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DEVELOPING STEM CELL-BASED THERAPIES FOR NEURAL REPAIR

Topic Editors
Clare Parish and Lachlan Thompson

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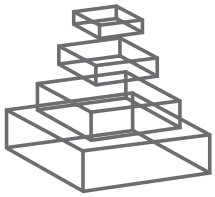
CELLULAR NEUOSCIENCE



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DEVELOPING STEM CELL-BASED THERAPIES FOR NEURAL REPAIR

Topic Editors:
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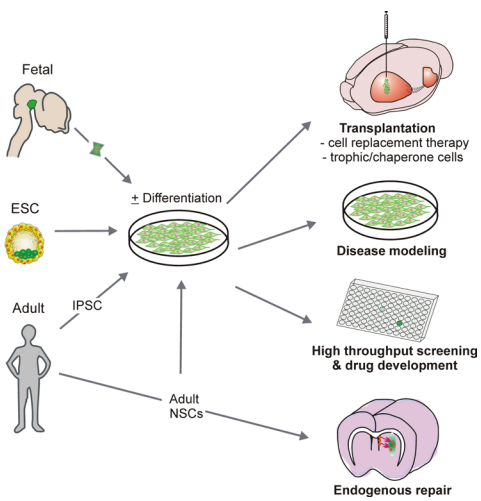

Figure 1: Various possible applications for stem cells in brain repair

Current pharmacotherapies and surgical intervention provide limited benefit in the treatment of neural injuries or halting disease progression and has resulted in significant hope for the successes of stem cell research. The properties of stem cells render them appropriate for cell replacement therapy, endogenous repair, disease modeling as well as high-throughput drug screening and development. Such applications will aide in increasing our knowledge and developing treatments for neurodegenerative disorders such as Parkinson's disease and Huntington's diseases as well as neural traumas including ischemic brain damage and traumatic brain injury. This Frontiers Research topic encouraged contributions from the general field of stem cell biology, with a particular emphasis on utilizing these cells to develop new therapies for neural repair. Related articles deal with issues such as: breakthroughs in stem cell proliferation/differentiation methodologies, using pluripotent and neural stem cells for transplantation and endogenous repair, the use of patient derived stem cells for disease modeling, using stem cells for drug discovery as well as the ethical issues related to the use of stem cells.

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Developing stem cell-based therapies for neural repair

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Keywords: stem cells, neural repair, transplantation, drug disovery, disease modelling, embryonic stem cells, pluripotent stem cells, nerve tissue proteins

Current pharmacotherapies and surgical intervention provide limited benefit in the treatment of neural injuries or halting disease progression. Significant advances in the area of stem cell biology in the last decade has lead to a special emphasis on the success of stem cell-based applications for the diagnosis and treatment of neurological conditions. The properties of stem cells render them appropriate for endogenous repair, disease modeling, high-throughput drug screening and development as well as neural transplantation procedures. Such applications will aide in increasing our knowledge and developing treatments for neurodegenerative disorders such as Parkinson's disease and Huntington's diseases as well as neural traumas including ischemic brain damage and traumatic brain injury. This Frontiers Research topic incorporates contributions from the general field of stem cell biology, with a particular emphasis on utilizing these cells to develop new therapies for neural repair. Related articles deal with issues including: promoting endogenous neurogenesis, breakthroughs in stem cell differentiation methodologies, using pluripotent and neural stem cells for transplantation, patient derived stem cells for disease modeling and using stem cells for drug discovery.

The first 3 papers focus on understanding and subsequently exploiting, endogenous neurogenesis in order to enhance neural repair. Within the adult mammalian brain, neurogenesis is largely restricted to two sites; the dentate gyrus of the hippocampus and the subventricular zone adjacent to the lateral ventricle. In order to "harness" these cells for repair it will be vital to understand the mechanisms regulating their proliferation, migration, differentiation and survival. Sui et al. (2013) examines the role of the neuropeptide cholecystokinin (CCK) and its receptor (CCK1R) in this context. It is now well recognized that existing rates of neurogenesis in the adult brain are insufficient to replace neurons lost to neurodegeneration or trauma. Taylor et al. (2013) provide an overview of the field of hippocampal neurogenesis, the consequences of ageing on these populations, as well as changes in mood-related disorders and touches on efforts to activate this largely quiescent cell population. Christie and Turnley (2013) provide a broader review of extrinsic and intrinsic factors that have been shown to regulate adult neurogenesis, and highlight those that have already shown evidence of preclinical efficacy.

The generation of stem cell lines provides valuable tools for disease modeling and drug development. Blastocyst screening for genetic disorders such as Huntington's disease and Down's syndrome have enabled the generation of embryonic stem cells carrying these mutations. In more recent years, inducible pluripotent stem (iPS) cell technology has allowed stem cell lines to be generated from somatic cells of patients suffering from a number of degenerative conditions including Parkinson's disease and motor neuron disease. Added to this has been the ability to generate neural stem cell lines from other sources such as olfactory ensheathing cells of patients. Combined, such cell lines provide new tools to examine the pathological events that lead to disease onset and progression as well as development and testing of novel drug targets. Here, Niclis et al. (2013) examines 2 Huntington disease derived ESC lines. Whilst they demonstrate these lines show many normal properties, including gene expression, that are typically dysregulated in the disease, subtle changes that may shed further insight into the disease are observed. An article by Mackay-Sim (2013) provides a review on the use of stem cells for high throughput, and high content screening of large chemical libraries in efforts to identify novel drug targets. Such targets can then be utilized for drug development and again tested on patient-derived stem cell lines in culture.

One of the most widely anticipated applications for pluripotent stem cells is their utilization in neural transplantation procedures. This will require robust and appropriate fate restriction into more specialized lineages and their survival and appropriate functional integration into existing host circuitry after transplantation. Kirkeby et al. (2013) contribute a detailed methodological paper on the generating regionally specified neurons, by way of controlling dorso-ventral and rostrocaudal patterning of stem cells. Arber and Li (2013) provide a more focused review of the literature on understanding cortical interneuron development and the progress in protocols to yield interneuron subpopulations from pluripotent stem cells for the purpose of transplantation into models of epilepsy. In an original research article, Yoshikawa et al. (2013) examine the potential of 2 commonly used anti-convulsant drugs to improve the differentiation and survival of iPSC-derived dopaminergic neurons *in vitro* and *in vivo*, with this knowledge having implications for improved grafting approaches for treatment of Parkinson's disease. Garcia et al. (2013) highlight advancement in tools and technology that enable us to more accurately assess the integration of transplanted neurons in the brain. Finally, Broughton et al. (2013) provide a review on the exogenous "chaperone" benefits that stem cell grafts may provide in neural repair. The focus of their review is on amnion cells; highlighting the non-tumorogenic, non-immunogenic and trophic benefits of an alternative stem cell source.

Collectively these works highlight the rapidly progressing stem cell field and draw attention to the current and on-going potential these cells have for improving our understanding of the healthy and diseased nervous system and their capacity to promote neural repair.

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Female mice lacking cholecystokinin 1 receptors have compromised neurogenesis, and fewer dopaminergic cells in the olfactory bulb

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Neurogenesis in the adult rodent brain is largely restricted to the subependymal zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the dentate gyrus (DG). We examined whether cholecystokinin (CCK) through actions mediated by CCK1 receptors (CCK1R) is involved in regulating neurogenesis. Proliferating cells in the SVZ, measured by 5-bromo-2-deoxyuridine (BrdU) injected 2 h prior to death or by immunoreactivity against Ki67, were reduced by 37 and 42%, respectively, in female (but not male) mice lacking CCK1Rs ($CCK1R^{-/-}$) compared to wild-type (WT). Generation of neuroblasts in the SVZ and rostral migratory stream (RMS) was also affected, since the number of doublecortin (DCX)-immunoreactive (ir) neuroblasts in these regions decreased by 29%. In the SGZ of female $CCK1R^{-/-}$ mice, BrdU-positive (+), and Ki67-ir cells were reduced by 38 and 56%, respectively, while DCX-ir neuroblasts were down 80%. Subsequently, the effect of reduced SVZ/SGZ proliferation on the generation and survival of mature adult-born cells in female $CCK1R^{-/-}$ mice was examined. In the OB granule cell layer (GCL), the number of neuronal nuclei (NeuN)-ir and calretinin-ir cells was stable compared to WT, and 42 days after BrdU injections, the number of BrdU+ cells co-expressing GABA- or NeuN-like immunoreactivity (LI) was similar. Compared to WT, the granule cell layer of the DG in female $CCK1R^{-/-}$ mice had a similar number of calbindin-ir cells and BrdU+ cells co-expressing calbindin-LI 42 days after BrdU injections. However, the OB glomerular layer (GL) of $CCK1R^{-/-}$ female mice had 11% fewer NeuN-ir cells, 23% less TH-ir cells, and a 38% and 29% reduction in BrdU+ cells that co-expressed TH-LI or GABA-LI, respectively. We conclude that CCK, via CCK1Rs, is involved in regulating the generation of proliferating cells and neuroblasts in the adult female mouse brain, and mechanisms are in place to maintain steady neuronal populations in the OB and DG when the rate of proliferation is altered.

Keywords: cholecystokinin 1 receptor, neurogenesis, subventricular zone, rostral migratory stream, olfactory bulb, subgranular zone, interneurons, survival

INTRODUCTION

Adult olfactory precursors divide primarily within the subventricular or subependymal zone (SVZ) of the lateral ventricle, where they differentiate into immature neurons. Neuroblasts then migrate tangentially along the rostral migratory stream (RMS) toward the main olfactory bulb (OB) (Curtis et al., 2007). When neuroblasts reach the OB, they migrate radially into the granular (GCL) and periglomerular (GL) layers of the OB, where they differentiate into local interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994). In the dentate gyrus (DG), neural progenitors in the subgranular zone (SGZ) proliferate and give rise to immature neurons (Altman and Das, 1965; Eriksson et al., 1998; Van Praag et al., 2002) that migrate a short distance to the granule cell layer (GrDG), where they functionally integrate into hippocampal circuitry (Kempermann et al., 2003).

In the young adult rodent, approximately 50% of adult-born cells that migrate into the OB differentiate to form interneurons that integrate into OB circuitry, while the other half undergo programmed cell death as progenitors, neuroblasts or young neuronal cells in the SVZ, RMS, or OB (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Lledo and Saghatelyan, 2005). Doublecortin (DCX), a neuron specific microtubule associated protein, is expressed by most dividing neuroblast cells and migrating postmitotic neuroblasts in the SVZ and RMS, and its down-regulation 10–14 days after the birth of a neuroblast coincides with the commencement of neuronal nuclei (NeuN) expression,

as the cells mature to become OB interneurons (Brown et al., 2003). Similarly, 50% of newly generated cells in the adult rodent GrDG die within 22 days of their birth (Dayer et al., 2003). DCX is expressed by adult-born cells on days 1–14 after birth (Brown et al., 2003), with some cells being NeuN-positive (+) on day 1 (Brandt et al., 2003), and the majority of surviving cells expressing NeuN (Brown et al., 2003) and calbindin (Brandt et al., 2003) 1 month after birth.

A wide range of molecular cues regulate neurogenesis in the adult brain (Lie et al., 2004; Abrous et al., 2005; Emsley et al., 2005; Ming and Song, 2005), and peptidergic systems, including neuropeptide Y (Hansel et al., 2001; Howell et al., 2005; Hökfelt et al., 2008; Stanić et al., 2008) and galanin (Mazarati et al., 2004), also participate in these processes. A neuropeptide that has remained largely unexplored within this context, and which may potentially regulate neurogenesis, is cholecystokinin (CCK). CCK is widely distributed in the mammalian CNS (Vanderhaeghen et al., 1975; Hökfelt et al., 1988) and so far two distinct CCK receptors have been cloned; the CCK 1 (CCK1R) and CCK 2 receptor (Hill et al., 1987; Wank et al., 1992). CCK modifies the migratory abilities, proliferation, and survival of tumor astrocytes (De Hauwer et al., 1998; Lefranc et al., 2002) and lymphocytes (Medina et al., 1998), and guides migrating gonadotropin-releasing hormone-1 (GnRH-1) neuroendocrine neurons into the brain (Giacobini et al., 2004). Moreover, immortalized rat brain neuroblasts express CCK1R and CCK2R mRNA (Langmesser et al., 2007). Exposure to CCK promoted proliferation of these cells, and improved their viability, indicating that CCK is an important regulator of proliferation.

We therefore examined whether CCK is involved in regulating neurogenesis in the adult brain. Using adult mice with genetic deletion of the CCK1 receptor ($CCK1R^{-/-}$), we investigated whether CCK1 receptors influence cell proliferation and neuroblast formation in the SVZ, RMS, and SGZ, and affect interneuron generation in the OB and DG. We report that female, but not male, $CCK1R^{-/-}$ mice have fewer proliferating cells, migratory neuroblasts, and tyrosine hydroxylase (TH)-immunoreactive (ir) OB interneurons than wild-type (WT) mice.

MATERIALS AND METHODS

ANIMALS

All experimental procedures conformed to the Australian National Health and Medical Research Council published code of practice, and were approved by the Florey Neuroscience Institutes' Animal Ethics Committee (#09-053 and #07-117). Twelve female and four male 16–20-week old mice lacking the CCK1R (Strain Name: 129-*Cckar*^{tm1Kpn}/J; Stock No. 006367; The Jackson Laboratory, Bar Harbor, ME) (Kopin et al., 1999) and 12 female and 4 male age-matched WT control mice, weighing between 20–25 g were used. All animals were maintained under standard conditions on a 12 h day/night cycle, with water and food *ad libitum*.

GENOTYPING

To obtain genomic DNA, 2–5 mm mouse tails were digested in 100 μL proteinase K solution (250 μL Tween 20, 500 μL 1M Tris, 2500 μL × 20 mg/ml proteinase K and MQ water to 50 ml). Mixtures were then incubated at 56°C for 60–90 min, followed by 10 min at 95°C. Tubes were centrifuged at maximum speed for 10 min, and subsequently stored at 4°C. For each PCR, 1 μl DNA template was added to 6 μl 2×Go Taq Green polymerase master mix (Promega, Madison, WI, Code No. 9PIM712), 1 μl of 10 μM mixture of each primer and 4 μl nuclease free water (Promega, Code No. P1193). PCR was performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following primer sequences (Geneworks, Hindmarsh, Australia): 5′-GCT GCA TAG CGT CAC TTG G-3′ for CCK1 receptor WT forward; 5′-GAT GGA GTT AGA CTG CAA CC-3′ for CCK1 receptor WT reverse; 5′-GAC AAT CGG CTG CTC TGA TG-3′ for $CCK1R^{-/-}$ forward. Cycling conditions were: 95°C for 5 min for initial denaturation, 35 cycles of 95°C for 30 s, 63°C for 60 s, and 72°C for 60 s, followed by final amplification at 72°C for 5 min. Final DNA products were visualized under UV after electrophoresis in 1.5% agarose gel containing 0.5 μL SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, Code No. S33102) per 10 ml. The expected fragments yielded by PCR were 507 bp for WT, 970 + 507 bp for CCK1R ± and 970 bp for $CCK1R^{-/-}$.

BrdU ADMINISTRATION

5-bromo-2-deoxyuridine (BrdU) (ICN Biomedicals Inc, Aurora, OH, Cat No. 100171) was administered intraperitoneally to $CCK1R^{-/-}$ and WT mice to study the proliferation and survival of adult-born cells in the SVZ, OB and DG of the hippocampal formation. Two different protocols were used to identify either proliferating cells in the SVZ and SGZ, or "mature" cells that had survived or integrated into the OB or GrDG: (1) To enable identification of proliferating cells in the SVZ/SGZ, a single dose of BrdU (150 mg/kg, i.p.) was injected 2 h prior to their death (**Figure 1A**); and (2) To label mature adult-born cells that migrate to, integrate and survive in the GCL, GL or GrDG, BrdU (50 mg/kg, i.p.) was administered twice daily for 5 consecutive days, and mice killed 42 days later (**Figure 1B**).

TISSUE PREPARATION

All animals were deeply anaesthetized using pentobarbitone sodium (Lethabarb, Virbac, Milperra, NSW, Australia, 100 mg/kg i.p.) and perfused through the heart via the ascending aorta with 20 ml Ca^{2+}-free Tyrode's buffer (37°C), followed by 20 ml of a mixture of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and 0.2% picric acid (Sigma) diluted in 0.16 M phosphate buffer (pH 6.9, 37°C) (Pease, 1962; Zamboni and De Martino, 1967) and 50 ml of the same fixative at 4°C, the latter for approximately 5 min. The brains were dissected out and postfixed in the same fixative for 90 min at 4°C, and finally immersed for 48 h at 4°C in 10% sucrose dissolved in phosphate buffered saline (PBS, pH 7.4) containing 0.01% sodium azide (Sigma) and 0.02% bacitracin (Sigma), before rapid freezing by CO_2. Sections were cut using a cryostat (Leica CM1850, Wetzlar, Germany) at: (1) a thickness of 14 microns, and thaw-mounted on slides coated with 0.5% gelatin (Sigma) and 0.05% chromium(III) potassium sulphate dodecahydrate (Merck, KGaA, Darmstadt, Germany); or (2) a thickness of 30 microns, and stored in a cryoprotectant solution [30% v/v ethyleneglycol (Merck); 15% w/v sucrose; 35% v/v 0.1 M phosphate buffer; 35% v/v distilled H_2O], at −20°C.

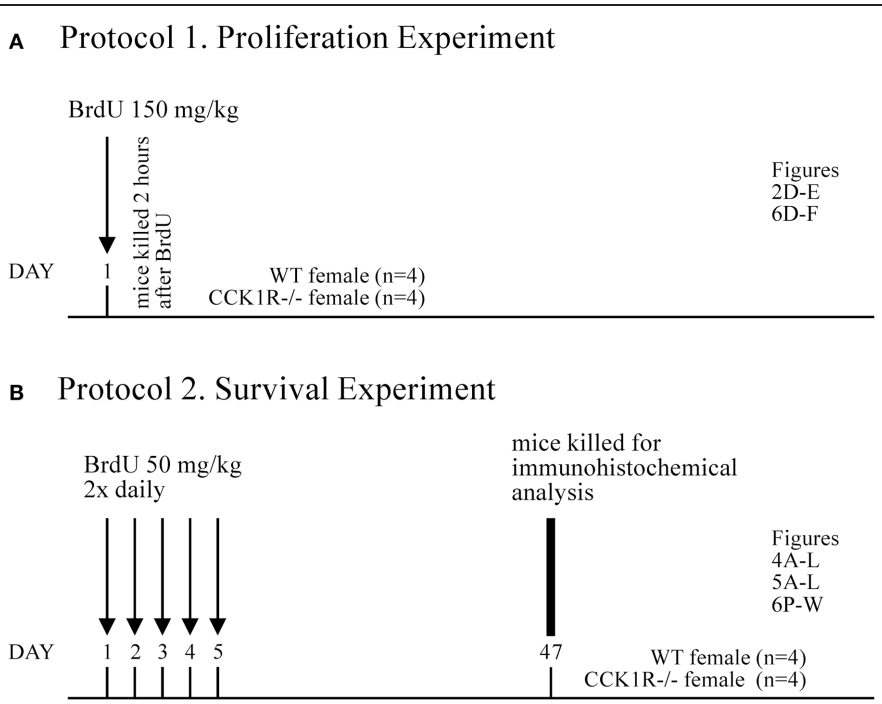

FIGURE 1 | BrdU Experimental Design. (A) *Protocol 1*. To identify proliferating cells in the SVZ and SGZ, a single dose of BrdU (150 mg/kg i.p.) was administered 2 h prior to death ($n = 4$ for each experimental group). From these animals, the number of BrdU+ cell bodies in the SVZ (**Figures 2D,E**) and SGZ (**Figures 6A–C**) was estimated. **(B)** *Protocol 2*. To label mature adult-born cells that migrate to, integrate and survive in the GCL and GL of the OB, and the GrDG of the hippocampal formation, BrdU (50 mg/kg, i.p.) was administered twice daily for 5 consecutive days, and mice killed 42 days later (i.e., 47 days after first BrdU administration; $n = 4$ for each experimental group). From these animals, the number of BrdU+ cell bodies in the GCL (**Figures 4A,B**) and GL (**Figures 5A,B**) of the OB was estimated, as was the number of: BrdU/GABA (**Figures 4C–E**), BrdU/NeuN (**Figures 4G–I**), and BrDU/calretinin (**Figures 4J–L**) co-expressing cells in the GCL of the OB; BrdU/TH (**Figures 5C–F**), BrdU/GABA (**Figures 5G–I**), and BrdU/calbindin (**Figures 5J–L**) co-expressing cells in the GL; BrdU+ cells in the GrDG (**Figures 6P–R**); and BrdU/calbindin co-expressing cells in the GrDG (**Figures 6S–W**).

