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Supplemental Information

Human Neural Stem Cell Transplantation Rescues Functional Deficits in R6/2 and Q140 Huntington's Disease Mice

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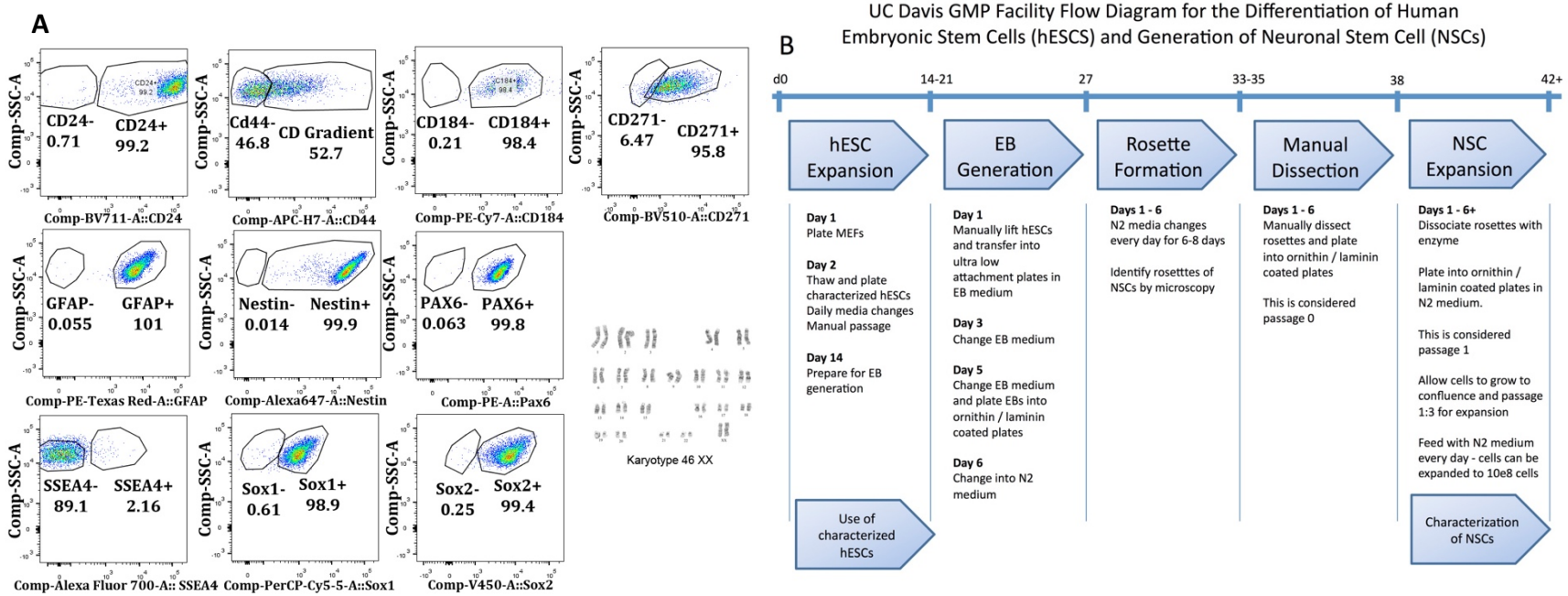
Supplemental Data/Supplemental Figures



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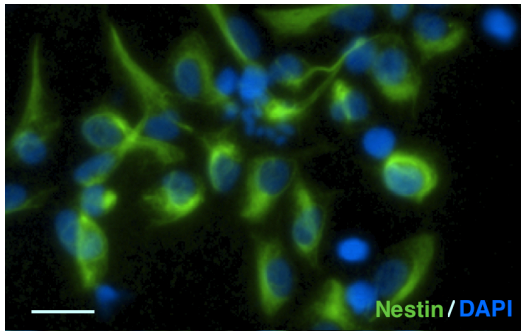


Fig. S1

Fig. S1: A) Characterization of ESI-017 hNSCs by Single Color Flowcytometry: ESI-017 hNSCs stain positive for CD24, SOX1, SOX2, Nestin and Pax6 NSC markers. ESI-017 hNSCs stain negative for the pluripotent marker SSEA4. Karyotyping on ESI-017 hNSCs was performed and metaphases were visualized by Giemsa staining of condensed chromosomes. The final Karyotype was shown to have a high mitotic index with a 46,XX normal profile. **B) Flow Diagram of the NSC manufacturing process:** hNSCs are generated by embryoid body (EB) formation, followed by plating of the generated EBs into poly-ornithin-laminin (Poly-O) coated plates with subsequent neural rosette formation. Rosettes are manually dissected and transferred into fresh Poly-O plates, where they are allowed to attach. Expanded neural rosettes are then enzymatically dissected, followed by plating into fresh Poly-O plates. There the cells are allowed to grow to confluence and are passaged enzymatically into larger number of Poly-O plates. Final harvest and cryopreservation of generated hNSCs is performed after expansion to sufficient numbers. **C) Cultured ESI-017 hNSC Immunocytochemistry** shows positive NSC staining for neuralectodermal stem cell marker Nestin in green and DAPI nuclear staining in blue. Scale bar equals 30 μm.

