coronally on a freezing microtome at 40µm thickness. Free-floating sections were then placed back in 0.01M PBS and 0.02% NaN_3 for storage until used for histology.

Tissue Processing for Biochemical Analyses

Right hemispheres, previously frozen on dry ice and stored at -80˚ were microdissected and whole hippocampus was removed and homogenized in a solution of T-PER (Pierce) with phosphatase and protease inhibitor cocktails (Thermo Scientific & Roche) and then spun at 10,000 x g for 15 minutes at 4˚. The supernatant (soluble fraction) was extracted and stored at -80˚ for further biochemical analysis. For Aβ analysis the insoluble pellet was homogenized with 70% formic acid and spun at 100,000g for 1 hour. Supernatant (insoluble fraction) was removed and stored at -80˚ until further analysis. Prior to biochemical analyses volumes of insoluble fraction were neutralized with buffer containing 0.5M Tris-base, 0.25M Na_2HPO_4, and 5N NaOH.

Immunohistohemistry

Fluorescent immunohistochemical analysis followed previously described established protocols [Goldberg et al., 2015; Marsh et al., 2016]. Briefly, sections were incubated in primary antibodies overnight followed by detection with appropriate Alexa Fluor® conjugated secondary antibody (Invitrogen; Carlsbad, CA) and coverslipped using Fluoromount-G with or without DAPI (Southern Biotech).

Antibodies utilized were: Ku80 (human nuclei, Abcam; Cambridge, MA; ab80592 & ab79220), GFAP (Millipore; Billerica, MA; MAB360), GFAP (Dako; Carpinteria, CA; Z0334), Doublecortin (Santa Cruz Biotechnology; Dallas, TX; sc-8066), Olig2 (Millipore; MABN50), APC (CC-1) (Millipore; OP80-100UG), PSD95 (Abcam; ab13552), Vimentin (Dako; M0725), CD44 (Stem Cell Technologies; Vancouver, BC; #60068), S100β (Abcam; ab4066), LIN28 (Cell Signaling; Danvers, MA; #8706), NeuN (Millipore; ABN78), Ki67 (Abcam; ab16667). Negative controls were performed with omission of primary and secondary antibodies to verify the specificity of each antibody.

Confocal Microscopy and Immunohistochemical Analysis

Immunofluorescent sections were visualized and images captured using an Olympus FX1200 confocal microscope. To avoid non-specific bleed through each laser line was excited and detected independently. All images shown represent either single confocal *z*-slice or *z*-stacks. All image analyses were conducted by a blind observer using coded images

Analysis of HuCNS-SC differentiation was performed using Fluoview software (Olympus) by first overlaying images with 3x3 grid (~175µm x 175µm) followed by digital zoom to analyze one grid square at a time. Using both maximum projection images and orthogonal view the total number of Ku80+ and double-labeled HuCNS-SC were counted one grid square at a time. There was no significant difference between total number of Ku80+ cells counted between genotypes in either of the regions analyzed (data not shown) and average number of Ku80+ cells counted per region was ≥79.

High magnification confocal images of post-synaptic density (PSD95) immunolabeled sections were visualized with an Olympus FX1200 confocal microscope using the 40x oil objective with 5x digital zoom. 1-µm z-stack images were captured using 0.25µm step size in 4 randomly selected ROIs within the stratum oriens of the hippocampus. Post-synaptic puncta were counted using IMARIS (Bitplane; Concord,

MA) image analysis software using the "Spot" function. Identical settings used for spot analysis were for analysis of all images and all capture and analysis was performed by blind observer using coded images.

Light-level immunohistochemistry & Unbiased Stereological Quantification of HuCNS-SC Engraftment

To quantitatively measure HuCNS-SC engraftment following transplant every sixth coronal section was collected and incubated with an antibody against the human nuclear marker Ku80 (Abcam) overnight followed by incubation with biotin-conjugated secondary antibody. Antibody labeling was visualized using Vectastain Elite ABC kit (Vector Labs; Burlingame, CA) followed by 3,3'-diaminobenzidine (DAB) with Nickel Peroxidase (HRP) Substrate Kit (Vector Labs) before being mounted and coverslipped using DPX (DBH) mounting medium (VWR, Radnor, PA).

All unbiased stereological analysis was performed using Stereo Investigator software (MBF Bioscience; Williston, VT) on a Zeiss AXIO Imager.M2 microscope. Protocol for HuCNS-SC engraftment was adapted from previously published stereological protocols used for neuronal and stem cell quantification [Baglietto-Vargas et al., 2010; Ager et al., 2015].

Briefly, brain regions were outlined using the 2.5x/0.075 objective and all cell counts were performed using the 100x/1.4 objective. Briefly an optical fractionator probe was utilized to estimate the total number of engrafted HuCNS-SC. Stereological quantification was performed throughout the entire rostral/caudal extent of stem cell engraftment within the hippocampus and dorsal cortex. A counting frame of 50 x 50μm in a sampling grid of 350 x 350 μm was used for cell quantification. Guard zone height for both top and bottom was set at 3 μm with an optical dissector height of 10 μm. Using the optical fractionator formula, in which $N = 1/ssf.1/asf.1/hsf.\sum Q$, where ssf represents the section sampling fraction, asf is the sampling fraction, which is calculated by dividing the area sampled with the total area of the layer, hsf stands for the height sampling fraction, which is calculated by dividing the height sampled (10 μm in this study) with the section thickness, and $\sum Q$ is the total count of nuclei sampled for the entire area. The accuracy of the individual estimation was expressed by the total coefficient of error (CE) calculated using the CEs in each individual animal, with acceptable CE ranged between 0.02 and 0.09.

Multiplex ELISAs

Quantitative biochemical analysis of Aβ and BDNF were conducted using commercially available electrochemiluminescent multiplex assay system (Meso Scale Discovery (MSD); Gaithersburg, MD). Hippocampal lysates were analyzed using Human Aβ triplex for simultaenous measurement of Aβ38, Aβ40, and Aβ42 and a BDNF MSD ELISA assay was used to measure BDNF levels.

Independent histopathology examination by a contract research organization

To provide independent assessment of the HuCNS-SC-derived clusters located within the ventricles, both labeled and unlabeled sections were sent to Charles River, a contract research organization (CRO). Raw confocal image files for all images shown in Figure 6 and a software viewer were also provided. A Charles River veterinary pathologist performed standard Hematoxilin/Eosin staining of unlabeled sections and also examined a subset of the existing slides and images.