IMMUNOHISTOCHEMISTRY

Incubation protocol (immunofluorescence)

Sections were washed using 0.01 M PBS (3 × 10 min) and incubated for 24 h at 4°C with a rat anti-BrdU (1:300, Axyll, Westbury, NY, Code No. OBT0030), rabbit anti-calbindin (1:10,000, Swant, Marly, Switzerland, Code No. CB-38a), goat anti-calretinin (1:4000, Swant, Code No. CG1), goat anti-DCX (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, Code No. SC-8066), rabbit anti-γ-aminobutyric acid (GABA) (1:2000, Sigma, Code No. A2052), rabbit anti-glial fibrillary acidic protein (GFAP) (1:400, Dako, Glostrup, Denmark, Code No. Z0334), mouse anti-NeuN (1:1000, Millipore, Billerica, MA, Code No. MAB377), rabbit anti-tyrosine hydroxylase (TH) (1:1000, Pel-Freeze, Rogers, Ar, Code No. P40101-0) or sheep anti-TH (1:400, Pel-Freeze, Code No. P60101-0) antibody, diluted in 0.01 M PBS containing 0.3% Triton X-100 and 0.5% BSA. Sections were then washed in TNT buffer [0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20 (Sigma)] for 15 min and incubated in TNB buffer [0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking reagent (PerkinElmer, Boston, MA, Code No. FP1020)] for 30 min at room temperature (RT). Immunoreactivity was visualized using Alexa Fluor 594-conjugated goat anti-rat, Alexa Fluor 488-conjugated donkey anti-goat, Alexa Fluor 594-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 594-conjugated goat anti-mouse or Alexa Fluor 488-conjugated donkey anti-sheep (1:200, Molecular Probes, Eugene, OR), as appropriate, in TNB buffer for 2 h. Finally, sections were washed in TNT (3 × 10 min) and coverslipped using a fluorescent mounting medium (Dako). Hoechst 33342 (1:1000, Invitrogen) was applied to sections immunostained with GFAP for 1.5 min during the third TNT wash, to provide a nuclear counter stain. Prior to commencing immunoreactivity for BrdU, antigen retrieval and DNA denaturation was performed, where sections were incubated in 50% formamide (BDH Laboratory Supplies, England) in 0.01 M PBS at 65°C for 2 h, 2 M HCl for 30 min at 37°C, and 0.1 M sodium borate (Borax, Sigma, B-3545) buffer for 10 min at RT.

To visualize calbindin and calretinin immunoreactivity, sections were processed using a commercial kit (TSA$^+$, NEN Life Science Products, Inc., Boston, MA). Briefly, following 24 h incubation in primary antisera, sections were washed in TNT buffer (15 min), incubated with TNB buffer (30 min) and incubated with horse-radish peroxidase (HRP)-conjugated swine anti-rabbit (1:200, Dako) or HRP-conjugated donkey anti-goat (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA), as appropriate, diluted in TNB buffer for 30 min. Sections

were then washed in TNT buffer (3 × 10 min) and incubated in a biotinyl tyramide-fluoroscein (BT-FITC) conjugate (NEN) diluted 1:100 in amplification diluent for 10 min at RT, followed by washes in TNT (3 × 10 min).

For double-immunofluorescence experiments, antigen retrieval and DNA denaturation was performed first, followed by incubations of the anti-BrdU and either the anti-calbindin, calretinin, DCX, GABA, NeuN, or TH antibodies, according to the above concentrations and protocols.

Incubation protocol [diaminobenzidine (DAB)]

Sections were rinsed (3 × 10 min) in 0.01 M PBS, followed by incubation in blocking diluent [0.01 M PBS containing 5% normal goat serum (NGS) and 0.3% Triton X-100 (Sigma)] for 30 min, and rabbit anti-Ki67 antibody (1:15,000, Thermo Fisher Scientific, Fremont, CA, Code No. RM-9106-s1) diluted in 0.01 M PBS, 1% NGS and 0.3% Triton X-100 for 48 h at 4°C. Sections were then incubated in biotinylated goat anti-rabbit (1:1000, Dako) diluted in 0.01 M PBS, 1% NGS and 0.3% Triton X-100 for 3 h at RT, and then avidin peroxidase (1:5000 in 0.01 M PBS and 0.075% Triton X-100) for 1 h, followed by DAB (1:100, Sigma) for 20 min. Three percent Hydrogen peroxidase (Merck) was added to the DAB solution for substrate precipitation and the reaction terminated 2 min later by rinsing sections in 0.01 M PBS. Sections were counter stained with neutral red, dehydrated in a series of graded ethanol (50–100%), cleared in X3B solvent (Shell Chemicals, Hawthorn East, Australia), and then coverslipped with DePeX (VWR International, Poole, England). Rinses using 0.01 M PBS (3 × 10 min) were performed between each step.

IMAGE PROCESSING

After processing, sections were examined using a Leica DMLB2 fluorescence microscope (Leica, Wetzlar, Germany), equipped with a dark field condenser and epi-polarization, and epifluorescence with appropriate filter combinations, and with objective lenses of ×10 (N.A. 0.45), ×20 (N.A. 0.70), ×40 (N.A. 0.75), ×60 oil (N.A. 1.40), and ×100 oil (N.A. 1.30). Photographs were taken using a Microfire digital camera (2.3A, Optronics, Goleta, CA) attached to the microscope, operated through Picture Frame software (v2.3, Optronics). For confocal analysis, an Olympus FV1000 confocal laser scanning microscope equipped with ×10 (N.A. 0.4), ×20 (N.A. 0.75), ×40 oil (N.A. 1.30) and ×60 oil (N.A. 1.35) objectives was used. The AlexaFluor 488 and FITC labeling was excited using the 473 nm diode laser. For the detection of AlexaFluor 594, a 559 nm diode laser was used. Z-stack images were captured with multiple images, each separated by a stepwise depth of 1.0 um in the z-plane. Digital images from the microscopy were slightly modified to optimize for image resolution, brightness and contrast using Adobe Photoshop CS5 software (Adobe Systems Inc., San Jose, CA), so as to best represent the immunohistochemistry observed at the microscope.

STEREOLOGY

For quantification of cell bodies in the SVZ and RMS, the level at which the anterior commissure (AC) converged through the midline [Bregma +0.14 mm (see Paxinos and Franklin, 2001)] was used as a reference to define the caudal boundary of the SVZ. Serial sections rostral to this point were acquired, with sections between 0–1400 μm rostral to the AC convergence (i.e., Bregma +0.14 to +1.54 mm) defined as containing the SVZ, and sections from 1500 to 4100 μm (i.e., Bregma +1.6 to +4.2 mm) regarded as having the RMS. Analysis of the OB was performed on sections rostral to Bregma +2.6 mm. Fourteen μm-thick sections, each 280 μm apart, were analysed, and guard zones of 1 μm (top) and 1 μm (bottom) were employed. For quantification of proliferating cells and neuroblasts in SVZ, only the lateral wall of the lateral ventricle was analyzed, as these cells are largely absent in the medial and dorsal walls (Doetsch et al., 1997).

Regions of the DG from which cell bodies were quantified corresponded to Bregma −1.30 to −3.10 mm (see Paxinos and Franklin, 2001). DCX-ir and Ki67-ir cell bodies in the SGZ/GrDG were counted on 14 μm-thick sections, each 140 μm apart, and GFAP-ir and calbindin-ir cell bodies on 14 μm-thick sections, each 280 μm apart. BrdU+ in the SGZ/GrDG, and BrdU+ cell bodies expressing calbindin-like immunoreactivity (LI), were quantified on 30 μm-thick free floating sections, each 180 μm apart, with guard zones of 1 μm (top and bottom). For quantification of Ki67-ir and BrdU-positive proliferating cells in the SGZ, the SGZ was recognized as the border between the polymorph and granule cell layers of the DG, including one cell body width of the GrDG and the equivalent of two granule cell body widths within the polymorph layer (Cameron and McKay, 2001).

Estimates of the number of proliferating cells, neuroblasts and mature neurons in the SVZ, RMS, OB, and DG were made using a fractionator sampling design according to optical dissector rules (Gundersen et al., 1988; West et al., 1991; Stanic et al., 2003; Parish et al., 2005). Regular predetermined *x*, *y* intervals and counting frame dimensions for all estimates were derived by means of a grid program (Stereoinvestigator v.7.0, MicroBrightField, Williston, VT, viewed through a microscope, Leica) and are outlined in **Table 1**.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). All comparisons were conducted by student *t*-tests, and ANOVA with Tukey multiple comparisons test where indicated, and a value of $p < 0.05$ was considered statistically significant. Values are expressed as the mean ± SEM.

RESULTS

We examined whether the number of proliferating cells in the SVZ and SGZ was altered in adult mice lacking the CCK1R. Subsequently, stereological quantification was performed to estimate: (1) the number of migrating neuroblasts in the SVZ and RMS; (2) the number of mature interneurons present in the OB; and (3) the survival of adult-born cells in the OB. Also, we examined whether the number of proliferating cells, neuroblasts and mature neurons was altered in the DG of CCK1R$^{-/-}$ mice.

ADULT FEMALE CCK1R$^{-/-}$ MICE HAVE A LOWER NUMBER OF PROLIFERATING CELLS IN THE SVZ AND RMS

Immunoreactivity against Ki67, which labels cells in all phases of mitosis, except G1, was used to identify dividing cells in the SVZ

Table 1 | Counting frame dimensions and *x, y* co-ordinates for estimates of proliferating cells (Ki67, BrdU 2 h), migrating neuroblasts (DCX), interneurons, and mature cells (NeuN, TH, calbindin, calretinin, GABA, BrdU 42 days) in the SVZ, RMS, OB, SGZ, and GrDG.

Antibody	Region analysed	Counting frame size (μm)	Fractionator *x, y* coordinates (μm)
BrdU (2 h)	SVZ	30 × 30	30 × 30
Ki67	SVZ/RMS	30 × 20	40 × 100
DCX	SVZ/RMS	30 × 20	70 × 150
GFAP	SVZ	40 × 40	40 × 40
NeuN	GCL	20 × 20	70 × 300
NeuN	GL	30 × 30	70 × 300
Calretinin	GCL	80 × 80	150 × 300
Calbindin, Calretinin	GL	80 × 80	100 × 400
GABA, TH, GABA/TH	GL	40 × 40	100 × 400
BrdU (42 days)	GCL	50 × 50	100 × 200
BrdU (42 days)/GABA	GCL	50 × 50	100 × 200
BrdU (42 days)/NeuN	GCL	50 × 50	100 × 200
BrdU (42 days)/Calretinin	GCL	50 × 50	100 × 200
BrdU (42 days)/DCX	GCL	50 × 50	100 × 200
BrdU (42 days)	GL	170 × 135	170 × 270
BrdU (42 days)/TH	GL	170 × 135	170 × 270
BrdU (42 days)/GABA	GL	170 × 135	170 × 270
BrdU (42 days)/Calbindin	GL	170 × 135	170 × 270
BrdU (42 days)/DCX	GL	170 × 135	170 × 270
BrdU (2 h)	SGZ	170 × 135	170 × 135
BrdU (42 days)	GrDG	170 × 135	170 × 135
BrdU (42 days)/Calbindin	GrDG	170 × 135	170 × 135

and RMS. There were 42% fewer Ki67-ir cell bodies in female CCK1R$^{-/-}$ mice than in female WT mice (**Figures 2A,B**). In contrast, the number of Ki67-ir proliferating cells was similar in male CCK1R$^{-/-}$ and male WT mice (**Figure 2A**). ANOVA with Tukey multiple comparisons test indicated a statistical difference between WT and female CCK1R$^{-/-}$ mice and female and male CCK1R$^{-/-}$ mice, and a statistical similarity between WT mice and male CCK1R$^{-/-}$ mice, and male and female WT mice (**Figure 2A**).

The rate of cell proliferation in the SVZ was further examined by injecting BrdU (150 mg/kg i.p.) into mice 2 h prior to their death, to label cells in S-phase of the cell cycle (**Figure 1A**). The number of BrdU-positive (BrdU+) cell bodies in the SVZ of female CCK1R$^{-/-}$ mice was 37% lower than the number in female WT mice (**Figures 2D,E**).

LOWER NUMBERS OF DCX-IR NEUROBLASTS IN THE SVZ AND RMS OF FEMALE CCK1R$^{-/-}$ MICE

Because cell proliferation in the SVZ and RMS of female CCK1R$^{-/-}$ mice was reduced, we next examined whether the number of neuroblasts in the SVZ and RMS were altered. Neuroblasts in these regions were identified by immunoreactivity against DCX (Francis et al., 1999; Gleeson et al., 1999; Brown et al., 2003). The number of DCX-ir neuroblasts in the SVZ and RMS decreased by 29% in CCK1R$^{-/-}$ mice (**Figures 2G–I**).

NO CHANGE IN THE NUMBER OF GFAP-IR ASTROCYTES IN THE SVZ OF FEMALE CCK1R$^{-/-}$ MICE

Immunohistochemistry for GFAP was performed to determine whether reduced cell proliferation in the SVZ had an effect on the number of astrocytes (Merkle et al., 2004). The number of GFAP-ir cell bodies found in the SVZ of CCK1R$^{-/-}$ and WT mice was similar (**Figures 2C,F**), suggesting that reduced proliferation in the SVZ leads principally to the generation of fewer cells of neural lineage.

FEWER PROLIFERATING PRECURSOR CELLS IN THE SVZ AND RMS OF CCK1R$^{-/-}$ FEMALE MICE LEAD TO A REDUCED NUMBER OF MATURE NEURONS IN THE GL OF THE OB

As cells born in the SVZ migrate along the RMS toward the OB, where they differentiate into local interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994), the effect of reduced proliferation in the SVZ on the number of mature interneurons in the OB was examined. Mature OB interneurons were identified using an antibody against NeuN. Despite the reduction in the number of proliferating precursors in the SVZ of CCK1R$^{-/-}$ mice, the number of NeuN-ir cell bodies in the GCL was similar to the number found in WT mice (**Figures 3A,B**). In the GL, however, the modest 11% decrease in NeuN-ir cell bodies in female CCK1R$^{-/-}$ mice differed statistically from WT controls (**Figures 3C,D**).

SUBTYPES OF INTERNEURONS IN THE OB OF FEMALE CCK1R$^{-/-}$ MICE REMAIN UNAFFECTED, EXCEPT FOR PERIGLOMERULAR TH-IR INTERNEURONS

Subclasses of granular and periglomerular cells in the OB can be identified by expression of GABA, TH (Betarbet et al., 1996), calbindin, and calretinin (Rogers, 1992; Rogers and Resibois, 1992; De Marchis et al., 2007). We examined whether the reduction of cell proliferation in the SVZ of CCK1R$^{-/-}$ mice affected the number of interneurons in each of these subclasses.

In the GCL of female CCK1R$^{-/-}$ and WT mice, the number of calretinin-ir cell bodies was similar (**Figures 3E,F**). In the GL of female CCK1R$^{-/-}$ mice, the number of calretinin-ir (**Figures 3G,H**), calbindin-ir (**Figures 3I,J**) and GABA-ir (**Figures 3K,L**) cell bodies was also similar to the number estimated in WT mice. However, the 23% reduction in the number of TH-ir cell bodies in the GL of CCK1R$^{-/-}$ mice was statistically different from WT mice (**Figures 3M,N**). Double-labeling experiments revealed that the number of cells in the GL that co-expressed GABA- and TH-LI was similar in CCK 1R$^{-/-}$ and WT mice (**Figures 3O,P**), as was the proportion of GABA-ir cells that co-expressed TH-LI (**Figure 3Q**). This suggests that the decreased number of TH-ir cell bodies in the GL of female CCK1R$^{-/-}$ mice is not due to a reduced number of GABA/TH co-expressing cells, nor a decrease of TH expression in GABA-ir cells.

NO CHANGE IN THE NUMBER OF NEWBORN CELLS IN THE OB OF CCK1R$^{-/-}$ FEMALE MICE

We next examined the effect of reduced SVZ precursor proliferation on the number of newborn cells that migrate to, integrate and survive in the GCL and GL of the OB. BrdU (50 mg/kg i.p.) was administered twice daily for 5 consecutive days. The mice were killed 42 days after the last BrdU administration, a

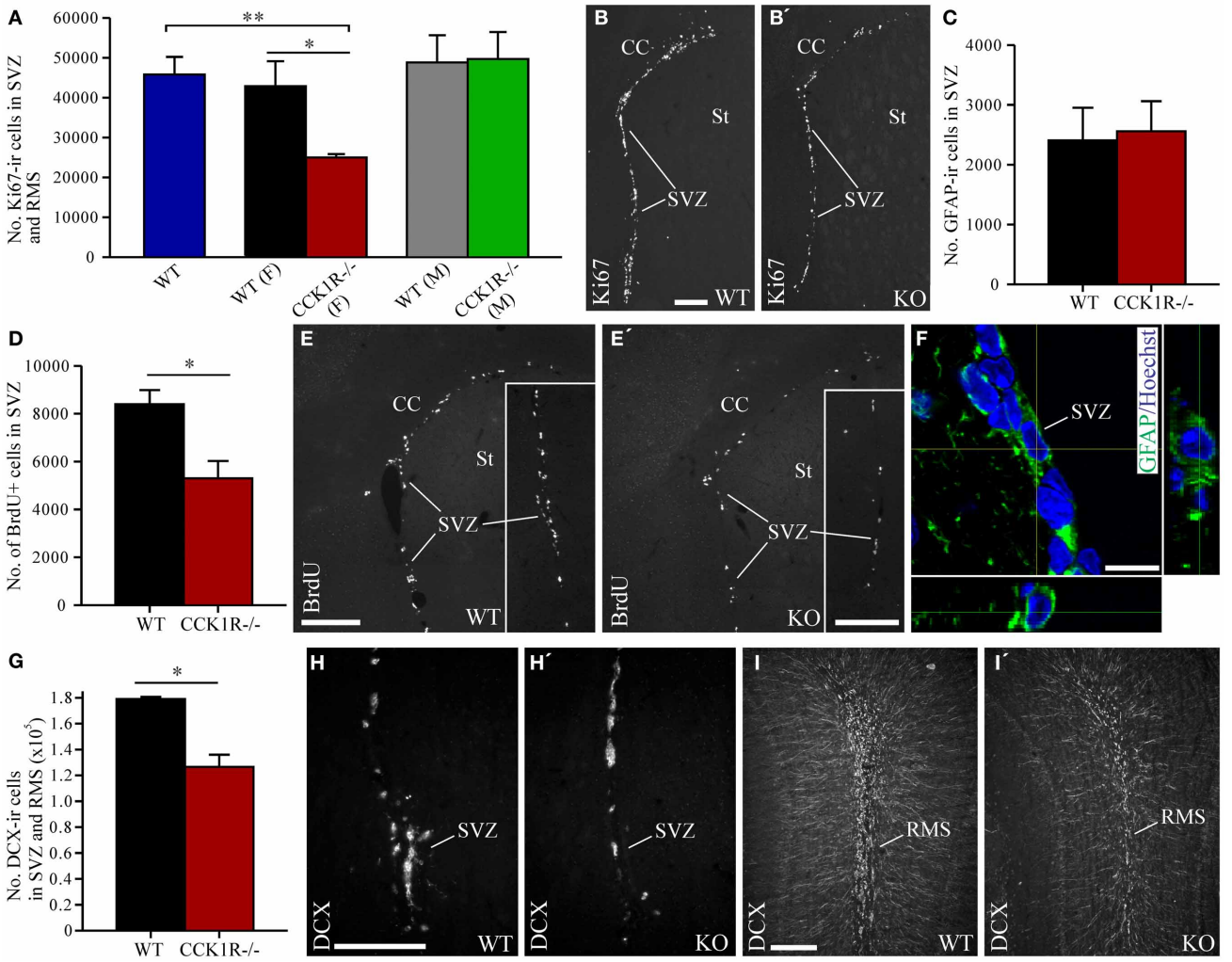

FIGURE 2 | Estimates of proliferating cells, neuroblasts, and astrocytes in the SVZ and RMS of CCK1R$^{-/-}$ and wild-type mice. **(A,B)** Estimated number of Ki67-ir cell bodies in the SVZ and RMS of CCK1R$^{-/-}$ and wild-type mice **(A)**. In **(A)** WT blue bar = wild-type (male and female combined, $n = 8$); WT (F) black bar = female wild-type ($n = 4$); CCK1R$^{-/-}$ (F) red bar = female CCK1R$^{-/-}$ ($n = 4$); WT (M) grey bar = male wild-type ($n = 4$); and CCK1R$^{-/-}$ (M) green bar = male CCK1R$^{-/-}$ ($n = 4$). Photomicrographs of Ki67-LI in the SVZ of female **(B)** wild-type and **(B′)** CCK1R$^{-/-}$ mice. **(D,E)** Estimated number of BrdU+ cell bodies in the SVZ of female CCK1R$^{-/-}$ and wild-type mice **(D)** (see *Protocol 1*, **Figure 1A**). Photomicrographs of BrdU-LI in the SVZ of female **(E)** wild-type and **(E′)** CCK1R$^{-/-}$ mice. **(C,F)** Estimated number of GFAP-ir astrocytes in the SVZ of female wild-type and CCK1R$^{-/-}$ mice **(C)**. Double-immunofluorescence confocal micrograph of GFAP-LI (green) and Hoechst staining (blue) in the SVZ of female CCK1R$^{-/-}$ mouse **(F)**, the latter providing a nuclear counter stain. **(G–I)** Estimated number of DCX-ir neuroblasts in the SVZ and RMS of female wild-type and CCK1R$^{-/-}$ mice **(G)**. Photomicrographs of DCX-ir neuroblasts in the **(H,H′)** SVZ and **(I,I′)** RMS of female wild-type and CCK1R$^{-/-}$ mice, respectively. In plots **(A,C,D,G)**, black bars = female wild-type animals ($n = 4$), and red bars = female CCK1R$^{-/-}$ mice ($n = 4$). CC, corpus collosum; ir, immunoreactive; KO, CCK1R$^{-/-}$; RMS, rostral migratory stream; St, striatum; SVZ, subventricular zone; WT, wild-type; +, postive. Scale bars: **(B)** = 200 μm, applies **(B,B′)**; **(E)** = 200 μm, applies **(E,E′)**; **(E′)** inset = 200 μm, applies **(E)** inset, **(E′)** inset; **(F)** = 10 μm; **(H)** = 200 μm, applies **(H,H′)**; **(I)** = 200 μm, applies **(I,I′)**. *corresponds to $P < 0.05$ (student *t*-test); **corresponds to $P < 0.05$ (ANOVA with Tukey multiple comparisons test).

suitable period for assessing the number of newly born cells that have matured and survived in the OB (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Lledo and Saghatelyan, 2005) (**Figure 1B**).