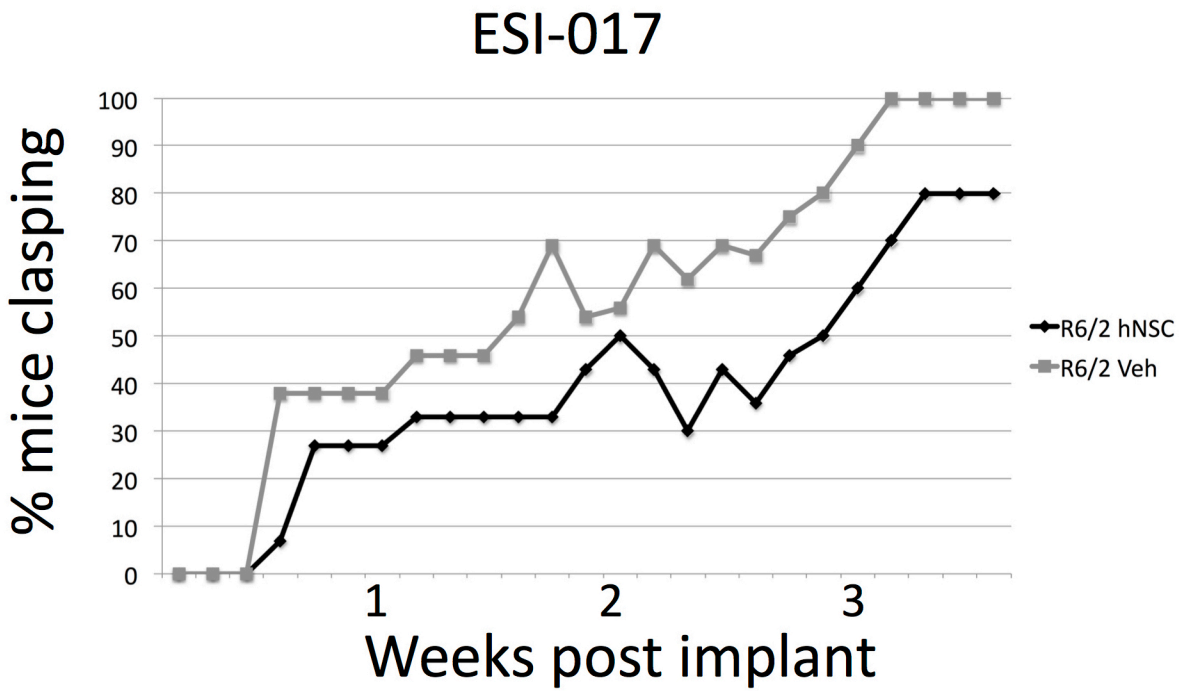
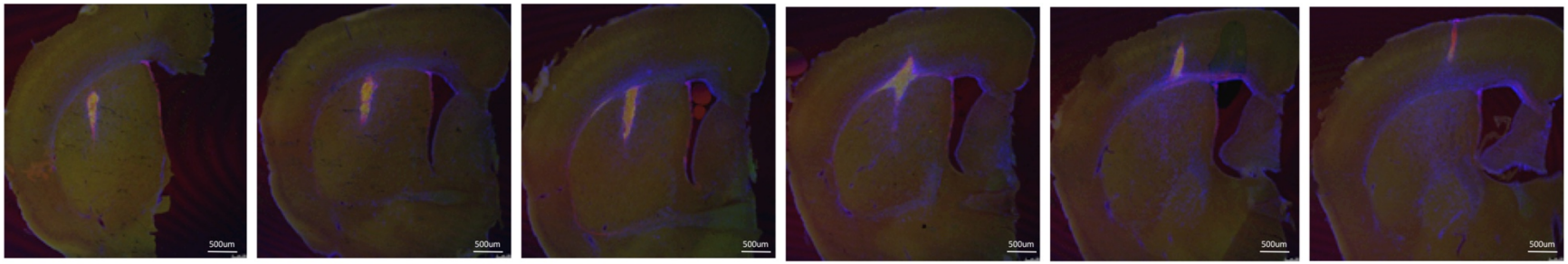


Fig. S2

Fig. S2: Clasping behavior: R6/2 mice treated with ESI-017 hNSCs (n=15) show delayed clasping behavior post implant. Non-transgenic (NT) mice do not demonstrate this phenotype. Mice were tested daily for the phenotype and graphs depict percentage of each group clasping over the course of the study. Significance in the clasping assay was determined by Fisher's exact probability test.



S1 S2 S3 S4 S5 S6

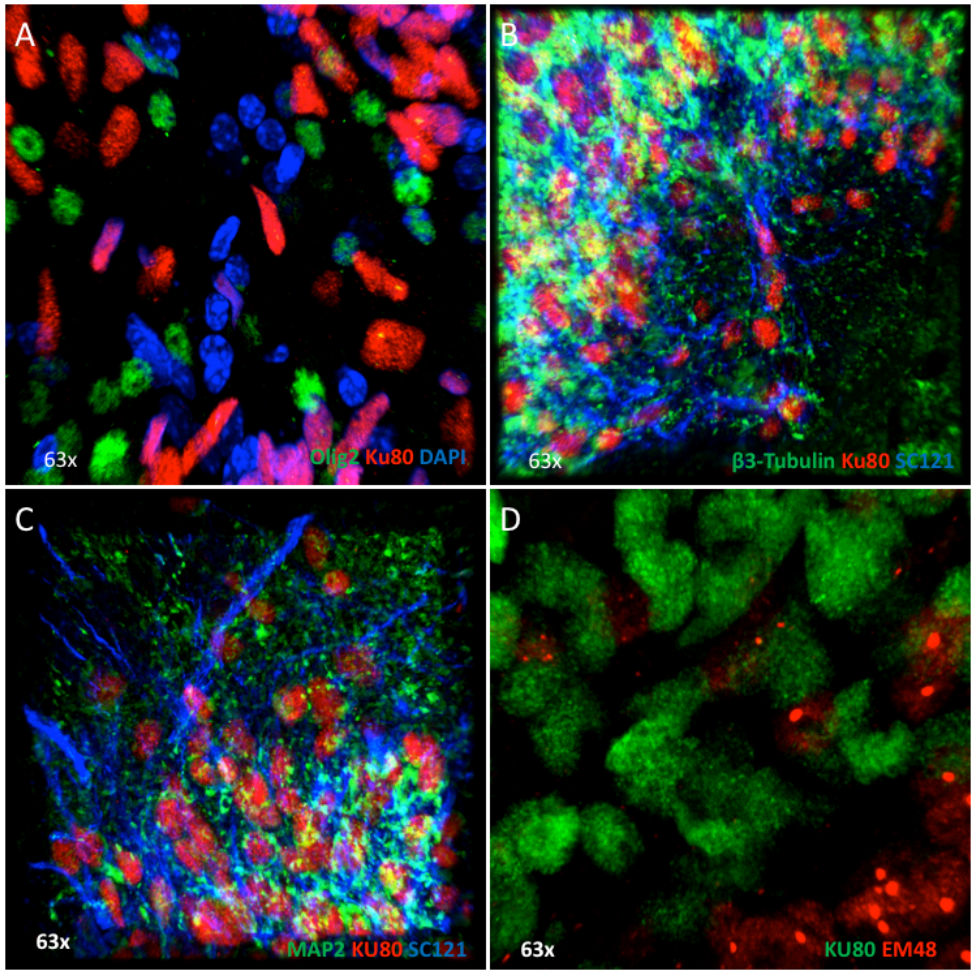
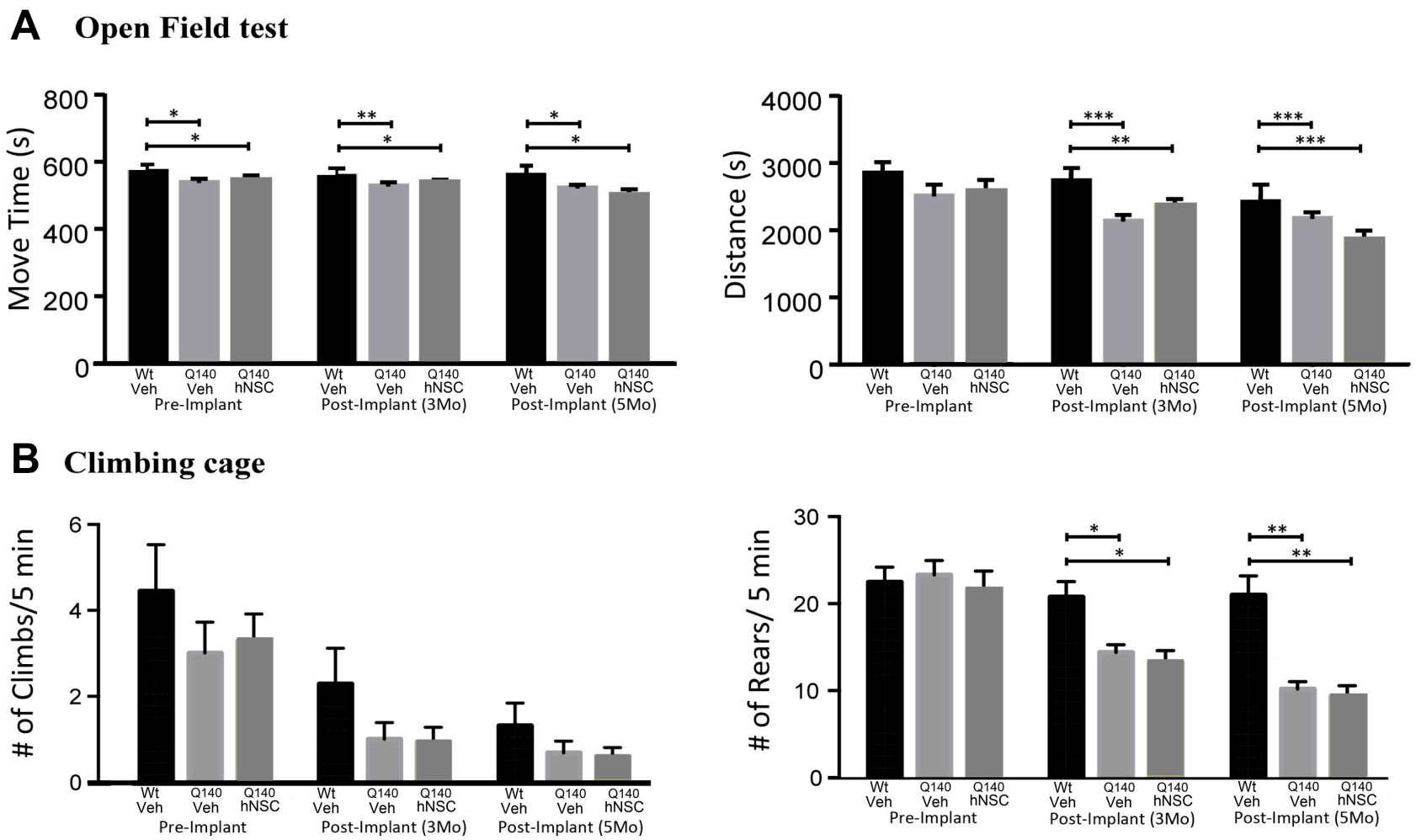