In the GCL, 6% fewer BrdU+ cell bodies were observed in CCK1R$^{-/-}$ female mice, however, this was not statistically different from WT (**Figures 4A,B**). Because interneurons in the GCL predominantly express GABA, double-immunofluorescence histochemistry for GABA and BrdU was performed (**Figure 4E**) to examine whether reduced SVZ proliferation in CCK1R$^{-/-}$ mice led to a change in the number of BrdU+ newborn cells that had differentiated into GABA-ir interneurons. Forty-two days after the last BrdU administration, the number of cells in the GCL that were BrdU+ and contained GABA-LI was 5% lower in CCK1R$^{-/-}$ mice, but not statistically different to WT mice (**Figure 4C**). The proportion of BrdU+ cell bodies in the GCL

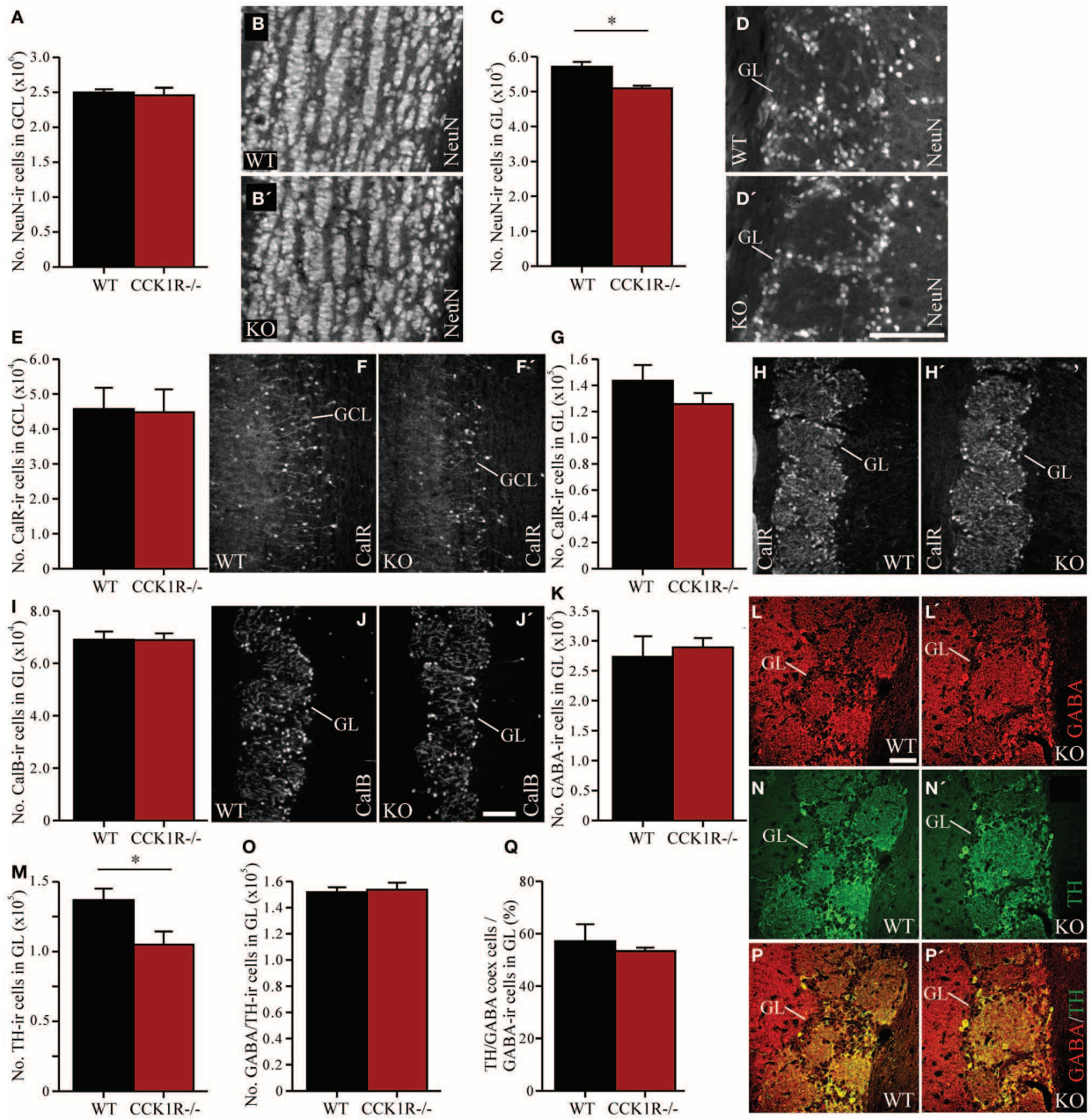

FIGURE 3 | The effect of reduced precursor proliferation in the SVZ of CCK1R$^{-/-}$ female mice on subclasses of interneurons in the GCL and GL. (A,B) Estimated number of NeuN-ir cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(A)**. Photomicrographs of NeuN-LI in the GCL of **(B)** wild-type and **(B′)** CCK1R$^{-/-}$ mice. **(C,D)** Estimated number of NeuN-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(C)**. Photomicrographs of NeuN-LI in the GL of **(D)** wild-type and **(D′)** CCK1R$^{-/-}$ mice. **(E,F)** Estimated number of calretinin-ir cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(E)**. Photomicrographs of calretinin-LI in the GCL of **(F)** wild-type and **(F′)** CCK1R$^{-/-}$ mice. **(G,H)** Estimated number of calretinin-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(G)**. Photomicrographs of calretinin-LI in the GL of **(H)** wild-type and **(H′)** CCK1R$^{-/-}$ mice. **(I,J)** Estimated number of calbindin-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(I)**. Photomicrographs of calbindin-LI in the GL of **(J)** wild-type and **(J′)** CCK1R$^{-/-}$ mice. **(K,L)** Estimated number of GABA-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(K)**. Photomicrographs of GABA-LI in the GL of **(L)** wild-type and **(L′)** CCK1R$^{-/-}$ mice. **(M,N)** Estimated number of TH-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(M)**. Photomicrographs of TH-LI in the GL of **(N)** wild-type and **(N′)** CCK1R$^{-/-}$ mice. **(O)** Estimated number of GABA and TH double-labeled cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice. **(P)** Double-immunofluorescence photomicrographs of TH-LI (green, **N**,**N′**) and GABA-LI (red, **L**, **L′**) in the GL of **(P)** wild-type and **(P′)** CCK1R$^{-/-}$ mice. **(Q)** Estimated number of GABA-ir/TH-ir co-expressing cell bodies expressed as a proportion of the total number of GABA-ir cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice. In all plots, black bars = wild-type female animals ($n = 4$), and red bars = CCK1R$^{-/-}$ female mice ($n = 4$). CalB, calbindin; CalR, calretinin; GABA, γ-aminobutyric acid; GCL, granular cell layer of OB; GL, glomerular cell layer of OB; ir, immunoreactive; KO, CCK1R$^{-/-}$; TH, tyrosine hydroxylase; WT, wild-type. Scale bars: **(D′)** = 100 μm, applies **(B,B′,D,D′)**; **(J′)** = 200 μm, applies **(F,F′,H,H′,J,J′)**; **(L)** = 50 μm, applies **(L,L′,N,N′,P,P′)**. *corresponds to $P < 0.05$.

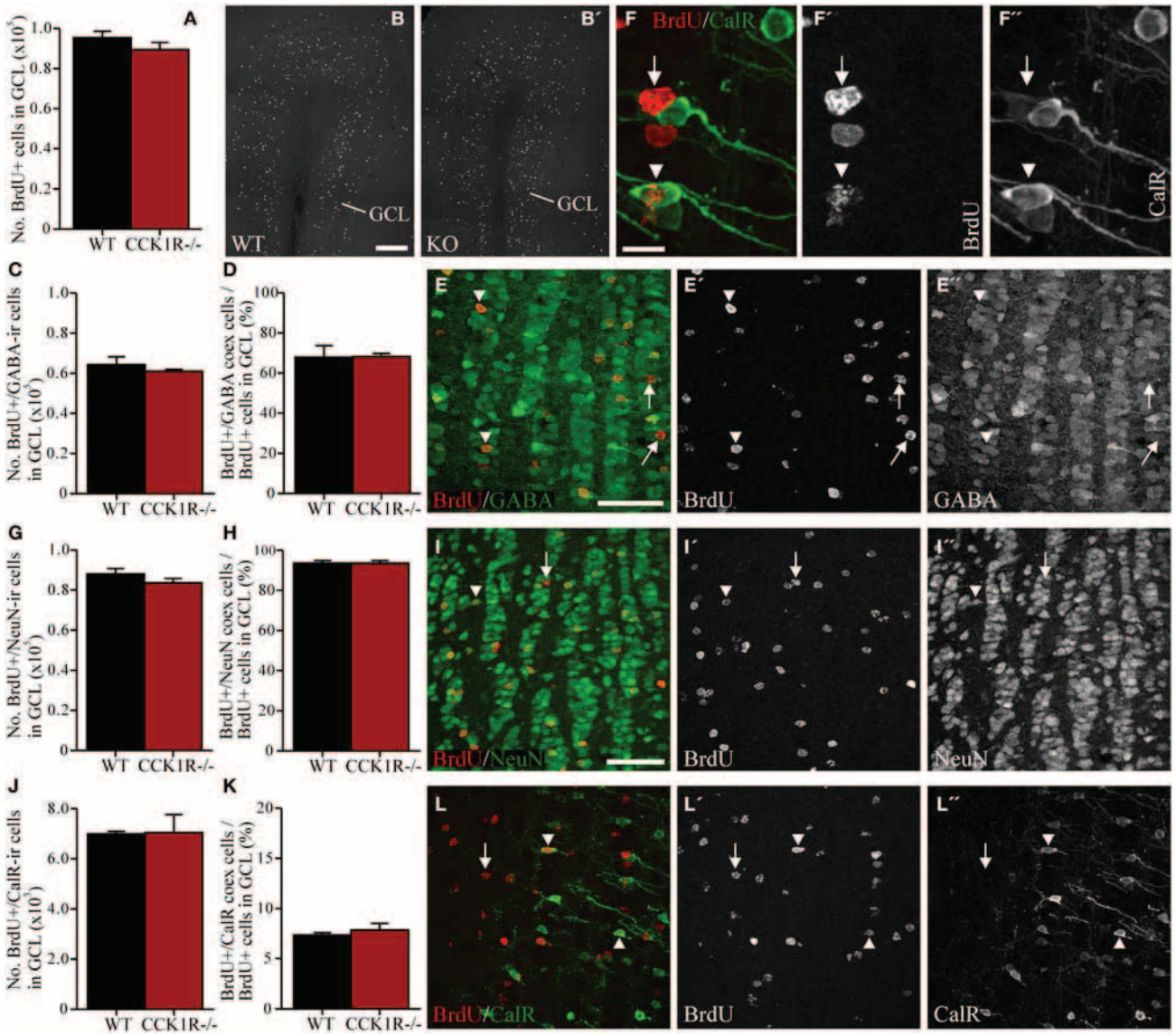

FIGURE 4 | Estimates of adult-born cells in the GCL of female CCK1R$^{-/-}$ and wild-type mice. BrdU (50 mg/kg i.p.) was administered twice daily for 5 consecutive days, and mice killed 42 days after the last BrdU administration (see *Protocol 2*, **Figure 1B**). **(A,B)** Estimated number of BrdU+ cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(A)**. Photomicrograph of BrdU+ cell bodies in the GCL of **(B)** wild-type and **(B′)** CCK1R$^{-/-}$ mice. **(C–E)** Estimated number of BrdU+ and GABA-ir double-labeled cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(C)**. **(D)** Estimated number of BrdU+/GABA-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice. **(E)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**E′**, red) and GABA-LI (**E″**, green) in the GCL of CCK1R$^{-/-}$ mouse. **(G–I)** Estimated number of BrdU+ and NeuN-ir double-labeled cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(G)**. **(H)** Estimated number of BrdU+/NeuN-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice. **(I)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**I′**, red) and NeuN-LI (**I″**, green) in the GCL of CCK1R$^{-/-}$ mouse. **(J–L,F)** Estimated number of BrdU+ and calretinin-ir double-labeled cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice **(J)**. **(K)** Estimated number of BrdU+/calretinin-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GCL of wild-type and CCK1R$^{-/-}$ mice. **(L)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**L′**, red) and calretinin-LI (**L″**, green) in the GCL of CCK1R$^{-/-}$ mouse, shown at higher magnification in **(F)**, **(F′)**, and **(F″)**, respectively. In all plots, black bars = female wild-type animals ($n = 4$), and red bars = female CCK1R$^{-/-}$ mice ($n = 4$). Arrowheads point to double-labeled cell bodies, and arrows to single labeled BrdU+ cell bodies. BrdU, 5-bromo-2-deoxyuridine; CalR, calretinin; GABA, γ-aminobutyric acid; GCL, granular cell layer of OB; ir, immunoreactive; KO, CCK1R$^{-/-}$; NeuN, neuronal nuclei; WT, wild-type; +, positive. Scale bars: **(B)** = 200 μm, applies **(B,B′)**; **(E)** = 50 μm, applies **(E,E′,E″)**; **(F)** = 10 μm, applies **(F,F′,F″)**; **(I)** = 50 μm, applies **(I,I′,I″,L,L′,L″)**.

that co-expressed GABA-LI was also similar in WT (68%) and CCK1R$^{-/-}$ mice (68%) (**Figure 4D**).

Double-immunofluorescence for BrdU and NeuN was also performed (**Figure 4I**). Forty-two days after the last BrdU administration, the number of cells in the GCL that were BrdU+ and contained NeuN-LI was statistically similar in CCK1R$^{-/-}$ and WT mice (**Figure 4G**), as was the proportion of BrdU+ cell bodies in the GCL that co-expressed NeuN-LI (**Figure 4H**). Likewise, the number of cells in the GCL that were BrdU+ and contained calretinin-LI was similar in CCK1R$^{-/-}$ and WT mice (**Figures 4F,J,L**), as was the proportion of BrdU+ cell bodies in the GCL that co-expressed calretinin-LI (**Figure 4K**).

In the GL, the number of BrdU+ cell bodies observed in female CCK1R$^{-/-}$ mice was 15% lower, but not statistically different to the number observed in WT mice (**Figures 5A,B**). Because interneurons in the GL express TH, GABA and calbindin, double-immunofluorescence histochemistry for BrdU and these interneuronal subtypes was performed. Forty-two days after the last BrdU administration, the number of BrdU+ cells in the GL that co-labeled TH-LI in CCK1R$^{-/-}$ mice was 38% lower than in WT mice (**Figures 5C,E,F**), and the proportion of BrdU+ cells co-expressing TH-LI reduced from 11% in WT mice, to 9% in CCK1R$^{-/-}$ mice (**Figure 5D**). There were 29% fewer BrdU+ cells in the GL of CCK1R$^{-/-}$ mice that co-labeled GABA-LI (**Figures 5G,I**), although the proportion of BrdU+ cell bodies that co-expressed GABA-LI remained similar to levels observed in WT mice (**Figure 5H**). No statistical change in the number BrdU+ cells in the GL that co-labeled calbindin-LI (**Figures 5J,L**), nor the proportion of BrdU+ cells that co-expressed calbindin-LI (**Figure 5K**) was observed when comparing WT and CCK1R$^{-/-}$ mice. Finally, no BrdU+ cell bodies in GCL or GL of CCK1R$^{-/-}$ or WT mice were found to co-express DCX-LI 42 days after the last BrdU administration (data not shown).

ADULT FEMALE CCK1R$^{-/-}$ MICE HAVE A LOWER NUMBER OF PROLIFERATING CELLS IN THE SGZ

To examine whether CCK1R deletion leads to a change in the number of proliferating cells in the DG, immunoreactivity against Ki67 was performed to identify proliferating cells in the SGZ (i.e., cells bordering the polymorph and granule layers of the DG), who subsequently differentiate into granule cells in the DG (Eriksson et al., 1998; Cameron and McKay, 2001; Kee et al., 2002; Ming and Song, 2011; von Bohlen Und Halbach, 2011). Ki67-ir cell bodies in the SGZ of female CCK1R$^{-/-}$ mice were down 56% in comparison to female WT controls (**Figures 6A–C**). A statistical difference in the number of Ki67 cells in the SGZ was observed when comparing WT and female CCK1R$^{-/-}$ mice and female CCK1R$^{-/-}$ vs. male CCK1R$^{-/-}$ mice, while WT and male CCK1R$^{-/-}$ mice were statistically similar, as were male and female WT mice (**Figure 6A**, ANOVA with Tukey multiple comparisons test).

BrdU (150 mg/kg i.p.) was also injected into mice 2 h prior to their death (**Figure 1A**). BrdU+ cell bodies were found in the SGZ, where their number in female CCK1R$^{-/-}$ mice was 38% lower than in female WT mice (**Figures 6D–F**).

LOWER NUMBERS OF DCX-IR NEUROBLASTS IN THE DENTATE GYRUS OF FEMALE CCK1R$^{-/-}$ MICE

Because neural progenitors in the SGZ give rise to immature neurons (Van Praag et al., 2002; Ming and Song, 2005), we examined whether the number of DCX-ir neuroblasts in the DG were altered in female CCK1R$^{-/-}$ mice. DCX-ir cell bodies were observed within the SGZ and GrDG, with dendritic processes extending through the GrDG, and into the molecular layer (**Figure 6H**). There were 80% fewer DCX-ir neuroblasts in the SGZ and GrDG of CCK1R$^{-/-}$ mice than in WT mice (**Figures 6G–I**).

SIMILAR NUMBERS OF GFAP-IR ASTROCYTES IN THE DENTATE GYRUS OF FEMALE CCK1R$^{-/-}$ MICE

Immunohistochemistry for GFAP was performed to determine whether reduced proliferation in the SGZ also influenced the number of astrocytes in the DG. Strong GFAP-LI was observed in the DG of CCK1R$^{-/-}$ and WT mice (**Figures 6K,L**), and the number of GFAP-ir astrocytes in the SGZ and GrDG was quantified. We found 12% fewer GFAP-ir astrocytes in CCK1R$^{-/-}$ mice which was not statistically different from WT controls (**Figure 6J**), suggesting that reduced proliferation in the SGZ of CCK1R$^{-/-}$ female mice leads principally to the generation of fewer cells of neural lineage, without affecting the generation of astrocytes.

SIMILAR NUMBERS OF CALBINDIN-IR CELL BODIES IN THE DENTATE GYRUS OF FEMALE CCK1R$^{-/-}$ MICE

Newly generated neurons in the SGZ migrate a short distance to the GrDG (Ming and Song, 2005), where they mature into neurons that express calbindin (Sloviter, 1989; Markakis and Gage, 1999; Van Praag et al., 2002). Immunohistochemistry for calbindin was performed to determine whether reduced proliferation in the SGZ also led to fewer calbindin-ir neurons in the GrDG. Although the number of calbindin-ir cell bodies in CCK1R$^{-/-}$ mice was 10% fewer than in WT mice (**Figures 6M–O**), this change was not statistically different.

NO CHANGE IN THE NUMBER OF MATURE NEWBORN CELLS IN THE DENTATE GYRUS OF CCK1R$^{-/-}$ FEMALE MICE

We next examined the effect of reduced SGZ precursor proliferation on the number of newborn cells that integrate and survive in the GrDG. BrdU (50 mg/kg i.p.) was administered twice daily for 5 consecutive days, and the mice were killed 42 days after the last BrdU administration (**Figure 1B**), a suitable period for assessing the number of newly born cells that have survived and matured in the GrDG (Ming and Song, 2005).

In the GrDG, the number of BrdU+ cell bodies observed in CCK1R$^{-/-}$ female mice was similar to the number observed in WT mice (**Figures 6P–R**). Because the majority of mature neurons in the GrDG express calbindin-LI, double-immunofluorescence histochemistry for calbindin and BrdU was performed to examine whether reduced SGZ proliferation in CCK1R$^{-/-}$ mice led to a change in the number of BrdU+ newborn cells that had differentiated into calbindin-ir interneurons. Forty-two days after the last BrdU administration, the number of cells in the GrDG that were BrdU+ and contained calbindin-LI

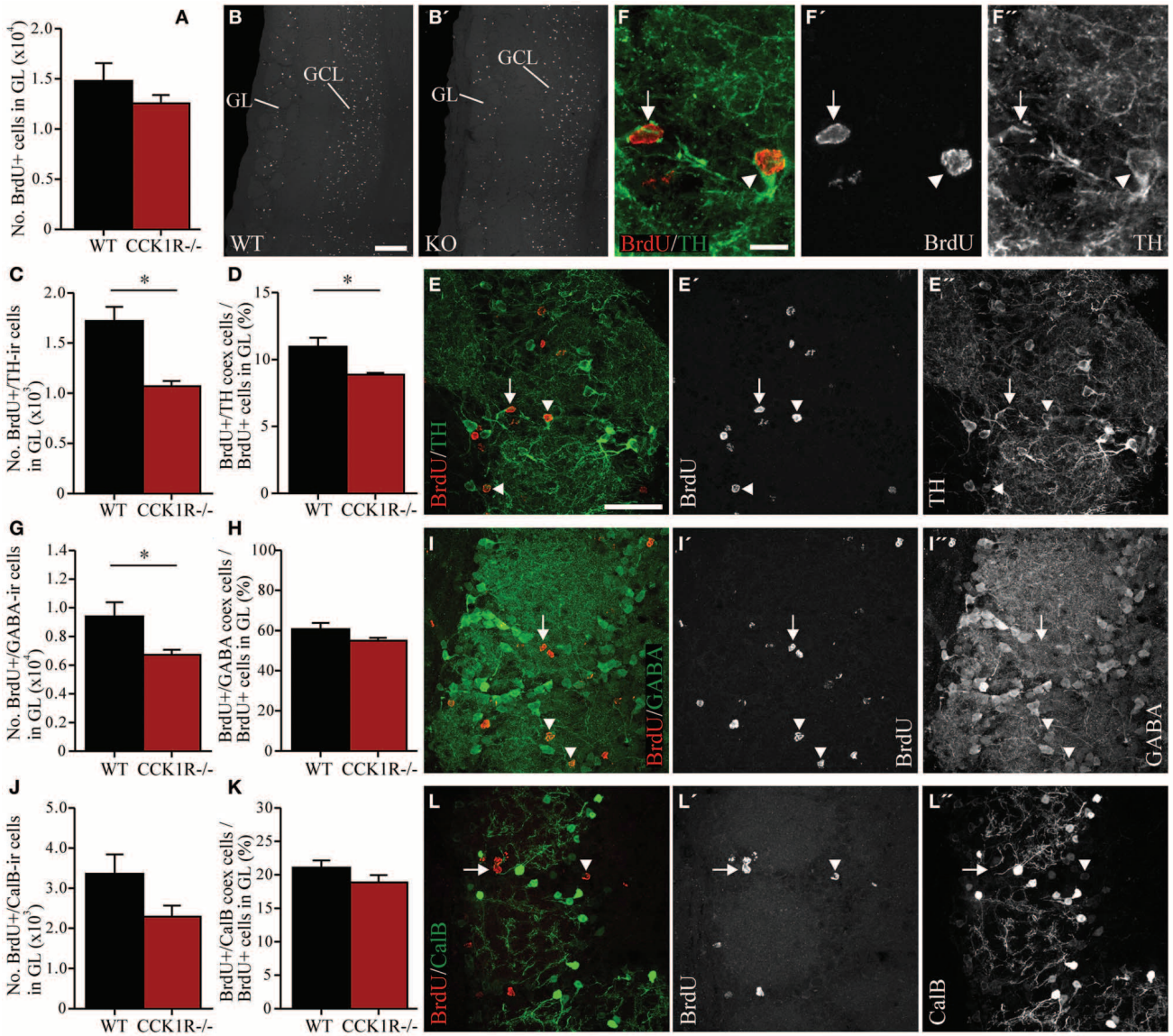

FIGURE 5 | Estimates of adult-born cells in the GL of female CCK1R$^{-/-}$ and wild-type mice. BrdU (50 mg/kg i.p.) was administered twice daily for 5 consecutive days, and mice killed 42 days after the last BrdU administration (see *Protocol 2*, **Figure 1B**). **(A,B)** Estimated number of BrdU+ cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(A)**. Photomicrographs of BrdU+ cell bodies in the GL of **(B)** wild-type and **(B′)** CCK1R$^{-/-}$ mice. **(C–F)** Estimated number of BrdU+ and TH-ir double-labeled cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(C)**. **(D)** Estimated number of BrdU+/TH-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice. **(E)** Double-immunofluorescence confocal micrograph of BrdU-LI (**E′**, red) and TH-LI (**E″**, green) in the GL of CCK1R$^{-/-}$ mouse, shown at higher magnification in **(F)**, **(F′)**, and **(F″)**, respectively. **(G–I)** Estimated number of BrdU+ and GABA-ir double-labeled cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(G)**. **(H)** Estimated number of BrdU+/GABA-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice. **(I)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**I′**, red) and GABA-LI (**I″**, green) in the GL of CCK1R$^{-/-}$ mouse. **(J–L)** Estimated number of BrdU+ and calbindin-ir double-labeled cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice **(J)**. **(K)** Estimated number of BrdU+/calbindin-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GL of wild-type and CCK1R$^{-/-}$ mice. **(L)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**L′**, red) and calbindin-LI (**L″**, green) in the GL of CCK1R$^{-/-}$ mouse. In all plots, black bars = female wild-type animals ($n = 4$), and red bars = female CCK1R$^{-/-}$ mice ($n = 4$). Arrowheads point to double-labeled cell bodies, and arrows to single labeled BrdU+ cell bodies. BrdU, 5-bromo-2-deoxyuridine; CalB, calbindin; GABA, γ-aminobutyric acid; GCL, granular cell layer of OB; GL, glomerular cell layer of OB; ir, immunoreactive; KO, CCK1R$^{-/-}$; TH, tyrosine hydroxylase; WT, wild-type; +, positive. Scale bars: **(B)** = 200 μm, applies **(B,B′)**; **(E)** = 50 μm, applies **(E,E′,E″,I,I′,I″,** and **L,L′,L″)**; **(F)** = 10 μm, applies **(F,F′,F″)**. *corresponds to $P < 0.05$.

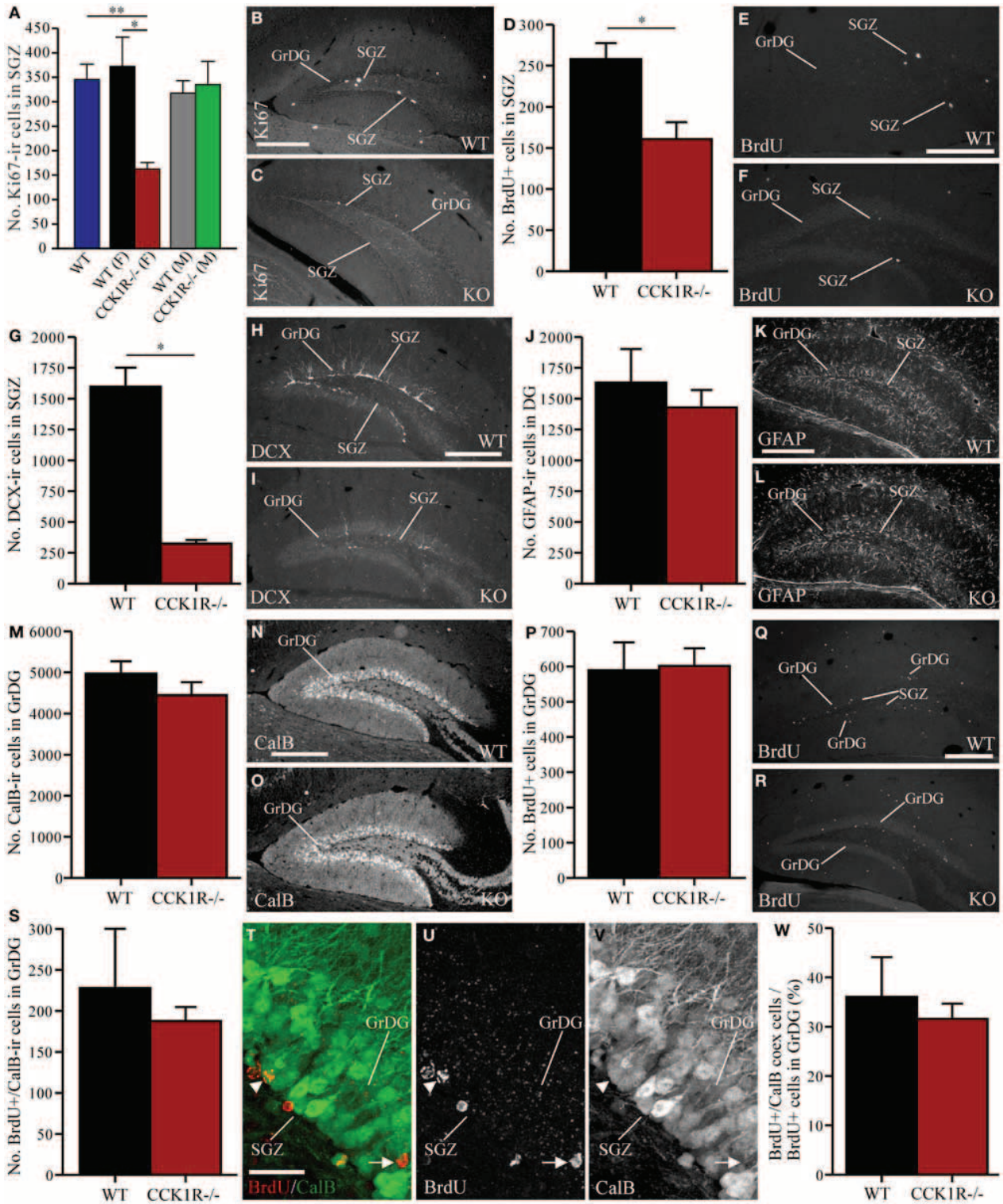

FIGURE 6 | Estimates of proliferating cells, neuroblasts, astrocytes, and mature neurons in the SGZ and GrDG of CCK1R$^{-/-}$ and wild-type mice. **(A–C)** Estimated number of Ki67-ir cell bodies in the SGZ of CCK1R$^{-/-}$ and wild-type mice **(A)**. In **(A)**: WT blue bar = wild-type (male and female combined, $n = 8$); WT (F) black bar = female wild-type ($n = 4$); CCK1R$^{-/-}$ (F) red bar = female CCK1R$^{-/-}$ ($n = 4$); WT (M) grey bar = male wild-type ($n = 4$); and CCK1R$^{-/-}$ (M) green bar = male CCK1R$^{-/-}$ ($n = 4$). Photomicrograph of Ki67-ir cell bodies in the SGZ of **(B)** female wild-type and **(C)** female CCK1R$^{-/-}$ mice. **(D–F)** Estimated number of BrdU+ cell bodies in the SGZ of female CCK1R$^{-/-}$ and female wild-type mice **(D)** (see *Protocol 1*, **Figure 1A**). Photomicrograph of BrdU+ cell bodies in the SGZ of

(Continued)

FIGURE 6 | Continued
(E) female wild-type and **(F)** female CCK1R$^{-/-}$ mice. **(G–I)** Estimated number of DCX-ir neuroblast cells in the SGZ of female CCK1R$^{-/-}$ and female wild-type mice **(G)**. Photomicrograph of DCX-ir neuroblasts in the SGZ of **(H)** female wild-type and **(I)** female CCK1R$^{-/-}$ mice. **(J–L)** Estimated number of GFAP-ir astrocytes in the DG of female CCK1R$^{-/-}$ and female wild-type mice **(J)**. Photomicrograph of GFAP-ir astrocytes in the DG of **(K)** female wild-type and **(L)** female CCK1R$^{-/-}$mice. **(M–O)** Estimated number of calbindin-ir cell bodies in the GrDG of female CCK1R$^{-/-}$ and female wild-type mice **(M)**. Photomicrograph of calbindin-ir cell bodies in the GrDG of **(N)** female wild-type and **(O)** female CCK1R$^{-/-}$ mice. **(P–R)** Estimated number of BrdU+ cell bodies in the GrDG of female wild-type and female CCK1R$^{-/-}$ mice **(P)**. BrdU (50 mg/kg i.p.) was administered twice daily for 5 consecutive days, and mice killed 42 days after the last BrdU administration (see *Protocol 2*, **Figure 1B**). Photomicrograph of BrdU+ cell bodies in the GrDG of **(Q)** female wild-type and **(R)** female CCK1R$^{-/-}$ mice. **(S–W)** Estimated number of BrdU+ and calbindin-ir double-labeled cell bodies in the GrDG of female wild-type and female CCK1R$^{-/-}$ mice **(S)**. **(T)** Double-immunofluorescence confocal micrograph showing BrdU-LI (**U**, red) and calbindin-LI (**V**, green) in the GrDG of female wild-type mouse. Arrowheads point to BrdU/calbindin double-labeled cell bodies. Arrows point to single-labeled BrdU+ cell bodies. **(W)** Estimated number of BrdU+/calbindin-ir co-expressing cell bodies expressed as a proportion of the total number of BrdU+ cell bodies in the GrDG of female wild-type and female CCK1R$^{-/-}$ mice. In all plots, black bars = female wild-type animals ($n = 4$), and red bars = female CCK1R$^{-/-}$ mice ($n = 4$). BrdU, 5-bromo-2-deoxyuridine; CalB, calbindin; DCX, doublecortin; GFAP, glial fibrillary acidic protein; GrDG, granule cell layer of DG; ir, immunoreactive; KO, CCK1R$^{-/-}$; SGZ, subgranular zone of DG; WT, wild-type; +, positive. Scale bars: **(B)** = 250 μm, applies **(B,C)**; **(E)** = 200 μm, applies **(E,F)**; **(H)** = 250 μm, applies **(H,I)**; **(K)** = 250 μm, applies **(K,L)**; **(N)** = 250 μm, applies **(N,O)**; **(Q)** = 200 μm, applies **(Q,R)**; **(T)** = 25 μm, applies **(T–V)**. *corresponds to $P < 0.05$ (student *t*-test); **corresponds to $P < 0.05$ (ANOVA with Tukey multiple comparisons test).

was 18% fewer in CCK1R$^{-/-}$ mice, but not statistically different to WT mice (**Figures 6S–V**). A 12% decrease in the proportion of BrdU+ cell bodies in the GCL that co-expressed calbindin-LI was found in CCK1R$^{-/-}$ mice (**Figure 6W**), which also was not statistically different to the proportion found in WT mice. Finally, 42 days after the last BrdU administration, no BrdU+ cell bodies in the GrDG of CCK1R$^{-/-}$ or WT mice were found to co-express DCX-LI (data not shown).

DISCUSSION

Using genetically modified mice, we provide evidence that CCK, by actions mediated through the CCK1R, can regulate cell proliferation in the adult mouse SVZ and SGZ. Female mice lacking these receptors were found to have fewer proliferating cells and less migratory neuroblasts in the SVZ, RMS and SGZ. Our data indicate that the reduced number of proliferating precursors in the SVZ and SGZ of CCK1R$^{-/-}$ female mice had a discrete effect on the number of mature neurons in the OB: the number NeuN-ir cell bodies and TH-ir interneurons in the GL of the OB was reduced, as was the number of BrdU+ cell bodies in the GL that co-expressed TH-LI or GABA-LI.

In general, our results point to a regulation of neurogenesis in the adult brain, so that a steady neuronal population is maintained in the OB and GrDG, irrespective of the number of proliferating cells in the SVZ or SGZ, or their rate of proliferation. Despite the reduction in proliferating cells and neuroblasts in the SVZ, RMS and SGZ of female CCK1R$^{-/-}$ mice, the number of adult-born BrdU+ cell bodies in the GL, GCL and GrDG was similar to WT mice 42 days after the last BrdU pulse. This coincided with a similar number of adult-born BrdU+ neurons in CCK1R$^{-/-}$ and WT mice that expressed: NeuN-, GABA or calretinin-LI in the GCL; calbindin-LI in the GCL; and calbindin-LI in the GrDG. The capacity for maintaining the number of adult-born cells that survived and integrated into the circuitry of the OB and GrDG, despite the lower availability of adult-born cells, led to the number of mature NeuN-ir and calretinin-ir cell bodies in the GCL, calbindin-ir, calretinin-ir and GABA-ir cell bodies in the GL, and calbindin-ir cell bodies in the GrDG remaining the same in CCK1R$^{-/-}$ and WT mice.

Our findings are in contrast with previous reports that used agents that cause permanent and often complete suppression of proliferation in the SVZ or SGZ, and that examined the effects of reduced SVZ/SGZ proliferation over a longer period. In previous work, genetic ablation of newly formed neurons in adult mice led to a progressive reduction in the number of DCX-ir neuroblasts in the SVZ and a gradual decrease in OB granule cells 3–12 weeks after ablation (Imayoshi et al., 2008). Similarly, x-ray irradiation that reduced adult-born cells in the SVZ by 96% led to a 20% decrease in OB granule cells 8 weeks after irradiation (Valley et al., 2009). Here, we report a ∼40% reduction in the number of proliferating cells and 29% fewer DCX-ir neuroblasts in the SVZ/RMS of female CCK1R$^{-/-}$ mice, but no consequent change in the number of OB granule cells.

A key difference in models used previously is the almost complete suppression of proliferating cells and neuroblasts in the SVZ/RMS that was induced in adult animals (Imayoshi et al., 2008; Valley et al., 2009). This compares to the permanent, but less vigorous, reduction of proliferating cells and neuroblasts that arises from the developmental deletion of the CCK1R, which allows for the generation of a lower than normal number of adult-born cells that still have the capacity to migrate to and integrate into OB circuitry. Thus, an explanation for our observation of no change in OB granule cells when SVZ proliferation is reduced, is a greater rate of survival of adult-born cells generated in the SVZ and RMS of CCK1R$^{-/-}$ mice. In support of this notion, the number of mature BrdU+ interneurons in the GCL that expressed GABA-LI, calretinin-LI or NeuN-LI was similar in CCK1R$^{-/-}$ and WT mice 42 days after the last BrdU pulse.

Under normal conditions, adult-born cells generated in the SGZ add to the number of neurons in the GrDG over time (Bayer et al., 1982; Dayer et al., 2003; Imayoshi et al., 2008), whereas ablation of neurogenesis prevents such an increase so that the number and density of neurons in the GrDG remain constant (Imayoshi et al., 2008). If the same principles were to apply here, we would expect the number of neurons in the GrDG of CCK1R$^{-/-}$ mice to be lower than the numbers in WT mice. However, we found a similar number of calbindin-ir cell bodies in the GrDG of CCK1R$^{-/-}$ and WT mice, despite the reduction in proliferating cells (38 and 56% fewer BrdU+ and Ki67-ir cell bodies, respectively) and DCX-ir neuroblasts (80%) in the SGZ. Again, this may be attributable to the increased rate of survival of adult-born

cells generated in the SGZ, where 42 days after the last injection of BrdU, a similar number of BrdU+ cell bodies and BrdU cell bodies that expressed calbindin-ir was observed in the GrDG of CCK1R$^{-/-}$ and WT mice.

The situation was different in the GL of the OB, where fewer TH-ir interneurons and BrdU+ cell bodies that expressed either TH-LI or GABA-LI were found in CCK1R$^{-/-}$ mice. No change in the number of calbindin-ir cell bodies, or BrdU+/calbindin-ir interneurons was observed in the GL however, suggesting that CCK1R deletion and/or reduced SVZ proliferation has a limited influence on calbindin-expressing interneurons, most of which are generated early in life (De Marchis et al., 2007). In contrast, TH-ir and calretinin-ir cell bodies in the GL are predominantly generated in the adult (McLean and Shipley, 1988; Winner et al., 2002; De Marchis et al., 2007) and the number of TH-ir cell bodies was affected in adult female CCK1R$^{-/-}$ mice. [N.B. the non-statistical trend for a reduction in calretinin-ir cell bodies in the GL of CCK1R$^{-/-}$ mice, and previous qualitative results showing reduced numbers of calretinin-ir cell bodies in CCK1R$^{-/-}$ mice (Stanić et al., 2008).] Recently, we also found changes to the numbers of calretinin-ir and TH-ir, but not calbindin-ir, cell bodies in the GL of adult mice following an induced reduction of proliferation in the SVZ (Sui et al., 2012), and odor deprivation reduces TH expression in the GL, without affecting the GABA, calbindin and calretinin phenotypes (Stone et al., 1991; Baker et al., 1993; Bastien-Dionne et al., 2010). Our results therefore suggest that the CCK1 receptor may play an important role in modulating the generation and/or survival of TH (and calretinin) interneurons in the GL of the OB.