Fig. S3

Fig. S3: Low magnification Immunohistochemistry of ESI-017 hNSC implanted R6/2 mice: hNSCs (human marker SC121, green) implanted in R6/2 mice co-localize (yellow) with marker for neuron restricted progenitors (doublecortin DCX, red). S1-6 shows coronal sections collected and immuno-stained starting at bregma 1.70mm, 40um per section. To screen for hNSC, IHC is performed on sections #34, 37, 40, 43, 46, and 49 (equivalent to Bregma 0.38mm, 0.26mm, 0.14mm, 0.02mm, -0.10mm, and -0.22mm, respectively). S2 is a re-use of the image shown in figure 1D for a comparison to other coronal sections. **ESI-017 hNSC implant in R6/2 mice Immunohistochemistry**: **A)** hNSCs (human marker Ku80, red) implanted in R6/2 mice do not co-localize with an oligodendrocyte marker (Olig2, green) mouse cell nuclei shown with DAPI in blue. High magnification (63x) showing differentiation: **(B)** hNSCs (human nuclear marker Ku80, red and cytosolic marker SC121 blue) shows co-localization (lt. blue) with neuron restricted progenitors (BIII-tubulin, green). **C)** hNSCs (human nuclear marker Ku80, red and cytosolic marker SC121 blue) shows co-localization (lt. blue) with neuron restricted progenitors (MAP-2, green). **D)** hNSCs (human nuclear marker Ku80, green) do not co-localize with huntingtin marker (EM48, red).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Open Field test
Move Time (s)
800
600
400
200
0
Distance (s)
4000
3000
2000
1000
0
Wt Veh
Q140 Veh
Q140 hNSC
Pre-Implant
Post-Implant (3Mo)
Post-Implant (5Mo)
B
Climbing cage
# of Climbs/5 min
6
4
2
0
# of Rears/ 5 min
30
20
10
0

Fig. S4

Fig. S4: ESI-017 hNSCs implanted into the striatum did not improve deficits in Open field or Climbing cage tests in Q140 mice. Mice were tested in the open field (**A**) for 15 minutes and climbing cage for 5 minutes (**B**) at 0.5 months pre-implant, or 3 and 5 months post implant. Data are represented as the mean ± SEM; Wt Veh (n=18), Q140 Veh (n=18), and Q140 hNSC (n=17). Two-way ANOVA with Bonferroni post-test *$p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to same time point of Vehicle-treated Wt mice.

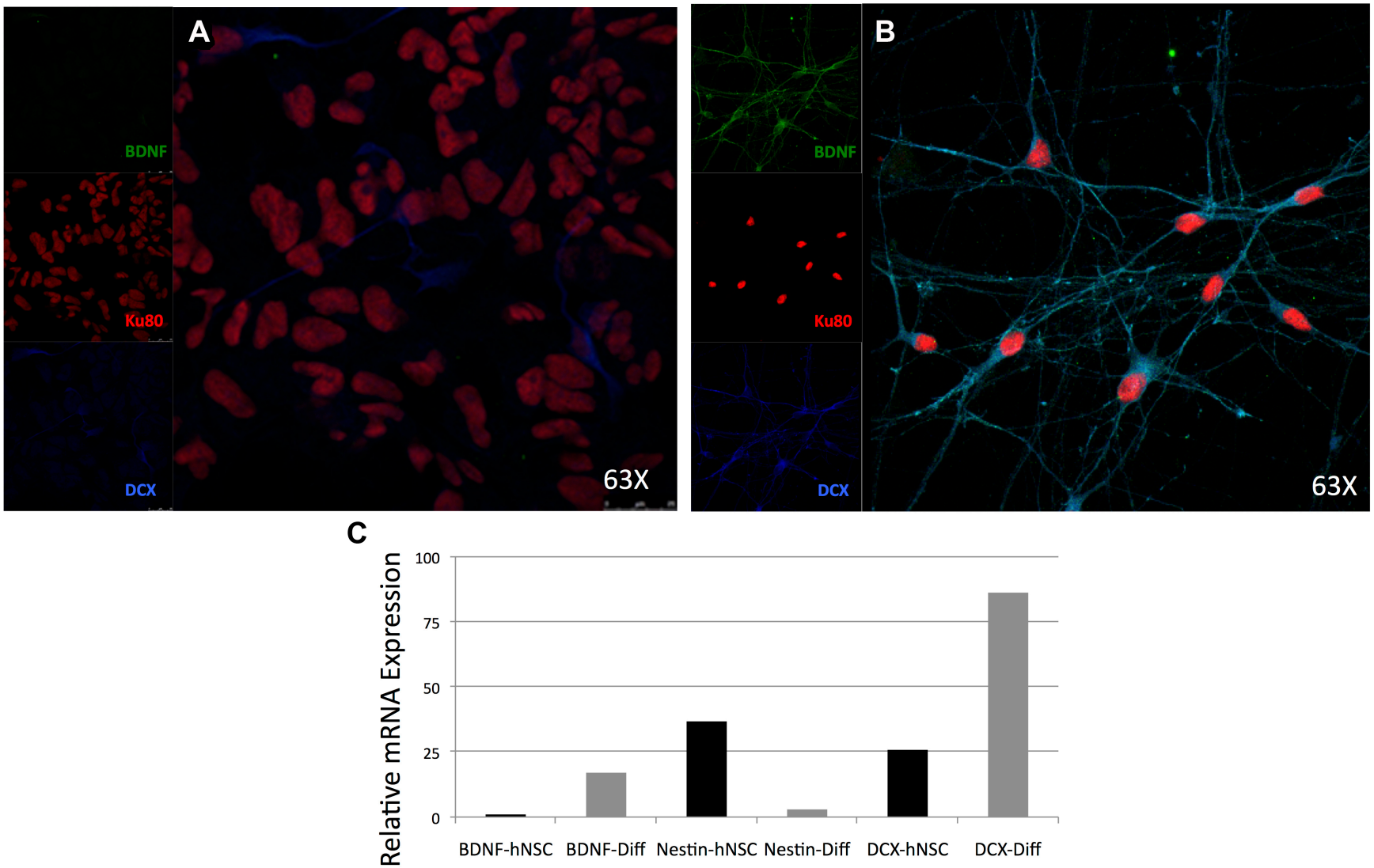
A
BDNF
Ku80
DCX
63X
B
BDNF
Ku80
DCX
63X
C
Relative mRNA Expression
100
75
50
25
0
BDNF-hNSC
BDNF-Diff
Nestin-hNSC
Nestin-Diff
DCX-hNSC
DCX-Diff