While plasticity in the population of TH-ir interneurons may reflect their ability to adapt to continuously changing odor environments (Doetsch and Hen, 2005), changes in subtypes of interneurons present in the OB may alter the complex circuitry that exists within the OB. This includes the intricate arrangement of dendrites in the external plexiform layer that are derived from mitral, granule and tufted cells that engage in dendro-dendritic reciprocal synaptic interactions with each other (Rall et al., 1966; Shepherd, 1972; Jackowski et al., 1978; Shipley et al., 1996; Stanić et al., 2010), and the interactions of periglomerular cells in the glomerular layer (Shipley and Ennis, 1996; Kosaka and Kosaka, 2005). Because less TH-ir interneurons were integrated into OB circuitry, functional properties of mitral and tufted cells [e.g., their odorant-evoked firing properties (Nagayama et al., 2004)] and the timing of the transmission of olfactory information and bulbar output may be altered. Thus, it would be interesting to determine whether deficits in olfactory functioning, e.g., short- and long-term odor memory, odor discrimination and fear conditioning (Gheusi et al., 2000; Rochefort et al., 2002; Lazarini et al., 2009; Valley et al., 2009), exist in adult CCK1R$^{-/-}$ female mice.

Proliferating cells and neuroblasts were reduced only in female CCK1R$^{-/-}$ mice. It is possible that this sex difference is related to estrous cycle influences (Ormerod and Galea, 2001) because, for example, estrus induction is associated with increased numbers of dividing cells in the SVZ/RMS of prairie voles (Smith et al., 2001), and adult female rats produce more cells during proestrus, compared with estrus and diestrus (Tanapat et al., 1999). Furthermore, levels of CCK fluctuate in the brain during a normal estrous cycle (Hilke et al., 2007). Thus, lower levels of CCK during pro-estrus, combined with absence of the CCK1R, may reduce the rate of cell division in neurogenic regions of the female mouse brain. However, we do not favor this explanation because, in C57BL6 mice, proliferation or neurogenesis in the SGZ is not influenced by the estrous cycle or after ovariectomy (Lagace et al., 2007), and no gender differences in hippocampal proliferation or neurogenesis was observed here, or previously in mice (Lagace et al., 2007; Manning et al., 2012). Thus, it is unlikely that the estrous cycle and fluctuating estradiol levels contributed to the lower numbers of proliferating cells and neuroblasts observed in female CCK1R$^{-/-}$ mice. The neurochemical mechanisms underlying reduced proliferating cells and neuroblasts in female CCK1R$^{-/-}$ mice remains unclear and awaits future investigations.

In conclusion, we show a reduction of proliferation in the SVZ and SGZ of adult female CCK1R$^{-/-}$ mice that does not alter the number of mature neurons in the OB and GrDG, except for TH-ir interneurons in the GL of the OB. Despite these mice having fewer proliferating cells and neuroblasts, we proposed that the numbers of mature neurons are maintained in the OB and GrDG due to the enhanced survival of neuroblasts and mature neurons that integrate into the circuitry of the OB and DG. Further investigations are needed to understand the role of these "longer-surviving" adult-born cells on the circuitry and function of the OB and DG.

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The therapeutic potential of endogenous hippocampal stem cells for the treatment of neurological disorders

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While it is now well-established that resident populations of stem and progenitor cells drive neurogenesis in the adult brain, a growing body of evidence indicates that these new neurons play a pivotal role in spatial learning, memory, and mood regulation. As such, interest is gathering to develop strategies to harness the brain's endogenous reservoir of stem and progenitor cells, with the view that newborn neurons may help overcome the loss of neural and cognitive function that occurs during neurodegenerative disease and psychiatric illness. Here we review evidence for the presence of endogenous stem cell populations in the adult hippocampus, especially large pools of latent stem and precursor cells, and the ways in which these populations can be stimulated to produce new neurons. While the translation of this research from animal models to human application is still in its infancy, understanding in detail the cellular and molecular mechanisms that regulate endogenous neurogenesis, offers the potential to use this innate reservoir of precursors to produce neurons that may be able to mitigate against cognitive decline and mood disorders.

Keywords: hippocampus, neural stem cell, neural precursor cell, stem cell therapy, endogenous adult neurogenesis

INTRODUCTION

Stem cells have the remarkable ability to self-renew, divide and differentiate into diverse cell types. We are starting to realize the vast implications embryonic and adult stem cells may have for regenerative medicine. In particular, there is a great degree of interest in the development of strategies to harness the therapeutic potential of endogenous neural stem cells in the adult brain for their use in the treatment of neurological disorders. With precise and controlled manipulation, stem cells may have the capacity to replace damaged neurons, halt the progression of neurodegenerative diseases, and ultimately recover function in damaged area(s) of the brain. Since 1992, it has been know that endogenous stem and precursor cells reside in the brains of adult animals (Reynolds and Weiss, 1992; Richards et al., 1992), primarily, though by no means exclusively, in two major areas of the adult brain—the subgranular zone (SGZ) of the hippocampus, and the subventricular zone of the lateral ventricles—and have been shown to continuously generate functional new neurons throughout adulthood. Furthermore, considering recent studies demonstrating the crucial role of newborn neurons in learning and memory formation (Deng et al., 2010; Marin-Burgin and Schinder, 2012) and mood regulation (Sahay and Hen, 2007), the enhancement of hippocampal neurogenesis has immense potential for the treatment of age-associated cognitive decline, dementia, and mental health disorders.

Adult hippocampal neurogenesis in the SGZ is a tightly orchestrated process, involving the maintenance, activation and proliferation of the stem cells; differentiation and migration of the intermediate progenitors; and maturation of the newborn neurons (Bergami and Berninger, 2012; Jhaveri et al., 2012; Lugert et al., 2012). Following successful integration into the neuronal network, these neurons are able to contribute to hippocampal function, as well as sculpt the existing circuitry (Song et al., 2012). A host of intrinsic and extrinsic factors regulates each of these developmental stages (Zhao et al., 2008; Ming and Song, 2011; Jhaveri et al., 2012). To exploit the brain's endogenous reservoir of hippocampal neural stem cells as a potential therapeutic strategy, it is necessary to examine (1) the properties of stem cells that enable them to proliferate, and (2) the methods that can be employed to stimulate their proliferation and neuronal differentiation.

THE QUIESCENT POPULATION OF STEM AND PROGENITOR CELLS IN THE ADULT HIPPOCAMPUS

As mentioned, a *bona fide* stem cell must have the defining characteristics of self-renewal through mitotic division, and the ability to differentiate into different cell types. On the other hand, if a cell has limited self-renewal and restricted lineage potential, it is classified as a progenitor cell (Bonaguidi et al., 2011). Without specific techniques to distinguish between these two cell types, stem and progenitor cells are often collectively referred to as precursor cells. Nevertheless, with our advancing understanding of the transcription factors and markers expressed during the different stages of neurogenesis (Hodge and Hevner, 2011; von Bohlen und Halbach, 2011), the introduction of constitutive reporters and inducible transgenic mouse lines (Dhaliwal and Lagace, 2011), and morphological criteria, successful identification of stem and progenitor cells is becoming somewhat easier.

Nevertheless, because unequivocal identification of the potential of a single precursor *in vivo* is still very difficult, characterization and enumeration of stem and progenitor cells in the

hippocampus has largely arisen from *in vitro* studies utilizing the neurosphere assay (Bull and Bartlett, 2005; Walker et al., 2008; Jhaveri et al., 2010). When dissociated precursor cells from adult hippocampal tissue are exposed to mitogens, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), they proliferate to form a ball of cells called a neurosphere. We have found the size of a neurosphere reflects the proliferative capacity of the original precursor cell; for instance, a stem cell with great proliferative and self-renewal potential can generate a neurosphere over 250 μm in diameter, whereas a progenitor cell with limited self-replication generates a neurosphere ∼50 μm in diameter (Walker et al., 2008; Jhaveri et al., 2010). Using this *in vitro* approach, our laboratory was the first to report the presence of a large and previously unidentified population of predominantly quiescent stem cells in the adult mouse hippocampus (Walker et al., 2008). In this study, dissociated hippocampal cells were cultured with depolarizing levels of potassium chloride (KCl), which activated a quiescent population of precursor cells to proliferate and produce a more than two-fold increase in number of neurospheres compared to control conditions. Furthermore, a small number (approximately eight per hippocampus) of very large neurospheres (>250 μm in diameter) were obtained, which displayed the stem cell properties of self-renewal and multipotentiality. When the active and rapidly dividing precursor population (i.e., precursors that formed neurospheres under non-depolarizing conditions) were ablated with the anti-mitotic agent AraC (cytosine-b-D-arabinofuranoside), the quiescent pool of precursor cells remained intact, and responded to high KCl stimulation (Walker et al., 2008). Recently, we have shown that stimulation with the monoaminergic neurotransmitter, norepinephrine, directly activated another population of latent stem and precursor cells, via β3-adrenergic receptors, in the adult hippocampus, which is quite distinct from the KCl-activated population (Jhaveri et al., 2010). This raises the tantalizing prospect that these distinct populations may have give rise to subtly different populations of neuronal progeny. Importantly, a recent *in vivo* clonal analysis, using sparsely labeled and inducible Nestin-positive cells, confirmed the existence of a small population—of similar size to that we identified *in vitro*—of quiescent stem cells in the hippocampus that are also capable of self-renewal and multipotent differentiation (Bonaguidi et al., 2011).

The existence of the quiescent stem cell population in the hippocampus has important ramifications for the use of neurogenesis as a therapeutic strategy for functional recovery following brain injury and disease. Given that stem cells in the quiescent pool are usually dormant, and have a seemingly limitless capacity for proliferation, this population offers an untapped resource for the activation and augmentation of neurogenesis. Thus, the most direct and robust method of increasing the rate of neurogenesis will be to activate the quiescent stem cell pool; however, unless self-renewal in the quiescent population is precisely maintained by a number of factors, including Notch signaling (Pierfelice et al., 2011), its reservoir of stem cells may become depleted, leading to the premature loss of neurogenesis. Furthermore, the mechanisms regulating neuronal differentiation, survival and integration must also be well-managed to obtain successful neurogenesis.

In terms of origin, there may exist a reciprocal relationship between the quiescent and active stem cells in the hippocampus, in that they are able to give rise to one another (Suh et al., 2007; Lavado et al., 2010). Nevertheless, while these two subpopulations are kept in a delicate equilibrium by a number of factors, the precise mechanisms underlying how these pools are regulated and maintained, and the flux between their quiescent and active states, requires further characterization. Nonetheless, we believe that molecular signals may "prime" quiescent precursor cells to be able to respond to proliferative signals coming from the hippocampal niche (see **Figure 1**). Whether these signals come from newborn neurons, mature granule cells or surrounding glial cells as a result of neuronal activity is just beginning to be understood.

ACTIVATION OF THE QUIESCENT STEM CELL POPULATION

The SGZ of the hippocampus is enriched with a vast number of projections arising from a number of areas throughout the brain, which modulate neuronal activity in the dentate gyrus. Neurotransmitters released from these projections have been shown to affect multiple stages of hippocampal neurogenesis (Vaidya et al., 2007). Furthermore, growth factors and signaling molecules that are upregulated following high-intensity neuronal activity may also modulate hippocampal neurogenesis (e.g., Balkowiec and Katz, 2002; Chen et al., 2006). For instance, environmental novelty and neuronal activity-mediated activation of noradrenergic terminals in the dentate gyrus have been shown to result in the release of norepinephrine (Harley, 2007; King and Williams, 2009; Neugebauer et al., 2009), which in turn has been shown to directly activate the Hes5-expressing quiescent pool of stem and precursor cells in the adult hippocampus (Jhaveri et al., 2010). While there is a burgeoning number of putative candidates that regulate neurogenesis, we focus here on the role of neuronal activity in the activation of the quiescent stem cell pool in the adult hippocampus.

ELECTROPHYSIOLOGICAL STIMULATION

Learning-related electrophysiological stimulation of the hippocampus has been widely shown to promote precursor cell proliferation and the survival of newborn neurons in the SGZ (Bruel-Jungerman et al., 2006; Chun et al., 2006; Toda et al., 2008; Kitamura et al., 2010; Stone et al., 2011; Kameda et al., 2012). Nevertheless, the mechanisms underlying how the precursor population responds to the induction of LTP have not yet been definitively revealed. We have shown recently that activation of the precursor cell population occurs only following the successful induction of LTP in the dentate gyrus *in vivo* (Kameda et al., 2012). These precursors appear to be the same population that responds to KCl stimulation *in vitro*, as high KCl did not further increase the number of neurospheres in the LTP-induced hippocampal cells. The LTP-mediated activation of precursor cells, and the increase in the production of doublecortin (DCX)-positive neurons observed *in vivo*, was specific to the induction of NMDA-dependent late-LTP, as this effect was blocked by the NMDA receptor antagonist CPP [(6)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid], administered shortly before the induction of LTP. Similarly, protocols that delivered low-frequency stimulation, or high-frequency stimulation

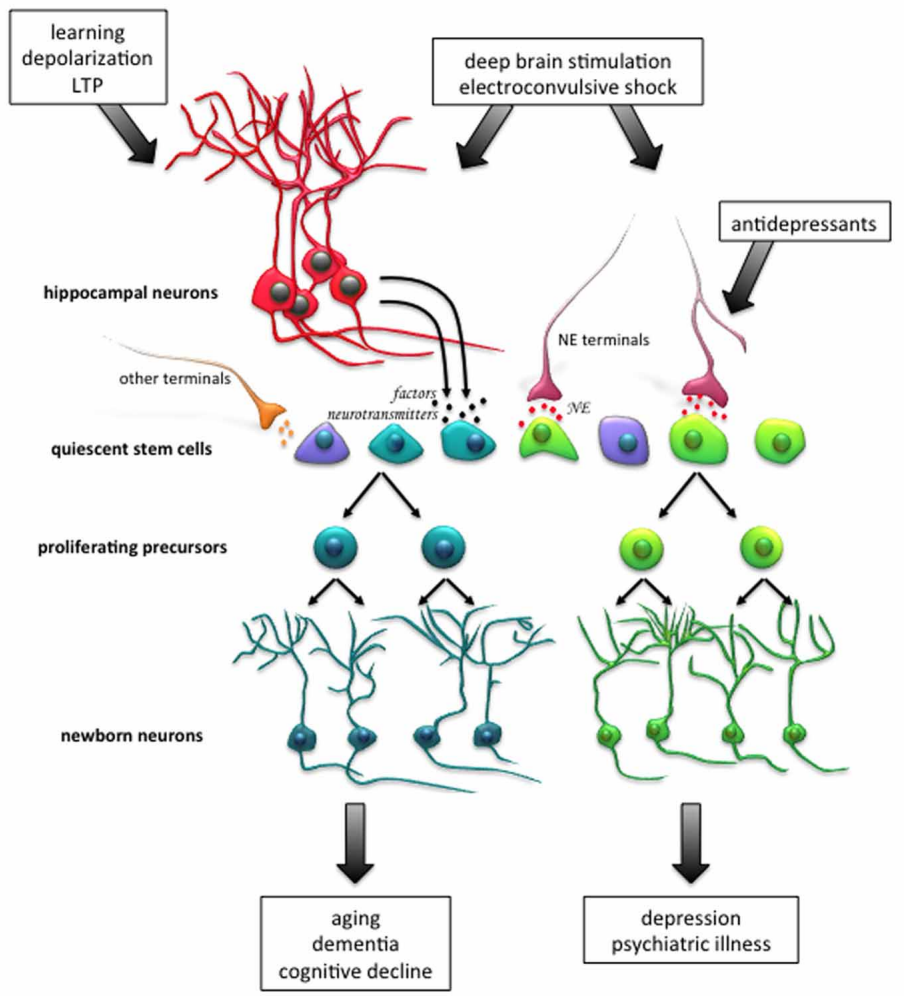

FIGURE 1 | Activation of the endogenous population of quiescent stem cells in the adult hippocampus as a therapeutic strategy for neural injury and disease. Enhancement of neurogenesis, through activation of the quiescent stem cell pool, may facilitate neuronal repair and recovery of cognitive function following aging, neuronal disorders, psychiatric conditions, and neurodegenerative diseases. For instance, neuronal activity, such as that which occurs during learning, the induction of long-term potentiation (LTP) and *in vitro* depolarization, potently activates quiescent stem cell proliferation. A different subpopulation of stem cells is activated following exposure to norepinephrine (NE), and it is thought that this population responds to NE-mediated antidepressant treatment which can alleviate clinical depression. Patterns of electrical stimulation, such as deep brain stimulation and electroconvulsive shock, which are currently used to treat many neurodegenerative and psychiatric conditions in human patients, have also been shown to promote proliferation in animal models. Newborn neurons arising from the distinct progenitor cell pools may have differential roles in the functional outcome of neurodegenerative disease and psychiatric illness. Abbreviations: NE, norepinephrine; LTP, long-term potentiation.

that failed to induce LTP or only induced early-LTP, did not affect precursor activation or proliferation. Interestingly, the magnitude of LTP strongly correlated with the extent of precursor cell activation, suggesting a dose-dependent-like mechanism underlies this effect. While speculative, it is possible that LTP induction somehow primes hippocampal precursor cells to respond to factors that directly activate proliferation. This effect may be temporally transient, given that when the hippocampus was dissociated 4 days following the induction of LTP, no significant increase in activation of precursor cells was seen *in vitro* (Kameda et al., 2012). Nonetheless, these observations are the first to provide direct evidence that patterns of synaptic activity associated with learning and memory can activate latent precursor cells in the adult hippocampus, as well as increase neuron production. More studies are required to investigate the mechanism of precursor cell activation following defined patterns of physiological stimulation, considering that synaptic plasticity, learning, and neurogenesis are intimately linked.

Other patterns of electrical stimulation have also been shown promote neurogenesis. Stimulation protocols used to induce electroconvulsive seizure (ECS) robustly increase the proliferation and survival of progenitor cells in rodents (Madsen et al., 2000; Ito et al., 2010) and non-human primates (Perera et al., 2007). Furthermore, recent clinical advances are starting to show that

deep brain stimulation (DBS) of the human brain can successfully treat many psychiatric and neurological conditions (reviewed in Cramer et al., 2011; Moreines et al., 2011; Hamani and Temel, 2012), and has been shown to affect neurogenesis in rodent models of disease (e.g., Khaindrava et al., 2011). While the link between electrical stimulation of the human brain and neurogenesis awaits investigation, a phase I clinical trial assessing the effect of DBS targeting the fornix/hypothalamus in patients experiencing mild Alzheimer's disease has revealed that some patients showed a slower rate of cognitive decline over a 12-month period (Laxton et al., 2010). Therefore, given the ability of electrical stimulation to enhance endogenous neurogenesis, and the strong links between neurogenesis in the SGZ and improvement in cognitive function (e.g., Deng et al., 2010; Marin-Burgin and Schinder, 2012), stimulating the quiescent population of stem and precursor cells to augment endogenous neurogenesis to recover or enhance cognitive function in neurodegenerative disease in an alluring prospect. Nevertheless, the identity of the latent precursor pool(s) that respond to the different patterns of electrical activity (e.g., LTP-inducing high-frequency stimulation, ECS and DBS) to generate new neurons remains to be addressed.

HIPPOCAMPAL NEUROGENESIS AND AGING

It is now well-established that hippocampal neurogenesis dramatically decreases with age. Aged animals show significantly less precursor and progenitor cell proliferation, neuronal differentiation and newborn neuron survival (reviewed by Lee et al., 2012). This is also true for the number of neurospheres that can be generated from mice as they age, which by 18 months in the hippocampus, declines by about 80% (Walker et al., 2008). The cause of this neurogenic decline over time is not well-understood, as many factors underlying the maintenance and regulation of neurogenesis are altered in the aged brain (Artegiani and Calegari, 2012; Lee et al., 2012). Nonetheless, given the negative impact of aging on hippocampal neurogenesis (Lee et al., 2012) and cognitive function (e.g., Rosenzweig and Barnes, 2003), stimulating the endogenous stem cell pool in the aged brain may be able to restore the memory and cognitive deficits observed in age-associated and mild cognitive impairment, and neurodegenerative diseases, such as Alzheimer's disease (e.g., Laxton et al., 2010).

To develop an effective strategy to enhance neurogenesis in the aged hippocampus, it is important to identify whether the age-related reduction in proliferation and neurogenesis results from an increase in stem cell quiescence over time (Lugert et al., 2010; Bonaguidi et al., 2011), or depletion of the stem cell pool (Encinas et al., 2011). A study from our laboratory has shown that the aged brain does in fact retain a population of quiescent stem cells, which have the capacity to respond to stimuli that activate proliferation *in vitro* (Walker et al., 2008). Indeed, physical exercise, which strongly increases stem cell proliferation and neuron production (van Praag et al., 1999; Kronenberg et al., 2003; Lugert et al., 2010), has been positively correlated with improved hippocampal-dependent cognitive performance in aged animals (van Praag et al., 2005; Marlatt et al., 2012). While a number of factors may underlie the neurogenic and cognitive restoration observed in aged animals following physical exercise (see Lee et al., 2012), a strong case has recently been made for the involvement of growth hormone in this process in the subventricular zone (SVZ) (Blackmore et al., 2009, 2012). Further investigation is required to show whether growth hormone mediates exercise-induced activation of the quiescent precursor cell population in the aged hippocampus.

We have shown recently that microglia are also very important in regulating the activation of precursors in the aging animal and after exercise (Vukovic et al., 2012). Removal of microglia from the hippocampal population in aged animals leads to a significant increase in precursor activation, indicating the low number of active precursors may be due to the inhibitory effects of microglia, probably through the release of cytokines. Conversely, microglia removed from hippocampal cultures of young animals has no effect, but their removal following running decreases neurosphere numbers, indicating microglia play a positive role in the activation process. We have shown that the ability of microglia to increase precursor activity is due to the release of the chemokine, CX3C (also known as fractalkine) from neurons, and that levels of this ligand decrease dramatically with age (Vukovic et al., 2012). These results open the possibility that raising CX3C levels may increase neurogenesis in the aging animal. In addition, we have found that interferon gamma, another cytokine found normally in the hippocampus that is raised during inflammatory conditions, is also a potent inhibitor of precursor activation. Interestingly, this inhibition can be partially blocked by factors released from microglia (Li et al., 2010). This environment of negative and positive regulators of hippocampal precursor activation poses some challenges, especially in the aged animal, as we try to stimulate the appropriate neurogenic production in the hippocampus in a way that leads to the enhancement of function in the aged brain suffering cognitive decline, as outlined in **Figure 1**.

HIPPOCAMPAL NEUROGENESIS IN MOOD-RELATED DISORDERS

Another area of research in which hippocampal neurogenesis has received considerable attention is in the investigation of animal models of depression, and specifically, the mechanisms underlying the actions of antidepressants (reviewed by Sahay and Hen, 2007). Changes in hippocampal neurogenesis have now been implicated in the etiology of depression; furthermore, current antidepressant treatments are thought to target these neurogenic changes (Santarelli et al., 2003; David et al., 2009; Snyder et al., 2011; Surget et al., 2011). Given the Australian National Survey of Mental Health and Wellbeing (2007) reported that about 20% of the population experience some form of mental disorder within a 12-month period, with mood-related disorders such as anxiety and depression being the most prevalent, a thorough understanding of the cellular and molecular mechanisms underlying these conditions is necessary to be able to identify novel therapeutic strategies.

Studies have shown an enhancement of hippocampal neurogenesis following chronic treatment with clinical antidepressants (Malberg et al., 2000; Duman, 2004; Perera et al., 2007). In particular, ECS, which initiate rapid onset of antidepressant action, have been shown to increase the proliferation

of GFAP- and Sox2-positive quiescent stem cells in the SGZ (Segi-Nishida et al., 2008). More recently, we have shown that norepinephrine, and antidepressants that block the reuptake of norepinephrine (such as reboxetine and atomoxetine), also increase proliferation by directly activating the quiescent population of precursors in the hippocampus (Jhaveri et al., 2010). In fact, the neurogenesis-depression hypothesis proposes that the functional effect of antidepressant treatment is mediated by the addition of new neurons to the hippocampal network (Kempermann and Kronenberg, 2003). This hypothesis is supported by the strong correlation that exists between the timeline of new neuron production and the delayed onset of the beneficial effects of antidepressant medication (Nestler et al., 2002; Sahay and Hen, 2007). Importantly, the blockade of hippocampal neurogenesis has been shown to inhibit some, but not all, of the therapeutic-like effects of antidepressants in rodent models (Santarelli et al., 2003; David et al., 2009); however, no anxiety/depressive-like behavior has been observed in animals lacking neurogenesis, suggesting impairment in neurogenesis alone is not sufficient to cause mood-related disorders. To better understand the etiology of depression, a detailed understanding of the interactions between the hippocampus and other brain areas is needed, with an emphasis on the precise molecular mechanisms by which newborn neurons in the hippocampus ameliorate depression.

The hippocampus is known to play an important role in regulating stress through the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 1989; Mizoguchi et al., 2003). While several studies have shown that chronic stress or an elevation of cortisol levels decreases neurogenesis (Gould et al., 1998; Murray et al., 2008; Lagace et al., 2010), two recent studies have suggested that newborn hippocampal neurons mediate the feedback inhibition of the HPA axis following stress, thereby regulating blood cortisol levels (Snyder et al., 2011; Surget et al., 2011). Snyder and colleagues (2011) have shown that in animals in which neurogenesis has been ablated, restraint stress leads to a longer-lasting elevation in cortisol levels compared to animals with intact neurogenesis. In addition, neurogenesis-ablated mice show increased food avoidance in a novel environment, indicating higher levels of anxiety. Importantly, Surget and colleagues (2011) have shown that fluoxetine treatment, in the absence of ongoing neurogenesis, fails to restore HPA axis activity and function in neurogenesis-ablated animals subjected to unpredictable chronic stress.

Taken together, it appears that functional hippocampal neurogenesis plays an important role in preventing the onset of mood-related disorders following chronic stress, and importantly, acts as a substrate for the therapeutic action of antidepressants. Enhancing the production of newborn neurons by harnessing the potential of endogenous precursor populations may therefore be an extremely useful therapeutic approach (see **Figure 1**). Given that at least two large latent precursor populations have been uncovered so far in the hippocampus (Walker et al., 2008; Jhaveri et al., 2010), driving neuronal production by enhancing the proliferative activity could indeed boost hippocampal neurogenesis. While the monoaminergic family of drugs remain the front line choice for the treatment of mood disorders, their ineffectiveness and distressing side effects warrants the discovery of new antidepressant treatments (Ellis, 2004). More effective, rapid-acting and long-lasting antidepressants may emerge from undertaking a detailed molecular characterization of these latent precursor pools, as well their neuronal progeny, which may lead to the identification of novel targets for drug development (Jhaveri et al., 2012). Finally, enhancing neuronal production may also prove beneficial for neurodegenerative diseases that involve significant cellular and functional loss in the hippocampus.

CONCLUSIONS

Resident populations of endogenous stem and progenitor cells drive the production of new neurons in the adult hippocampus. These precursor pools are carefully regulated and precisely maintained, such that upon pathological insult or homeostatic perturbations, proliferation can be rapidly enhanced to replace neurons lost due to damage or dysfunction. Nevertheless, enhancing proliferation alone is often not enough to generate sufficient numbers of new neurons and restore normal function. As such, various methods of further activating and amplifying stem and progenitor cells in the hippocampus have provided some positive results for the recovery of hippocampal-dependent behavioral functions in animal models.

While we have discussed the stimulation of endogenous neural stem cells in a number of disease states in rodent models, effective therapies for human application must be tailored to the endogenous microenvironment of the human neurogenic niche. Currently, there are limited studies providing evidence for ongoing hippocampal neurogenesis in the adult human brain (Eriksson et al., 1998; Blumcke et al., 2001; Sanai et al., 2004); furthermore, the cell cycle length in primates appears to be substantially longer than in rodents, indicating a much slower rate of proliferation and precursor cell turnover (Breunig et al., 2011; Curtis et al., 2012). Additional research is therefore required to examine the extent of adult neurogenesis in the human brain, for instance, using post-mortem brain tissue from age-matched controls, as well as patients suffering from mood and neurological disorders. Given that post-mortem brain tissue can only provide a temporal snapshot of neurogenesis, advances in live imaging techniques that can track cell proliferation in the human hippocampus at high resolution will open an exciting avenue for future investigation. In addition, the development of sophisticated drug delivery technology is needed to specifically target and activate proliferation of the endogenous populations of stem cells, and to facilitate the differentiation, migration, integration, and survival of newborn neurons. Nevertheless, a detailed understanding of the fundamental mechanisms regulating neurogenesis in animal models will be crucial in order to utilize the endogenous stem cell populations as a therapeutic approach in patients suffering from brain injury and disease.

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Regulation of endogenous neural stem/progenitor cells for neural repair—factors that promote neurogenesis and gliogenesis in the normal and damaged brain

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Neural stem/precursor cells in the adult brain reside in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. These cells primarily generate neuroblasts that normally migrate to the olfactory bulb (OB) and the dentate granule cell layer respectively. Following brain damage, such as traumatic brain injury, ischemic stroke or in degenerative disease models, neural precursor cells from the SVZ in particular, can migrate from their normal route along the rostral migratory stream (RMS) to the site of neural damage. This neural precursor cell response to neural damage is mediated by release of endogenous factors, including cytokines and chemokines produced by the inflammatory response at the injury site, and by the production of growth and neurotrophic factors. Endogenous hippocampal neurogenesis is frequently also directly or indirectly affected by neural damage. Administration of a variety of factors that regulate different aspects of neural stem/precursor biology often leads to improved functional motor and/or behavioral outcomes. Such factors can target neural stem/precursor proliferation, survival, migration and differentiation into appropriate neuronal or glial lineages. Newborn cells also need to subsequently survive and functionally integrate into extant neural circuitry, which may be the major bottleneck to the current therapeutic potential of neural stem/precursor cells. This review will cover the effects of a range of intrinsic and extrinsic factors that regulate neural stem/precursor cell functions. In particular it focuses on factors that may be harnessed to enhance the endogenous neural stem/precursor cell response to neural damage, highlighting those that have already shown evidence of preclinical effectiveness and discussing others that warrant further preclinical investigation.

Keywords: neural stem cell (NSC), neural precursor cell (NPC), neural stem/precursor cell (NSPC), neurogenesis, gliogenesis, traumatic brain injury, ischemic stroke, subventricular zone (SVZ)

INTRODUCTION

The concept that the adult mammalian brain contains populations of resident neural stem/progenitor cells (hereafter collectively referred to as NSPCs) was generally accepted two decades ago (Reynolds and Weiss, 1992; Richards et al., 1992), although first shown by Altman and Das in 1965 (Altman and Das, 1965). Adult neurogenesis occurs primarily in two neurogenic regions in the brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. To produce new neurons a NSPC must go through a process of proliferation, migration, differentiation, and integration to become a productive member of existing circuitry in the brain. Under normal physiological conditions adult NSPCs predominantly produce neurons, in particular interneurons in the olfactory bulb (OB) for SVZ-derived cells and dentate gyrus granule cell neurons for SGZ-derived cells. Following neural damage, however, SVZ-derived cells have the capacity to respond to the insult and produce neurons as well as astrocytes and oligodendrocytes. Harnessing this ability of NSPCs to produce new neuronal and glial cells as a means to replace cells damaged or lost following neural injury or disease to promote neural repair has been the focus of a substantial body of research. There are four main areas in the life cycle of a NSPC that can be targeted to try to enhance neural repair, namely proliferation, migration, differentiation (and specific cell type generation), and survival/integration. However, the field is full of conflicting reports on the benefit and ability of NSPCs to recover function following a range of injuries and diseases. While many labs have reported an increase in proliferation of neuroblasts and even migration to injured areas, a large part of the problem may lie in the failure of newly generated neurons to integrate into existing neural circuitry and survive past 4 weeks or so after their generation. Augmentation of all steps of the process of neurogenesis would ideally lead to an increased benefit following injury, but ultimately the cells must integrate and survive to have a functional benefit.

This review will cover some of the main factors known to be involved in neurogenesis and gliogenesis in the adult CNS and in particular those that have been shown to play a role in neural

repair. It will also explore how inflammatory mediators and other injury-related factors may modify the NSPC response to neural damage. We will not undertake a comprehensive review of factors regulating neurogenesis and gliogenesis in the normal developing or adult brain, as there have been several recent and comprehensive reviews covering this area (e.g., Guillemot, 2007; Ming and Song, 2011; Faigle and Song, 2012). Rather, we will highlight factors that play a role in regulation of normal adult NSPC function and which have been shown to be modulated to enhance repair following neural damage. **Table 1** summarizes the factors discussed, which NSPC populations are affected (SVZ or SGZ), the type of neural injury, if any and the NSPC function affected. **Figure 1** depicts the effects of extrinsic factors on the SVZ-derived NPC response to injury.

NSPC FATE: NORMAL NEUROGENIC NICHES vs. THE INJURED CNS ENVIRONMENT

Although the bulk of neurogenesis and gliogenesis occurs during embryonic and early postnatal development, NSPCs continue to produce neural cells in the adult brain. Importantly for the purpose of harnessing adult NSPCs for neural repair, unlike during development, the vast majority of adult-derived cells are fated to a neuronal lineage, with a much smaller percent differentiating into oligodendrocytes in the normal adult brain. In general, the diversity of cell types and neuronal subtypes that can be spontaneously generated by adult NSPCs is substantially limited compared to embryonic cells. This is probably largely due to a less permissive adult environment rather than a constitutive feature of the NSPCs themselves, as under appropriate conditions, such as in neurospheres *in vitro* (Reynolds and Weiss, 1992) or following ablation of neuronal populations without inflammatory system activation (Magavi et al., 2000), the ability of adult NSPCs to produce different neural cell types has been demonstrated. The more restricted fate of adult NSPCs in the normal adult CNS reflects the relative paucity of growth and neurotrophic factors compared to during development. The presence of inhibitory/attractive substrates, such as in the RMS, to constrain migratory routes and the lack of available space in the adult circuitry to allow integration of newborn cells, in general restricts normal NSPC function to neurogenic regions and currently limits the ability of endogenous NSPCs to replace specific neuronal or glial types in different regions in the CNS.

To further complicate matters, the injured adult CNS is an entirely different environment to the normal adult neurogenic niche or the developing nervous system, with substantial influence on NSPC function that in some instances appear to override the normal program of NSPC fate. This is particularly the case for SVZ NSPCs, which, as detailed further below, can be induced away from their normal migratory route to the OB toward the site of neural damage, a process largely induced by inflammatory mediators. After successful migration to the correct location, new neuroblasts must differentiate into the proper phenotype of neuron and integrate into local circuitry. The local circuitry to be repaired will depend on the type of damage, be it ischemic, traumatically injured or neurodegenerative, with some common factors and others specific to the site and type of damage. Effects of exogenous factors have been variably examined in each of the above types of neural damage and are described below for the relevant factors.

The majority of research on ectopic migration and neural differentiation of SVZ-derived NSPCs following neural damage has been performed by use of ischemia models and has demonstrated that cells do indeed reach the injured parenchyma (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003; Sundholm-Peters et al., 2005; Ohab et al., 2006; Yamashita et al., 2006; Cayre et al., 2009; Young et al., 2011). It appears that the cells in general no longer migrate in a chain formation and carry on individually, with some reports describing an increase in progenitor numbers without an effect on numbers of cells in the RMS (Zhang et al., 2001b), while others report that the response to injury is at the expense of the RMS population (Jin et al., 2003; Goings et al., 2004). This change in migration is the direct result of chemoattractive cues expressed from the injury site. As detailed further below, chemokines and their receptors can attract neuroblasts from the RMS. For example, Stromal cell-derived factor-1/CXCL12 and its receptor CXCR4 are upregulated at the injury site (Imitola et al., 2004; Robin et al., 2006) and expression of several other chemokines and their receptors are upregulated on adult NSPCs by inflammatory cytokines, such as IFNγ and TNFα (Turbic et al., 2011).

In general ischemia models have demonstrated production of new neurons from the SVZ in damaged cortex or striatum, while injury of the cortex usually promotes the generation of astrocytes and microglia/macrophages at the site of injury, with few or no new neurons produced (Ramaswamy et al., 2005; Richardson et al., 2007; Kreuzberg et al., 2010; Blizzard et al., 2011; Zhang et al., 2011). Neurodegenerative disease models, such as Parkinson's disease (PD) models, have also demonstrated migration of SVZ NPCs to the site of damage, with production of neurons in some cases but not others (Cooper and Isacson, 2004; Kadota et al., 2009; Jing et al., 2012). The production of astrocytes and oligodendrocytes near the injury site may be a result of expression of repressors of neuronal fate (Kernie et al., 2001; Shear et al., 2004; Buffo et al., 2005). Further, as detailed below, administration of a variety of factors promotes neurogenesis and/or gliogenesis in these normally non-neurogenic sites. The SVZ-derived NSPC response and the new cells they produce is quite variable, depending on injury type, region of neural damage, species and likely a range of other factors that remain to be defined.

There is little/no evidence that SGZ hippocampal NSPCs can migrate away from their normal GCL fate; however they do respond to neural damage (Dash et al., 2001; Chirumamilla et al., 2002), even if the hippocampus is not directly affected by damage, such as in cortical impact models (Kernie et al., 2001). This leads to alterations in neurogenesis that can produce altered cognition and/or effects on memory and anxiety. Little is currently known about how this neurogenesis is altered, but will be touched on below.

TRANSCRIPTION FACTOR REGULATION OF NSPC FUNCTION

Factors intrinsic to the cell participate in multiple roles of neurogenesis from proliferation to differentiation. Generally, transcription factors are the most widely studied intrinsic factors

Table 1 | Extrinsic factors affecting SVZ and SGZ NSPC responses under basal conditions and following neural damage.

Factor	SVZ				SGZ				Injury/Disease	Neurogenesis/ Gliogenesis	Selected references
	P	M	D	S	P	M	D	S			
EGF	✓	✓			✓			X	Stroke, TBI	Both-variable	(Teramoto et al., 2003; Sun et al., 2010)
EGF+/−FGF2	✓				✓			✓	Ischemia, TBI, PD	N	(Nakatomi et al., 2002; Tureyen et al., 2005; Winner et al., 2008; Sun et al., 2009)
ATP	✓										*(Suyama et al., 2012)
VEGF	✓	✓									*(Wittko et al., 2009; Calvo et al., 2011)
IGF1		✓			✓			✓		N (SGZ)	*(Aberg et al., 2000; Cheng et al., 2001)
NGF								✓	Ischemia	N	(Zhu et al., 2011)
BDNF			✓	✓			✓	✓	Ischemia, TBI	N	(Gao and Chen, 2009; Im et al., 2010)
Wnt (β-catenin)	✓		✓?	✓?	✓				Ischemia, TBI, AD	both	(He and Shen, 2009; White et al., 2010; Shruster et al., 2012)
Shh	✓				✓				Ischemia, SCI	both	(Bambakidis et al., 2003, 2012; Sims et al., 2009; Wang et al., 2009)
BMPs (noggin)	✓		✓		✓		✓		Demyelination TBI, intra-ventricular hemorrhage	BMP: A, Noggin: N and O	(Hampton et al., 2007; Cate et al., 2010; Dummula et al., 2011; Sabo et al., 2011; Lei et al., 2012)
Epo	✓	✓	✓		✓			X	Stroke, TBI, PD	N and O	(Tsai et al., 2006; Wang et al., 2006; Kadota et al., 2009; Meng et al., 2011; Ning et al., 2011; Xiong et al., 2011; Zhang et al., 2012)
SOCS2			✓					✓		N	*(Turnley et al., 2002; Ransome and Turnley, 2008b)
Chemokines	✓	✓		✓	✓						(Imitola et al., 2004; Gordon et al., 2009)
Eph/ephrins	✓	✓	✓						TBI, ischemia, PD	N	(Theus et al., 2010; Doeppner et al., 2011; Jing et al., 2012)
Nitric oxide	✓				✓				Ischemia		*(Packer et al., 2003; Park et al., 2003; Moreno-Lopez et al., 2004) (Zhang et al., 2001a; Zhu et al., 2003; Sun et al., 2005b)
Endo-cannabinoids	✓	✓			✓	✓				N	*(Aguado et al., 2005, 2007; Jiang et al., 2005; Goncalves et al., 2008; Gao et al., 2010; Hill et al., 2010; Wolf et al., 2010; Oudin et al., 2011a,b)
PSA-NCAM, Slit-Robo, integrins		✓									*(Cremer et al., 1994; Hu et al., 1996; Jacques et al., 1998; Wu et al., 1999; Chazal et al., 2000; Murase and Horwitz, 2002)
Reelin		✓				✓			Demyelination	N-migration	(Courtes et al., 2011)

(Continued)

Table 1 | Continued

Factor	SVZ				SGZ				Injury/Disease	Neurogenesis/ Gliogenesis	Selected references
	P	M	D	S	P	M	D	S			
Vasculature		✓							Ischemia	N-migration	(Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007; Kojima et al., 2010)
NMDA receptors				✓		✓		✓		N	*(Platel et al., 2010; Namba et al., 2011)

*P, Proliferation; M, Migration; D, Differentiation; S, Survival; N, Neurogenesis; O, Oligodendrogliogenesis; A, Astrogliogenesis; TBI, traumatic brain injury; PD, Parkinson's disease; AD, Alzheimer's disease; SCI, spinal cord injury; *references for basal non-injury conditions.*

researched in adult NSPCs and many are similar to programs for neurogenesis during development. However, while a substantial amount of information is known about the role that different transcription factors play in modulation of NSPC biology, much less is known about extrinsic factors that can be used to alter expression of the transcription factors to produce desired specific functional outcomes. This section will discuss the expression and roles of some transcription factors that may be useful therapeutic targets. Later sections will discuss effects of different extrinsic growth factors and cytokines, with particular reference to their role following neural damage, although there are currently few links described between these factors and expression of specific transcription factors in the adult NSPC or newborn neural cells.