Fig. S5

Fig. S5: ESI-017 hNSC BDNF expression *in vitro*. ESI-017 hNSCs were cultured in neural stem cell media (**A**) or differentiated (**B**) then stained for BDNF (green) human nuclear marker Ku80 (red) and doublecortin DCX (blue). (**C**) qPCR comparing RNA levels from cultured ESI-017 hNSCs show BDNF expression increased with differentiation. For comparison the stem cell marker nestin decreased with differentiation and DCX increased.

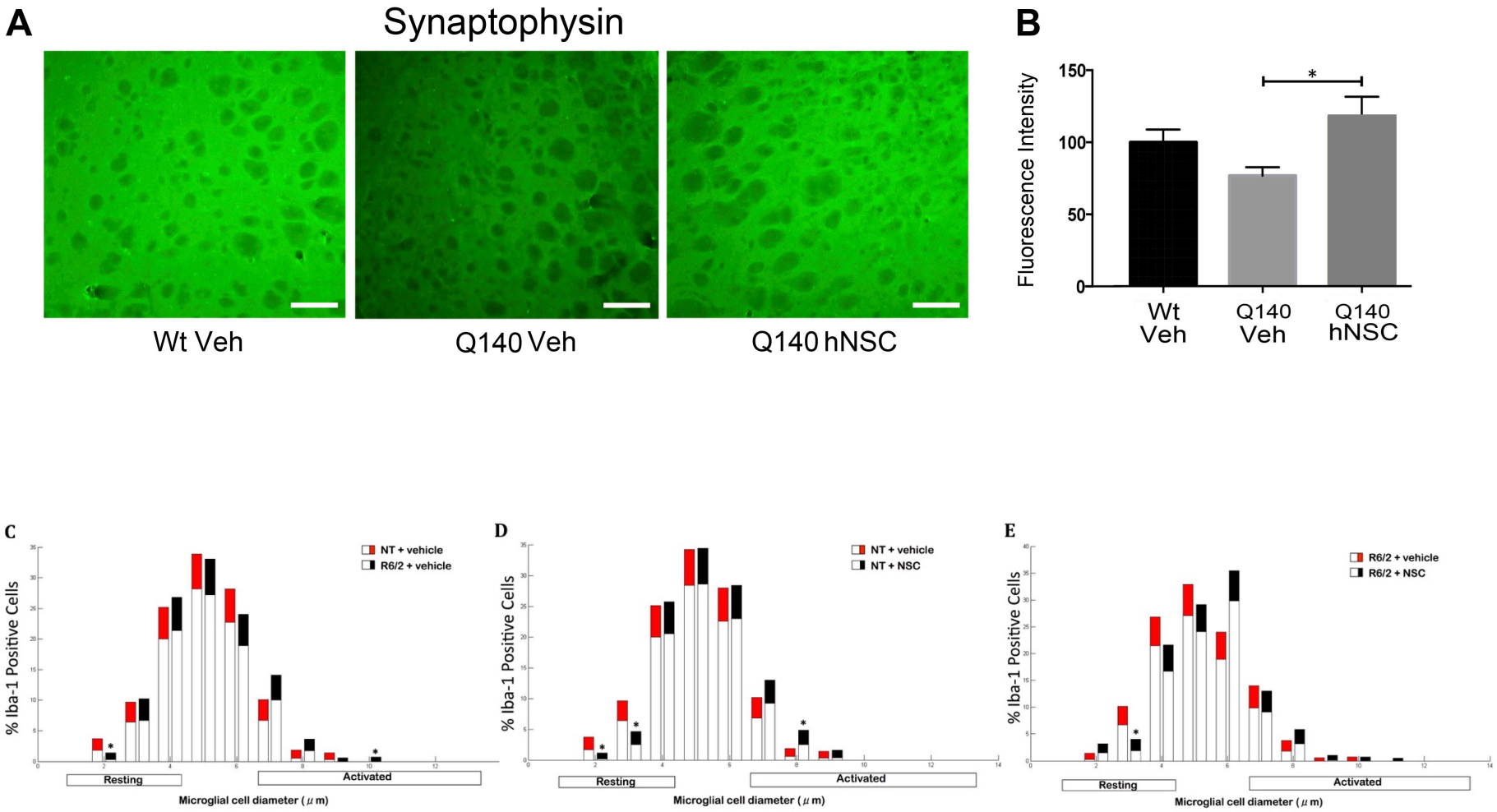
A
Synaptophysin
Wt Veh
Q140 Veh
Q140 hNSC
B
Fluorescence Intensity
150
100
50
0
*
Wt Veh
Q140 Veh
Q140 hNSC
C
% Iba-1 Positive Cells
NT + vehicle
R6/2 + vehicle
Resting
Activated
Microglial cell diameter (μm)
D
% Iba-1 Positive Cells
NT + vehicle
NT + NSC
Resting
Activated
Microglial cell diameter (μm)
E
% Iba-1 Positive Cells
R6/2 + vehicle
R6/2 + NSC
Resting
Activated
Microglial cell diameter (μm)