SVZ

Under basal conditions, NSPCs produced in the SVZ go on to form neuroblasts that migrate from the lateral ventricles along the rostral migratory stream (RMS) to the OB where they differentiate into neurons of the OB. The formation and proliferation of NSPCs is dependent on the Sox family of genes, in particular *Sox2*. The zinc-finger protein Ars2 (arsenite-resistant protein 2) controls the multipotent progenitor state of NSCs through activation of *Sox2* (Andreu-Agullo et al., 2012). c-Myb is required for maintenance of the neural stem cell niche, promoting expression of *Sox2* and *Pax6* and subsequent proliferation (Malaterre et al., 2008). Epigenetic pathways can also control proliferation; recently phosophorylation of the histone H2AX which was shown to limit proliferation and overall neurogenesis (Fernando et al., 2011). New neurons migrating from the RMS to the OB primarily become GABAergic granule neurons that provide lateral inhibition between mitral and tufted cells. A minority of the new neurons become periglomerular neurons that are involved in lateral inhibition between glomeruli, and a small number of these cells are dopaminergic. This differentiation is under transcriptional control and proneural basic-helix-loop-helix (bHLH) transcription factors control neuronal fate commitment in NSPCs. For example, type C cells of the SVZ fated to become GABAergic interneurons in the OB express *Ascl1* (Kim et al., 2007). *Ngn2* and *Tbr2* are expressed in dorsal SVZ progenitors that become glutamatergic juxtaglomerular neurons (Brill et al., 2009), while *Sp8* is required for parvalbumin-expressing interneurons in the OB (Li et al., 2011). Dopaminergic periglomerular cells in the OB express *Pax6* and *Dlx-2* (Doetsch et al., 2002; Hack et al., 2005; Brill et al., 2008).

SGZ

Hippocampal neurogenesis involves radial and horizontal NPCs first transitioning to intermediate progenitors and then on to immature dentate granule neurons. When the new neurons mature they make large mossy fiber projections with CA3 pyramidal neurons (Freund and Buzsaki, 1996). *Sox2* has a role in maintaining the precursor pool via Sonic hedgehog (Shh) in adult SGZ and a role in proliferation of NPCs along with *Pax6* and CCAAT/enhancer binding protein β (C/EBPβ) (Maekawa et al., 2005; Favaro et al., 2009; Ehm et al., 2010). Transcription factors have a large role in the differentiation of SGZ NSPCs. *Neurog2* and *Tbr2* are expressed in NSCs destined to become glutamatergic neurons in the hippocampus (Kim et al., 2007, 2011a; Ozen et al., 2007), while over-expression of *Ascl1* produces oligodendrocytes (Jessberger et al., 2008).

Intrinsic factors are also necessary for the maturation and survival of newly born neurons. For example, *Prox1* (Lavado et al., 2010), *NeuroD* (Gao et al., 2009b; Kuwabara et al., 2009), and Kruppel-like factor 9 (Scobie et al., 2009) play important roles in survival, while cyclic response element binding protein (CREB) signaling is required for maturation and integration into the network. Interestingly, CREB activates miR-132 which regulates dendrite maturation in newborn dentate gyrus granular neurons (Magill et al., 2010). There is limited knowledge of the changes in SGZ intrinsic factors following injury; however neurogenesis is increased in the SGZ following injury. Following ischemia this increase can result from an upregulation of phosphorylated CREB (Boneva and Yamashima, 2012). In a primate model of ischemia, pro-neuronal transcription factors are expressed including *Emx2*, *Pax6*, and *Ngn2* (Tonchev and Yamashima, 2006). Intriguingly, in models of neurodegeneration SGZ proliferation is decreased; as shown in a rat model of Huntington's disease, SGZ progenitor cell proliferation is decreased due to an increase in *Sox2*-positive quiescent stem cells and a decrease in CREB signaling (Kandasamy et al., 2010).

Thus, many transcription factors have been shown to play a role in NSPC function, though few have been directly targeted by infusion of exogenous factors as a means to promote specific *in vivo* NSPC function and fate. Will this even be possible *in vivo* without genetic modification, such as by use of viral

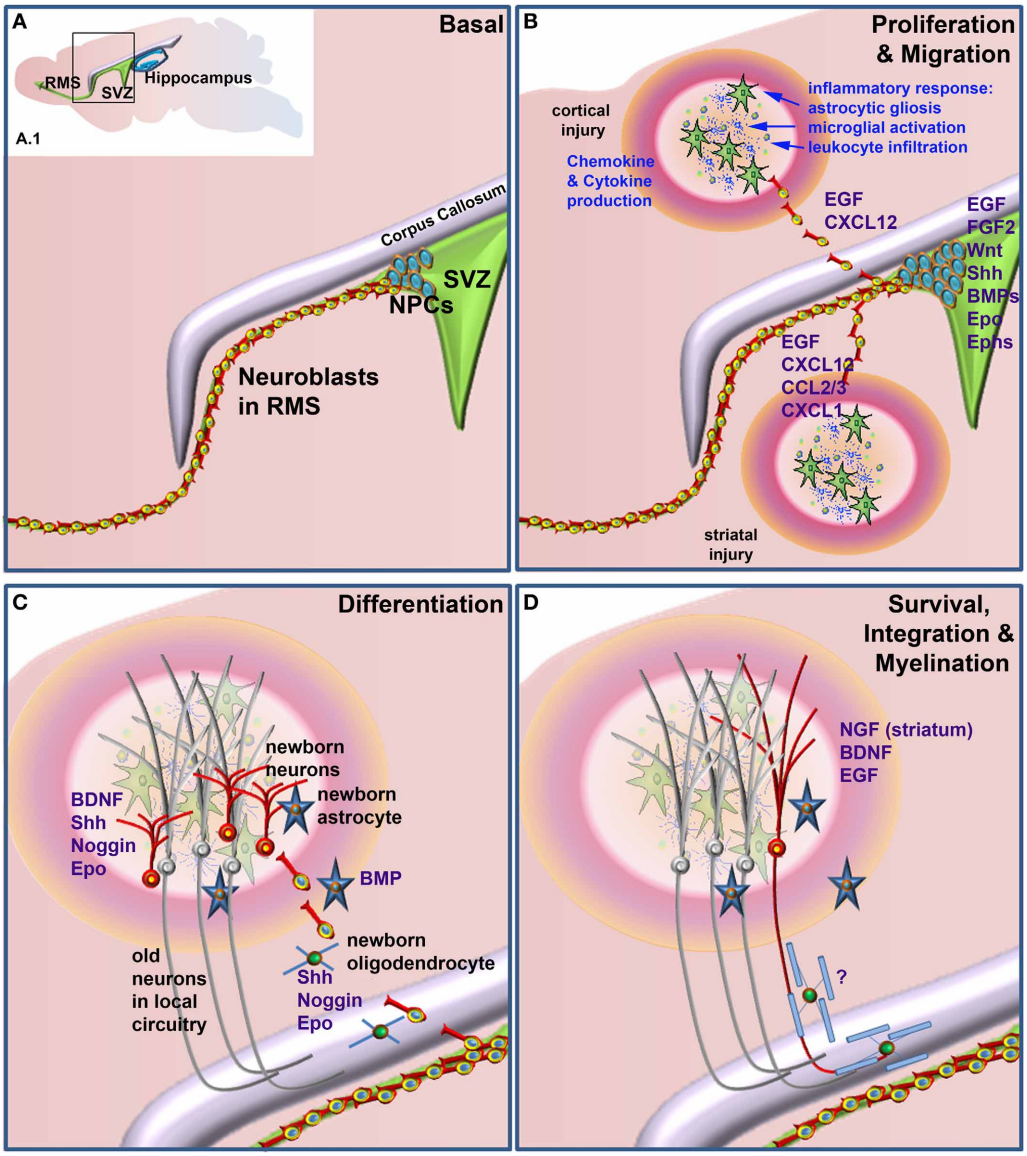

FIGURE 1 | Factors that when administered to the brain following neural damage regulate neural precursor cell responses. (A) Basal, non-damage conditions. Inset **(A.1)** depicts the two main neurogenic regions of the adult rodent brain, the subventricular zone (SVZ) and the hippocampus. The enlarged area bounded by the box in **(A.1)** depicts the general structure of the SVZ, which contains proliferative neural precursor cells (NPCs) that differentiate into neuroblasts that then migrate along the rostral migratory stream (RMS) to the olfactory bulb. Following neural injury **(B)** the inflammatory response induces astrocytic gliosis, microglial activation, and leukocyte infiltration from the periphery. Together, these produce a variety of chemokines, cytokines and other factors that can lead to the proliferation of the NPCs in the SVZ and redirect their migration to sites of damage. Once at or near the site of damage **(C)** these cells can differentiate into astrocytes, oligodendrocytes and astrocytes, depending upon which factors are administered. Unlike the normal predominance of neuronal differentiation under basal conditions, following injury SVZ NSPCs can generate neurons, astrocytes and/or oligodendrocytes. **(D)** Following differentiation cells need to survive and appropriately integrate into existing circuitry. This is a large bottleneck in harnessing the therapeutic potential of NSPCs, as the majority of newborn neurons die within a few weeks of their generation. For correct function, many new neurons also need to be appropriately myelinated although at present, few if any factors are known that enhance the final myelination stage of oligodendrocyte maturation following neural damage. Factors that have been shown to regulate the various stages of the NPC response when administered following neural injury are listed in blue text.

expression vectors? In many cases, particular exogenous factors were chosen for *in vivo* examination based on their known effects on induction of desired transcription pathways in other systems. However, in most instances, only single factors have been infused. This is in contrast to work being performed in the embryonic stem (ES) cell and induced pluripotent stem (iPS) cell field, in which specific transcription factors are induced by addition of extrinsic factors for defined periods of time and in a specific sequence to allow production of desired neural cell types. For example, such a system has been used to generate floor plate

cells which can subsequently generate mesencephalic dopaminergic neurons (Denham et al., 2012). Of course, such specific transcriptional regulation by exogenous factors is possible in the highly defined ES/iPS tissue culture environment but much more difficult *in vivo*, where there are many competing endogenous factors regulating NSPC function under normal conditions and even more so following neural injury. Further, the question of which transcription factors would be worth targeting for potential therapeutic effects is still open. Promoting a glial vs. a neuronal fate is relatively easy; for example infusion of BMP4 induces SMAD1/5/8 phosphorylation and subsequent astroglial fate (Cate et al., 2010). Induction of specific neuronal fates has largely not been addressed. SVZ NSPCs predominantly generate OB interneurons under normal conditions; however following neural injury the SVZ NSPCs can alter their normal fate, even in the absence of exogenous factor delivery, to become glial cells or adopt the specific neuronal type lost due to damage, such as striatal medium spiny neurons (Parent et al., 2002). Therefore, factors in the local damaged environment may be sufficient to direct final neuronal fate under some conditions, suggesting that adult SVZ NSPCs are not necessarily fate restricted and that defining factors to induce specific transcription factors, such as bHLH proneural genes, may be beneficial.

EXTRINSIC FACTORS THAT REGULATE NSPC FUNCTION FOLLOWING NEURAL DAMAGE

While many extracellular factors, ranging from growth factors to morphogens to extracellular matrix to cell:cell/receptor-mediated interactions have been shown to play a role in adult NSPC biology *in vitro* and/or *in vivo*, some have been more well characterized than others. This section will cover the predominant factors, as well as some less well characterized factors, shown to regulate normal adult NSPC biology and those shown to play a role following neural damage.

GROWTH FACTORS

Following ischemia and traumatic brain injury, expression of various growth factors such as epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), fibroblast growth factor 2 (FGF2), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1), pituitary adenylate cyclase-activating polypeptide (PACAP), and vascular endothelial growth factor (VEGF) are increased and modulate neurogenesis and NSPC biology. Additional exogenous application further augments NSPC proliferation and sometimes survival (Watanabe et al., 2004; Baldauf and Reymann, 2005; Tureyen et al., 2005; Dempsey and Kalluri, 2007; Schabitz et al., 2007; Johanson et al., 2011; Lu et al., 2011). This section will focus on factors that have been shown to regulate NSPC responses *in vivo* and commence with an examination of two of the most potent mitogens for increasing numbers of NSPCs, EGF, and FGF2.

EGF

EGF is a NSPC mitogen that is commonly used to promote neurosphere growth and maintenance in cell culture (Reynolds and Weiss, 1992). Neurospheres are primarily derived from the transit-amplifying (Type C) progenitor cells in the SVZ that normally produce the neuroblasts that migrate to the OB. These cells express the EGF receptor (EGFR) and when exposed to exogenous EGF can be induced to a multi-potent state capable of generating neuronal and glial cells (Doetsch et al., 2002).

Local production of EGF in the SVZ appears to be required for normal maintenance of the proliferative NSC pool in the SVZ, and this expression is maintained by dopaminergic innervation of striatal neurons (O'Keeffe et al., 2009; O'Keeffe and Barker, 2011). Activation of dopamine receptors using the dopamine receptor agonist pramipexole (PPX) augmented neurogenesis in a PD model by upregulating expression of the EGFR (Winner et al., 2009). Furthermore, the injury-induced proliferative response of SVZ NSPCs at least partly involves upregulation of the EGFR on these cells, thus enhancing their responsiveness to EGF (Alagappan et al., 2009). However, transforming growth factor alpha (TGFα), which also binds to the EGFR, is expressed at higher levels in the adult brain than EGF (Seroogy et al., 1993) and so TGFα may be the normal ligand for regulating NSPC function *in vivo*.

Given the mitogenic properties of EGF for NSPCs, infusion of EGF into the lateral ventricles following neural damage has been used to increase proliferation of NSPCs and attempt to increase their contribution to neural repair. This has led to mixed results, depending upon the injury/disease model used and possibly the species in which it was examined. EGF infusion for 6 days into the normal adult mouse forebrain increased the number of NSPCs at the SVZ, promoted their migration into the parenchyma (cortex, striatum, and septum), and resulted in 25% surviving for at least 7 weeks; however the majority of cells that differentiated were astrocytes with a small percentage of neurons and oligodendrocytes (Craig et al., 1996). Indeed, generally, EGF tends to promote an astroglial rather than neuronal fate, at least in rats (Kuhn et al., 1997). However, in mice, infusion of EGF into the lateral ventricles of the uninjured brain similarly increased SVZ NSPC proliferation and induced their migration into surrounding parenchyma, but most of the cells adopted an oligodendroglial fate, with a smaller percentage astroglial. This was further enhanced following a demyelinating lesion of the corpus callosum (Gonzalez-Perez et al., 2009). These cells may be a sub-population of SVZ NSPCs, the NG2+ oligodendrocyte progenitors, which express EGFR and are induced to migrate in response to EGF (Aguirre et al., 2005, 2007).

Conversely, in the damaged brain, such as following ischemia, EGF promoted SVZ NPC proliferation with subsequent production of neuroblasts in SVZ and striatum (Ninomiya et al., 2006), also it induced migration of doublecortin positive precursors and their subsequent long term (13 weeks) survival as parvalbumin-expressing interneurons (Teramoto et al., 2003). A similar infusion following traumatic brain injury also induced SVZ proliferation, as well as SGZ proliferation, at early timepoints but did not promote longer term (4 weeks survival) of the newborn SGZ cells, many of which had differentiated into astrocytes rather than neurons (Sun et al., 2010). Similarly, in a rat model of ischemia EGF alone induced proliferation and some NSPC migration but did not promote regeneration unless combined with a later administration of erythropoietin (Epo) (Kolb

et al., 2007), with similar results obtained for infusion of TGFα in a PD model (Cooper and Isacson, 2004). Part of the discrepancy between differentiation outcomes may lie in rat:mouse species differences. In general, mouse studies have indicated that EGF infusion can promote proliferation, migration and at least some neuronal differentiation while rat studies have shown proliferation, some migration and differentiation into glial cells, if they are found to differentiate at all. This may be due to EGF-induced dysplasia in rat but not mouse SVZ (Lindberg et al., 2012). In rats, the EGF-induced NSPC proliferative response generates SVZ/lateral ventricle polyps, which contain a higher percentage of cells expressing the transcription factors *Sox2*, *Olig2*, markers of proliferative NPCs, as well as Id1, an inhibitor of neuronal differentiation, concomitant with decreased expression of the neuroblast marker doublecortin. This suggests that in rat, EGF promotes proliferation of more glial fated NPCs that do not migrate to the same extent as mouse NPCs exposed to EGF. However, combination of EGF plus other growth factors appears to overcome some of the limitations of infusion of EGF alone to some extent.

EGF plus FGF2

FGF2 is another potent NSPC mitogen, commonly used to induce proliferation of these cells in culture, usually in combination with EGF. Infusion of FGF2 by itself into the lateral ventricles of normal adult rats promotes NSPC proliferation and enhances neurogenesis in the OB (Kuhn et al., 1997). Following traumatic brain injury it has been shown to enhance SVZ and SGZ NSPC proliferation and increase the 4 weeks survival of newborn neurons (Sun et al., 2009), while FGF2 knockout mice show decreased hippocampal neurogenesis following seizure or ischemia (Yoshimura et al., 2001). In addition, conditional deletion of FGFR1, the major receptor for FGF2, decreases hippocampal neurogenesis and impairs memory consolidation (Zhao et al., 2007). Quite a few studies have used a combination of EGF and FGF2 infusion, which has generally promoted neural repair. In a rat global ischemia model, which led to loss of CA1 hippocampal neurons, EGF+FGF2 promoted proliferation of SVZ-derived cells which repopulated the damaged CA1 hippocampal neurons and promoted functional recovery (Nakatomi et al., 2002). Similar studies reported increased numbers of neural progenitors in hippocampus, as well as SVZ and hypothalamus (Oya et al., 2008). Comparable results were obtained following transient middle cerebral artery occlusion in rat, including increased neuronal numbers and survival (Tureyen et al., 2005). However, in an endothelin model of transient ischemia, while co-infusion of EGF+FGF2 promoted increases in SVZ neurogenesis, it decreased hippocampal neurogenesis and increased infarct volumes (Baldauf and Reymann, 2005) but the reasons for the different outcomes in these models is not clear. Increased proliferation and migration to dopamine deficient areas have also been observed following EGF+FGF2 infusion in a PD model (Winner et al., 2008).

Other factors that can promote NSPC proliferation

Although EGF and FGF2 are possibly the most potent NSPC mitogens, particularly for SVZ-derived cells, other factors can also promote increases in NSPC numbers, although most have yet to be tested in brain injury/disease models. Infusion of ATP into the lateral ventricles of mice increased numbers of transit amplifying progenitor cells (Suyama et al., 2012). A mechanism by which this occurs may involve induction of FGF2 and TGF synthesis (Jia et al., 2011). VEGF can augment SVZ progenitor proliferation, as well as migration (Wittko et al., 2009; Calvo et al., 2011). Other factors have been shown to enhance proliferation induced by EGF and/or FGF2 without having a notable effect by themselves, such as growth hormone (GH) (McLenachan et al., 2009).

IGFs also play a role in several NSPC functions. IGF1 has different effects in the SVZ and dentate gyrus SGZ. Peripheral administration promotes hippocampal dentate gyrus NSPC proliferation and increases subsequent dentate gyrus neurogenesis (Aberg et al., 2000) and survival (Cheng et al., 2001). GH receptor knockout mice, which are serum-IGF1-deficient, do not show any alterations in hippocampal neurogenesis (Ransome and Turnley, 2008b), which may indicate that local hippocampal/dentate gyrus production of IGF-1 is important in supporting ongoing hippocampal neurogenesis (Sun et al., 2005a). However, peripheral administration of GH promoted hippocampal but not SVZ NSPC proliferation (Aberg et al., 2009). Indeed, in the SVZ IGF1 does not seem to regulate proliferation, rather being more important for NSPC migration; IGF1 null mice show an abundance of neuroblasts in the SVZ that have failed to migrate to the OB (Hurtado-Chong et al., 2009). IGF2 regulates proliferation in the dentate gyrus in an Akt-dependent manner (Bracko et al., 2012) and promotes survival of newborn hippocampal neurons as well as regulating hippocampal dependent fear extinction (Agis-Balboa et al., 2011).

NEUROTROPHINS

Neurotrophin signaling mediated by BDNF interacting with TrkB receptors and to a much lesser extent NGF binding to TrkA, regulates several aspects of NSPC function. In the adult brain, TrkB expression and effects of BDNF are widespread but TrkA expression is very limited, primarily restricted to basal forebrain cholinergic neurons which project to the NGF-expressing hippocampus. Therefore, effects of BDNF in NSPC biology have been the most widely studied, although NGF infusion has been shown to promote the survival of normal newborn dentate granule cell neurons but not proliferation of their progenitors (Frielingsdorf et al., 2007). Intranasal delivery of NGF following focal ischemia in rats similarly did not promote proliferation of SVZ progenitors but enhanced subsequent newborn neuronal survival in the ipsilateral SVZ and injured striatum (Zhu et al., 2011).

In vitro, SVZ-derived neurospheres express TrkB and little/no TrkA or TrkC and addition of BDNF promoted a transient increase in newborn neuron numbers due to enhanced differentiation and neurite outgrowth, rather than a proliferative effect on the NSPCs (Ahmed et al., 1995). Further, BDNF is required for cultured hippocampal progenitor cells to adopt a neuronal fate (Bull and Bartlett, 2005).