Fig. S6

Fig. S6. A&B) Synaptophysin levels are increased in the striatum of Q140 mice with ESI-017 hNSCs. A) Images were taken with a microarray scanner and quantified for fluorescence intensity. White scale bar equals 10 μm. (**B**) Data are shown as mean±SEM and statistical test used was One-way ANOVA with Bonferroni post-test *$p<0.05$, n=5 mice per group. **hNSC treatment in R6/2 mice does not alter microglial activation.** Data are represented as the mean +95% confidence interval (n=5 per group). Bars represent percent cells of each diameter and the colored portion represents the confidence interval. (**C**) Significant striatal microglial activation observed in R6/2 mice treated with vehicle (R6/2 Veh) compared to Non-transgenic control (NT Veh). (**E**) R6/2 mice treated with hNSCs (R6/2 NSC) showed no significant reduction of microglial activation in striatum compared to R6/2 Veh mice.

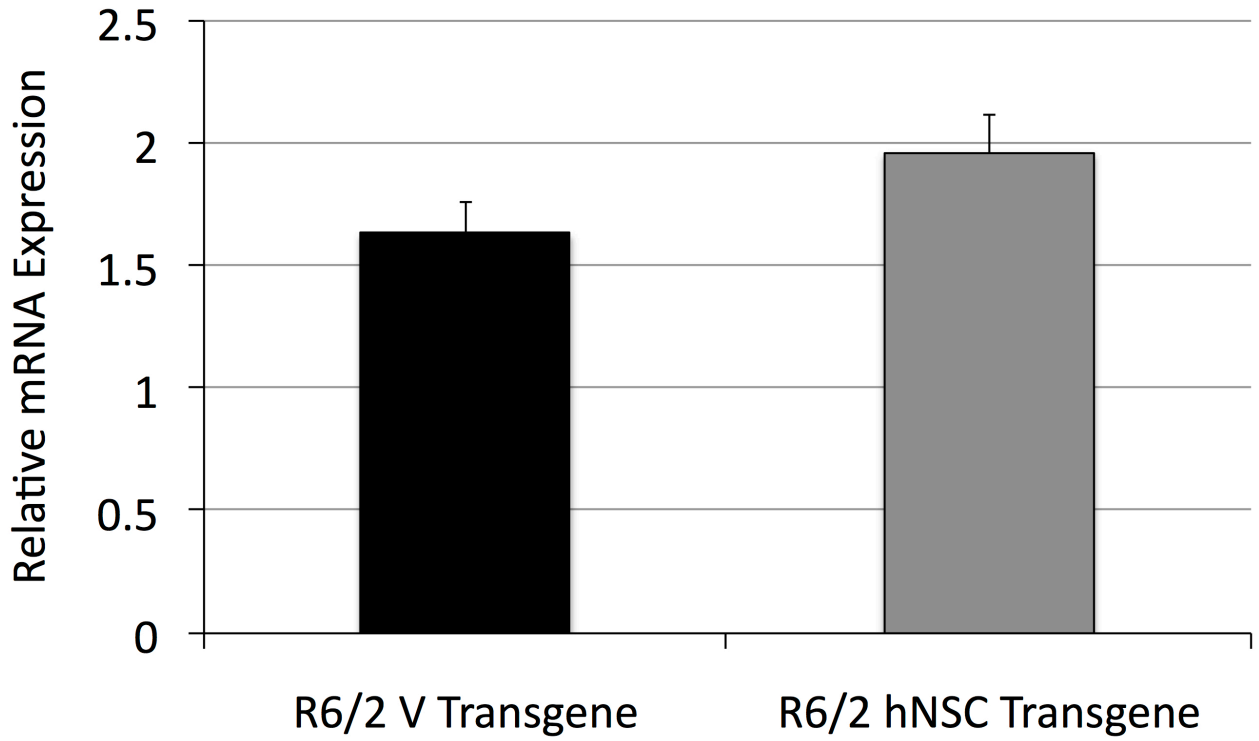

Fig. S7

Fig. S7: Real-time PCR of human HTT transgene expression in R6/2 mice. RPLPO (Large Ribosomal Protein) endogenous control was used to normalize gene expression differences in cDNA samples. No significance was observed as determined by one-way ANOVA with Bonferroni post-testing

Supplemental Experimental Procedures

hNSC isolation. Daily (D) culturing was as follows. D1: ESC colonies enzymatically "loosened" using Collagenase IV until colony edges began lifting. Colonies were manually scraped from wells, transferred to low attachment plates and cultured in EB medium (ESC medium minus bFGF) overnight. D2: EB culture media was supplemented with 500ng/ml Noggin plus 10μM SB431542 and cultured for 2 more days. D4: Media change. D5: EBs plated onto growth factor reduced matrigel coated 6-well plates in same medium. D6: Media changed and NP medium used to drive NPC differentiation. Media changed every two days until the twelfth day. D12-14: Rosettes visually isolated under dissecting scope, manually dissected out using an 18 gauge needle, and plated into growth factor reduced matrigel coated 6-well plates in NSC medium. 2-3 days later, rosettes were dissociated using accutase and plated onto polyornithine/laminin coated plates with NSC medium plus Y27632 compound. ESI-017 hNSC cytogenetic analysis found cells to be karyotypically stable with no observed abnormalities and single color flow-cytometry was performed for CD271 (Brilliant Violet 510-- BD Horizon cat# 563451), CD24 (Brilliant Violet 711-- BD Horizon cat# 563401), Pax6 (PE-- BD Pharmingen cat#561552), Nestin (Alexa Fluor 647-- BD Pharmingen cat#560341), SOX1 (PerCP CY5.5-- BD Pharmingen cat# 561549), SOX2 (V450--BD Horizon cat#561610), CD44 (APC-H7-- BD Pharmingen cat#560532), CD184 (PE-CY7-- Biolegend cat#306514) or SSEA4 (lexa Fluor 700-- Invitrogen cat#SSEA429).

Transplantation Surgery. Bilateral intra-striatal injections of hNSCs or vehicle were performed using a stereotaxic apparatus and the following coordinates relative to Bregma AP: 0.00 ML: +/- 2.00, and DV -3.25. Mice were placed in the stereotaxic frame and injected with either 100,000 hNSCs per side (2 μl/injection) or vehicle (2 μl HBSS with 20 ng/ml hEGF and hFGF) as a control treatment using a 5 μl Hamilton microsyringe (33-gauge) and an injection rate of 0.5 μl/min. R6/2 mice were anesthetized with isoflurane, Q140 mice were anesthetized with sodium pentobarbital (60 mg/kg Nembutal in sterile 0.9% saline, i.p.). For all mice; to maintain a surgical plane of anesthesia mice were administered isoflurane (1-2% in 100% oxygen, 0.5L/min) via a nose cone, oxygen was administered throughout surgery and

temperature of mice was maintained on an electronically controlled heat pad and monitored using a rectal probe thermometer (Physitemp). Accurate placement of the injection to the targeted region was confirmed for all animals by visualization of the needle tract within brain sections. Wounds were sealed using bone wax on the skull and closed with dermabond or with sutures. Mice were placed on heating pads in individual cages after surgery until they recovered from anesthesia. Single daily doses of the immunosuppressant CSA were administered i.p. at a concentration of 10 mg/kg beginning the day before surgeries to hNSC and vehicle implanted R6/2 and non-transgenic mice. To further immunosuppress the mice an additional regimen of i.p. weekly doses of a CD4 antibody (BioXcell, Lebanon, NH) was given at 10 mg/kg. Q140 mice or Wt littermates received immunosuppression by CSA (2mg/kg/day) administered by subcutaneous osmotic minipumps (Alzet#1004) that were changed monthly to ensure the continuous delivery of CSA during the entire study. Surgery to remove and replace minipumps was as follows. Mice were anesthetized with isoflurane (3% for induction and 1.75% for maintenance of anesthesia, in 100% oxygen). After sterilizing the incision site, the minipump was removed through a small incision in the back and new minipumps were implanted before the incision was sutured.

Behavioral Assessment.

R6/2: Mice were assigned to groups in a semi-randomized manner. The behavioral tests listed below were performed at 6, 7, 8, or 9 weeks of age depending on the task. Mice were weighed daily and no significant differences were observed with treatment. Researchers were blind to which mice had been hNSC transplanted during experiment testing and data collection. To minimize experimenter variability a single investigator conducted each behavioral test. Mice were obtained from breeding colonies at UCI using ovarian transplant female mice (Jackson labs).

The rotarod apparatus was used to measure fore and hind limb motor coordination and balance and mice were tested over 3 consecutive days using an accelerating assay for 300s. The rotarod test was performed every other week two times at ages 6 and 8 weeks. For the pole test mice were placed on the pole with their head pointing down and they then descended head first down the length of the pole. The

total time to descend from the starting point of placement was measured. The pole test was performed every other week two times at ages 7 and 9 weeks. An IITC Life Science instrument was used to measure the forelimb grip force via a digital force transducer, the unit gives readings in one gram increments. Grip was measured every other week two times at ages 7 and 9 weeks.

Q140: *Climbing test and Pole test.* To assess motor coordination and spontaneous activity during climbing mice were placed in the bottom of wire cylinder cages and spontaneous activity was videotaped. For pole test each mouse was positioned face-up at the top of the pole and timed to make a full body-turn into a downward position and timed to descend down the pole into its respective home cage.

Electrophysiology in R6/2 mice

Briefly, mice were anesthetized, transcardially perfused with high sucrose-based slicing solution then coronal slices (300 μm) transferred to incubating chamber containing ACSF. MSNs and NSCs were visualized using infrared illumination with differential interference contrast optics. All recordings were performed in or around the injection site (recorded MSNs were adjacent to the graft between 150-200um). Biocytin was added to the patch pipette for cell visualization. Spontaneous postsynaptic currents were recorded in the whole-cell configuration in standard ACSF. Membrane currents were recorded in gap-free mode. Cells were voltage-clamped at +10mV and spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in ACSF. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in ACSF at -70 mV (baseline) and in the presence of the $GABA_A$ receptor blocker, bicuculline methobromide (Tocris, Minneapolis, MN) to isolate glutamatergic excitatory events. Spontaneous synaptic currents were analyzed using the MiniAnalysis software (version 6.0, Synaptosoft, Fort Lee, NJ). Following recordings, slices were fixed then transferred to 30% sucrose at 4˚C until IHC processing. To identify biocytin-filled recorded cells and hNSCs, fixed slices were washed, permeabilized and blocked for 4 h, followed by incubation with SC121 (1:1000, StemCells, Inc.). After washing, slices were incubated in goat, anti-mouse Alexa-488 (1:1000, Life Technologies, Carlsbad, CA Catalog #:A-11001) and streptavidin

conjugated with Alexa-594 (1:1000, Life Technologies Catalog #: S11227). Slices were washed, mounted and cells visualized with a Zeiss LSM510 confocal microscope.

Biochemical, Molecular and Immunohistological analysis in R6/2 mice.

Confocal Microscopy and Quantification. Sections were imaged with a Bio-Rad Radiance 2100 confocal system using lambda-strobing mode. Images represent either single confocal Z-slices or Z-stacks. All unbiased stereological assessments were performed using StereoInvestigator software (MicroBrightField, Williston, VT). An optical fractionator probe was used to estimate mean cell numbers, diffuse aggregate numbers and inclusion body numbers. Guard zones were set at 3% of measured thickness with a minimum 14µm optical dissector height. Contour Tracing was done at 5x objective and counting was performed at 100x objective. For each section, tracing was done approximately 70µm away from the edges of the stem cell patches. Counting was done in every 3rd section (40µm coronal sections) for 6 sections throughout the striatum where Ku80 labeled cells could be seen between bregma 0.5 mm and Bregma -0.34mm. All counts were performed using a 50x50µm counting frame and 250x250µm sampling grid in only one brain hemisphere. The CEs value for each Individual mouse ranged between 0.03 and 0.06. Sections were stained for Ku80 using ABC kit and DAB substrate kit (Vector Laboratories) with nickel first, then for EM48 using only ABC and DAB kits. Sections were stained with cresyl violet for non-stem cell nuclear staining. Identical stereological parameters were used to count aggregates and cells on mice implanted with vehicle. Using this stereological assessment of Ku80 positive cells in implanted R6/2 brain sections, ESI-017 NSC implant survival numbers showed an average of 63,975 cells in male mice (n=3) and 18,673 cells in female mice (n=3), equivalent to 64% (males) and 18.6% (females) of initially transplanted cells. There is an overall average of 41,323 cells in mice (n=6, 3 males and 3 females) equivalent to ~41% of initially transplanted cells. The difference between males and females in number of implanted cells may be due to technical difficulties implanting the smaller females at 5 weeks.

Primary antibodies used for IHC; GFAP (Abcam ab4674), NeuN (Millipore MAB377), SC121 (STEM 121 a human specific cytosolic marker, Clontech AB-121-U-050), Ku80 (Abcam, Cambridge, United Kingdom ab80592), Doublecortin (Millipore AB2253), Olig2 (R&D Systems AF2418), BIII-tubulin (Abcam ab107216), MAP-2, (Abcam ab5392), BDNF (Icosagen, 329-100) and EM48 (Millipore MAB5374).

RNA Isolation and Real-Time Quantitative PCR. Brain tissues were homogenized in TRIzol (Invitrogen), and total RNA was isolated using RNEasy Mini kit (QIAGEN). DNase treatment was incorporated into the RNEasy procedure in order to remove residual DNA. RIN values were > 9 for each sample (Agilent Bioanalyzer). Reverse transcription was performed using oligo (dT) primers and 1 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR (qPCR) was performed as previously described (Vashishtha, Ng et al. 2013) and ddCT values were quantitated and analyzed against RPLPO. The primers used for amplifying R6/2-Htt transgene were: oIMR1594: 5'-CCCCTCAGGTTCTGCTTTTA-3', oIMR1596: 5'-TGGAAGGACTTGAGGGACTC-3'; RPLPO Forward: 5'-TGGTCATCCAGCAGGTGTTCGA-3', RPLPO Reverse: 5'-ACAGACACTGGCAACATTGCGG-3'. Other primers used were Nestin, F 5'TCAAGATGTCCCTCAGCCTGGA3' R 5'AAGCTGAGGGAAGTCTTGGAGC3' BDNF F 5'TATGCGCCGAAGCAAGTCTCCA3' R 5'CATCCAAGGACAGAGGCAGGTA3' and DCX As 5'GTAAAGCCAACCCTGTGTCG3' S 5'TCCGCTCCAAAATCTGACTC3'.

Immunohistological analysis in the Q140 mice.

Primary antibodies used for IHC: HNA (Millipore MAB1281), DCX (abcam ab18723), GFAP (Dako Z033401), or synaptophysin (Millipore 04-1019). IHC for the quantification of HTT aggregates used monoclonal antibody EM48 (Millipore MAB5374) as described (Menalled et al., 2003) and microglia used rabbit anti-Iba-1 (Wako 019-19741) as described (Watson et al., 2012). For cell counts, HNA+ cells were counted over the entire striatal area in 6 coronal sections. 2100 HNA-labeled cells were

quantified and the proportion of those cells that were double-labeled with neuronal (DCX, Abcam ab18723) or glial markers (GFAP, Dako Z033401). The final numbers were expressed as the mean of 5 mice per group ± SEM.