In the dentate gyrus, TrkB is expressed predominantly by maturing but not proliferating neuroblasts (Donovan et al., 2008), which correlates with findings that BDNF is important for neuroblast migration, survival, and integration of new neurons. BDNF increases the number and survival of newborn neurons

in the SVZ and OB (Kirschenbaum and Goldman, 1995; Zigova et al., 1998; Bath et al., 2008), in a p75 neurotrophin receptor-dependent manner (Young et al., 2007), and in striatum, caudate putamen and septum (Benraiss et al., 2001; Henry et al., 2007), dentate gyrus (Lee et al., 2002) as well as subcallosal neurons (Kim et al., 2011b). Knockdown of TrkB receptors and disruption of BDNF signaling resulted in decreased SGZ NSPC proliferation and neurogenesis, indicating that TrkB signaling may also have some proliferative effects in these cells (Li et al., 2008). Disruption of BDNF signaling also results in shorter dendrites and reduced spine formation, culminating in a lack of survival of newborn granule cells (Bergami et al., 2008; Gao et al., 2009a). In addition, conditional deletion of BDNF resulted in increased death of newborn neurons in mice following traumatic brain injury (Gao and Chen, 2009). However, conversely, enhanced long-term AAV-mediated BDNF expression in the hippocampus was shown to inhibit neurogenesis following ischemia in rats (Larsson et al., 2002). The reason for this discrepancy is unclear and further work is required to delineate the mechanisms by which BDNF regulates adult neurogenesis following neural damage. Given that neural activity promotes BDNF-mediated TrkB activation (Aloyz et al., 1999) and TrkB signaling is required for activity-dependent differentiation of hippocampal NSPCs, BDNF is likely to be a critical factor to promote NSPC-derived newborn neuron survival and integration into neural circuitry. However, if too much BDNF-stimulation is provided, this may be at the expense of NSPC numbers in some situations but this needs further study for confirmation.

BDNF combined with a mitogen is more effective in enhancing NSPC function and may be a better therapeutic option. Neurogenic NSPCs can be cultured from SVZ of adult humans but requires a combination of FGF2 and BDNF for robust growth (Pincus et al., 1998). This combination also appears to be required for creating a neurogenic environment in normally non-neurogenic regions *in vivo* (Chen et al., 2007a). In addition, co-infusion of BDNF plus EGF was more effective than either factor alone at promoting long term striatal neuron survival following ischemic injury in mice (Im et al., 2010).

MORPHOGENS

The morphogens Wnt, Shh, and Bone Morphogenic Proteins (BMPs) play a major role in neural development with some data to suggest they may also play a role in the NSPC response to neural damage.

Wnt signaling

While Wnts are endogenously expressed in adult neurogenic niches (Wexler et al., 2009), their expression is not upregulated following neuronal damage, such as stroke (Morris et al., 2007). Wnt signaling via β-catenin, promotes NSC proliferation to regulate their maintenance (Lie et al., 2005; Adachi et al., 2007). This appears to be by regulating symmetric vs. asymmetric division, promoting symmetric division during neural regeneration (Piccin and Morshead, 2011). Wnt signaling is negatively regulated by glycogen synthase kinase 3 beta (GSK3β) and inhibitors of GSK3β have been proposed as candidate targets for neural repair (Mao et al., 2009). Inhibition of β-catenin signaling by the amyloid beta peptide resulted in reduced neurogenesis from NPCs grown from Alzheimer disease brains, which also have increased levels of GSK3β (He and Shen, 2009). Further, down-regulation of β-catenin in a rat stroke model inhibited striatal neurogenesis (Lei et al., 2008), while overexpression of Wnt3a following focal ischemia promoted NSPC proliferation, neurogenesis and functional recovery (Shruster et al., 2012). However, following traumatic brain injury in mice, β-catenin activity was upregulated in cortical NG2+ oligodendrocyte/astrocyte progenitor cells, suggesting that it also plays a role in gliogenesis following injury (White et al., 2010).

Shh

Intrathecal administration of Shh to rats following ischemia promoted SVZ NSPC proliferation and improved behavioral recovery (Bambakidis et al., 2012), while following spinal cord injury it promoted proliferation of nestin+ NSPCs surrounding the central canal leading to increased numbers of oligodendrocyte precursors and neurons (Bambakidis et al., 2003). Shh expression is upregulated in the SVZ and hippocampus following ischemia, potentially playing a role in increased proliferation in a Notch dependent manner (Sims et al., 2009; Wang et al., 2009); however in the hippocampus its expression was also upregulated in mature CA3 neurons, suggesting effects in mature neurons as well as progenitors (Sims et al., 2009). The source of Shh in the adult brain appears to be astrocytes. In the normal brain, astrocytes in neurogenic regions produce Shh and when transplanted they induce neurogenesis in non-neurogenic regions of the brain, such as cortex (Jiao and Chen, 2008). Following brain injury activated astrocytes upregulate Shh expression in response to pro-inflammatory stimuli, which subsequently promotes increased numbers of Olig2+ NSPCs (Amankulor et al., 2009); this may be via activation of quiescent endogenous cortical NSPCs derived from astrocytes (Ahmed et al., 2012). However, in different neuroinflammatory conditions, such as experimental allergic encephalomyelitis (EAE) or multiple sclerosis, the inflammatory response induces Shh in astrocytes as above but inhibits Shh-induced NSPC differentiation by subsequent downregulation of the Gli1 transcription factor (Wang et al., 2008).

Bone morphogenic proteins (BMPs)

The role of BMP signaling in NSPCs and their role following CNS injury has recently been reviewed (Sabo et al., 2009) and so will only be covered briefly here. In general, BMP signaling inhibits neuronal and oligodendroglial differentiation of NPCs and promotes astrogliogenesis, during development (Gross et al., 1996) and in the adult (Lim et al., 2000), with somewhat different effects at different stages of development (Mehler et al., 2000). However, expression of the BMP inhibitor noggin in the SVZ (Lim et al., 2000) or SGZ (Bonaguidi et al., 2008) can obstruct this cascade and promote neurogenesis. Further, BMP signaling promotes non-neurogenic parenchymal astroglial cell fate while leukemia inhibitory factor (LIF), which also promotes astrogliogenesis, promotes cells of an astroglial progenitor cell phenotype (Bonaguidi et al., 2005). Demyelination induced expression of the BMP antagonist chordin in the SVZ, induced glial fate in neuroblasts to generate new oligodendrocytes in the corpus callosum

(Jablonska et al., 2010). Conversely, BMP signaling in SVZ NSCs but not NPCs is required to promote a neurogenic rather than oligodendrogliogenic cell fate (Colak et al., 2008).

BMP signaling inhibits NPC proliferation (Gajera et al., 2010) and while inhibition of the BMP pathway increases neurogenesis initially, it eventually results in depletion of the NSC pool, leading to decreased neurogenesis in the dentate gyrus (Mira et al., 2010). This indicates that a fine balance of BMP signaling vs. inhibition is required to regulate appropriate NSC numbers and subsequent cell fate.

Given that BMP signaling promotes an astroglial fate and inhibits oligodendrogliogenesis and neurogenesis, the effect of inhibition of BMP signaling by infusion of noggin into the lateral ventricles has been examined in various models of neural injury. In cuprizone-induced demyelination models noggin infusion inhibited the cuprizone induced upregulation of BMP4 and its signaling pathways, decreased SVZ astrocyte numbers, increased oligodendrocyte numbers and promoted remyelination of the corpus callosum (Cate et al., 2010; Sabo et al., 2011), possibly by regulation of Olig2 function (Chen et al., 2012). Noggin infusion also produced similar results in a model of intraventricular hemorrhage (Dummula et al., 2011). In a brain injury model, noggin was found to be produced by reactive astrocytes and similarly promoted oligodendrocyte fate (Hampton et al., 2007). It was also recently shown that Bcl2 regulates neurogenesis in a striatal injury model, by increasing β-catenin expression and decreasing BMP4 expression, in a noggin independent fashion (Lei et al., 2012).

ERYTHROPOIETIN (Epo)

Epo is a cytokine better known for its regulation of erythrocyte production. However it also has a number of functions within the CNS and on NSPC biology. Epo is expressed in the developing and adult SVZ (Shingo et al., 2001) and is required for endogenous embryonic and adult SVZ and SGZ neurogenesis (Tsai et al., 2006; Chen et al., 2007b). It promotes neurogenesis of SVZ NSPCs at the expense of multipotent progenitors in the normal rodent brain (Shingo et al., 2001) but in the SGZ this effect is transient, briefly increasing neuronal progenitor numbers but with no long term enhancement of survival (Ransome and Turnley, 2007). One of the mechanisms by which it may do this is by upregulation of Suppressor of Cytokine Signaling-2 (SOCS2) in NSPCs (Wang et al., 2004b). SOCS2, an intracellular regulator of cytokine signaling, promotes embryonic SVZ neurogenesis (Turnley et al., 2002; Scott et al., 2006), while in the hippocampus it promotes NSPC-derived newborn neuron survival (Ransome and Turnley, 2008b). It also promotes axonal growth of hippocampal neurons (Ransome and Turnley, 2008a), which may be a factor contributing to its enhanced neuroprotective effects following neural injury.

Following neural damage Epo has been found to be both neuroprotective and to promote neurogenesis (Wang et al., 2004a). Epo expression is induced/upregulated by hypoxia and thus many studies have examined its role post-ischemia or in ischemia/hypoxia models, although it has also been shown to be effective following traumatic brain injury. Conditional knockdown of the Epo receptor reduced post-stroke neurogenesis, with reduced proliferation and stroke-induced neuroblast migration to the cortex (Tsai et al., 2006). The Epo-induced NSPC migration appears to be indirect, rather inducing expression of matrix metalloproteases in endothelial cells, which in turn induced NSPC migration (Wang et al., 2006). Peripheral administration of Epo promotes NSPC proliferation, neurogenesis, oligodendrogenesis, and neurovascular remodeling following traumatic brain injury in rats, enhancing functional outcome (Lu et al., 2005; Zhang et al., 2010; Meng et al., 2011; Ning et al., 2011; Xiong et al., 2011). Inhibition of proliferation by infusion of AraC into the ventricles inhibited Epo-induced dentate gyrus neurogenesis and recovery of spatial learning (Zhang et al., 2012). Epo has also been shown to be neuroprotective in disease models, such as PD, in which it promoted SVZ NSPC proliferation and migration to the damaged striatum (Kadota et al., 2009).

One of the problems associated with use of Epo as a therapeutic for promotion of neural regeneration is that it generally increases the hematocrit at doses required for promotion of neurogenesis (e.g., 5000 U/kg). While this has not presented major issues for use in animal models, it raises concerns for potential clinical use. Therefore, several Epo derivatives have been developed to try to promote neuroprotective effects while avoiding effects on erythrocyte production. Carbamylated Epo does not bind to classical Epo receptor and does not stimulate erythropoiesis but does promote SVZ and dentate gyrus proliferation and neuronal differentiation of adult NSPCs (Wang et al., 2007; Leconte et al., 2011), while a different non-erythropoietic derivative asialo-Epo promoted SVZ-derived oligodendrogenesis (Kako et al., 2012). A peptide agonist of Epo, Epobis, has also recently been developed and shown to promote neuron survival and neurite outgrowth, but its effects on neurogenesis have yet to be determined (Pankratova et al., 2012).

CHEMOKINES AND CYTOKINES

Chemokines form a family of small (8–14 kD), mainly basic, secreted molecules that are primarily known for regulating chemoattraction of immune cells to sites of tissue damage. They have been reported to have widespread non-immunological effects in the CNS, including regulation of neural cell proliferation, migration, survival, and synaptic transmission and can act in a paracrine or autocrine manner (Bajetto et al., 2002; Cartier et al., 2005).

Pro-inflammatory cytokines, such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) appear to be major regulators of chemokine and chemokine receptor expression in many tissues (Hiroi and Ohmori, 2003; Suyama et al., 2005). In the adult brain there is basal expression of chemokines, especially in neurogenic regions, while treatment with IFNγ and TNFα can significantly increase the expression of specific chemokines including CXCL1, CXCL9, and CCL2 (Turbic et al., 2011).

Adult NSPCs express a range of chemokine receptors and chemokines are expressed in different brain regions, with the highest levels in the OB, suggesting an as yet largely unexplored role for chemokines in regulating basal adult NPC migration (Turbic et al., 2011). Neurospheres derived from adult mouse SVZ-derived NPCs have been shown to express a range

of chemokine receptors, including CCR1-8, 10 and CXCR1-6 (Tran et al., 2004). Functionally, specific chemokines, such as CXCL12/CXCR4 can promote NPC migration (Imitola et al., 2004; Tran et al., 2004; Dziembowska et al., 2005) and proliferation (Tran et al., 2004) or survival (Krathwohl and Kaiser, 2004; Dziembowska et al., 2005) *in vitro*. Following neural damage, NPC migration to site of injury is promoted by CXCL12 (Imitola et al., 2004; Itoh et al., 2009) and is mediated at least in part, by induction of metalloprotease expression in the NPCs (Barkho et al., 2008). CCL2, CCL3, and CXCL1 also promote NPC migration to striatum following quinolinic acid lesion (Gordon et al., 2009).

The gp130-associated cytokines, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), activate Janus kinase [JAK/signal transducer of transcription 3 (STAT3)], mitogen activated protein (MAP) kinase and PI-3K/Akt pathways following ligand binding. These cytokines have been shown to regulate NSC proliferation and differentiation (Turnley and Bartlett, 2000; Heinrich et al., 2003; Kamimura et al., 2003; Ernst and Jenkins, 2004). IFNγ, which signals via STAT1, and IFNβ which does not, both inhibit cultured adult NPC proliferation, but only IFNγ promotes neuronal differentiation (Wong et al., 2004; Lum et al., 2009). Specifically in the dentate gyrus, the activation of STAT3 from CNTF appears to be essential for the formation and maintenance of the NSCs (Muller et al., 2009).

OTHER FACTORS THAT PLAY A ROLE IN NSPC FUNCTION THAT MAY BE TARGETED FOR NEURAL REPAIR

There are factors involved in neurogenesis other than extrinsic growth factors and cytokines, as discussed above. Some of these include membrane bound molecules, neurotransmitters and their receptors and blood vasculature.

EPH/EPHRIN SIGNALING AND NSPCs—A NEW TARGET TO PROMOTE NSPC PROLIFERATION, SURVIVAL, AND MIGRATION

The Eph family of receptor tyrosine kinases and their ligands, the ephrins, are membrane bound molecules that signal bi-directionally, are generally involved in cell or axon guidance by repulsive mechanisms and which play a role following neural injury (reviewed in Goldshmit et al., 2006b). They have been receiving increasing interest of late as they have been shown to play major roles in inhibition of regeneration in the CNS following neural injury or disease, with deletion or blocking of various Ephs or ephrins promoting neural repair (Goldshmit et al., 2004, 2006b, 2011; Rodger et al., 2004; Liu et al., 2006; Fabes et al., 2007; Overman et al., 2012; Van Hoecke et al., 2012). EphA4 also plays a role in regulation of inflammation following neural injury (Munro et al., 2012), which could have secondary effects on NSPC responses.

Eph receptor signaling also regulates several aspects of NSPC biology but as yet has not been targeted to enhance NSPC responses following injury. The EphB2/ephrin-B2 pathway enables formation of the chain migration from the SVZ to the OB (Conover et al., 2000) and also controls the conversion of ependymal cells to astrocytic NSPCs (Nomura et al., 2010). EphB2 signaling via ephrin-B1 is also required for development of the dorsal half of the dentate gyrus, where it controls migration of the dentate progenitor cells (Catchpole and Henkemeyer, 2011). Ephrin-A2 signaling via EphA7 inhibits SVZ NSPC proliferation and subsequent neurogenesis (Holmberg et al., 2005), while conversely ephrin-A5 signaling via EphA7 induces their apoptosis (Depaepe et al., 2005). EphB2 and ephrin-B signaling however promotes SVZ NSPC proliferation, decreases migration, and promotes neuronal differentiation (Katakowski et al., 2005), with ephrin-B1 expression in SVZ NSPCs critical for maintenance of the proliferative NSPC state (Qiu et al., 2008), a role also played by EphA4 (Khodosevich et al., 2011). In addition, EphB3 is expressed by adult SVZ NSPCs and neuroblasts and EphB3 and ephrin-B3 knockout mice show increased neonatal and adult SVZ NSPC proliferation, indicating that this pathway normally inhibits their proliferation (Theus et al., 2010; Del Valle et al., 2011; Doeppner et al., 2011). Infusion of soluble ephrin-B3 into the lateral ventricles reversed the proliferation defect in ephrin-B3 knockout mice but not the EphB3 receptor knockout mice (Theus et al., 2010).

In the hippocampus, EphB1 and ephrin-B3 promote hippocampal NSPC proliferation and migration, as well as cell polarity (Chumley et al., 2007), while ephrin-A5 promotes hippocampal NSPC proliferation and neurogenesis, at least partly through regulation of the normal vascular system (Hara et al., 2010), which requires normal EphA4 (Goldshmit et al., 2006a) and EphB4 signaling (Colin-Castelan et al., 2011). Ephrin-B2, expressed by hippocampal astrocytes in the SGZ, induces neuronal differentiation of EphB4-expressing hippocampal NSPCs and this effect involves activation of β-catenin independent of Wnt signaling, with subsequent upregulation of proneural transcription factors (Ashton et al., 2012).

To date, little has been examined regarding the role of Eph/ephrin signaling following neural damage. Following traumatic brain injury, EphB3 expression was transiently downregulated, which may lift its inhibition of NSPC proliferation and this may be one of the mechanisms by which NSPCs are able to respond to injury, by increasing proliferation and survival (Theus et al., 2010). However, in ephrin-B3 knockout mice, while there was an increase in NSPC proliferation following ischemic injury, there was also enhanced cortical damage with an increased infarct volume (Doeppner et al., 2011) due to NSPC-independent effects of ephrin-B3, which will need to be taken into account when considering therapeutic use of Eph/ephrins. Conversely, in a model of PD infusion of soluble ephrin-A1 into the lateral ventricles, to activate EphA receptor/s, promoted SVZ NSPC proliferation and migration of these cells to striatum, where they subsequently differentiated in dopaminergic neurons (Jing et al., 2012).

OTHER FACTORS

Following injury, one of the early mediators of the injury response is upregulation of nitric oxide (NO). NO has been reported to have proliferative (Zhang et al., 2001a; Reif et al., 2004) or anti-proliferative (Packer et al., 2003; Park et al., 2003; Moreno-Lopez et al., 2004) effects on basal SVZ and SGZ NSPCs, as well as following injury (Zhang et al., 2001a; Zhu et al., 2003; Sun et al., 2005b). This apparent discrepancy in effect appears to lie largely in the source of NO production and the

subsequent signaling pathways induced. If the NO derives from neuronal nitric oxide synthase (nNOS) then the effect is largely anti-proliferative (Packer et al., 2003; Moreno-Lopez et al., 2004; Sun et al., 2005b), but if derived from inducible NOS (iNOS) (Zhu et al., 2003; Carreira et al., 2010) or endothelial derived NOS (eNOS) (Reif et al., 2004) then the effects are largely proliferative. This likely relates to the location of NOS as well as the concentration of NO produced. Further, infusion of NO donors that induce MAP kinase signaling (Carreira et al., 2010) or cyclic guanosine monophosphate (cGMP) signaling, promotes proliferation and neurogenesis in normal and ischemic rats (Zhang et al., 2001a). Indeed, both pathways appear to be involved in NSPC proliferation in response to NO, with activation of the MAPK pathway at shorter timepoints (6 h) and activation of cGMP at later timepoints (24 h) (Carreira et al., 2012). Recent work has indicated that the NO-cGMP pathway is also an important mediator of the neuro-proliferative effects of other factors, such as Neuropeptide Y in the hippocampus (Howell et al., 2005; Agasse et al., 2008; Cheung et al., 2012) and also explains the increased proliferation and neurogenesis observed with infusion of phosphodiesterase inhibitors, such as sildenafil and tadalafil, which lead to increased cGMP levels and improve outcome in stroke models (Zhang et al., 2003, 2006). cGMP levels also appear to regulate NSPC fate during embryonic development, with high levels promoting neuronal differentiation and low levels non-neuronal fate (Gomez-Pinedo et al., 2010), however, whether this is the case in adult remains to be determined.

Endocannabinoid signaling has been shown to regulate migration and neurogenesis in both the SVZ and dentate gyrus (Aguado et al., 2005, 2007; Jiang et al., 2005; Goncalves et al., 2008; Gao et al., 2010; Hill et al., 2010; Wolf et al., 2010; Oudin et al., 2011a,b). Other molecules involved in NPC migration include polysialated neural cell adhesion molecule (PSA-NCAM) (Cremer et al., 1994; Hu et al., 1996; Chazal et al., 2000), Slit-Robo (Wu et al., 1999), and integrins (Jacques et al., 1998; Murase and Horwitz, 2002). In basal conditions, reelin is a detachment signal for neuroblasts from the RMS at the OB (Hack et al., 2002), while overexpression of reelin in a demyelinating lesion led to an increase in ectopic migration of neuroblasts at the lesion site (Courtes et al., 2011). Many of these factors signal via the Rho kinase pathway, which is a downstream regulator of NPC migration (Leong et al., 2011) and targeting this pathway may circumvent the large number of external signals that converge on this pathway to allow more precise control of directed NPC migration and possibly neuronal differentiation and survival. In the brain parenchyma NSPCs interact with blood vessels in the neurovascular niche (Shen et al., 2008; Tavazoie et al., 2008), neuroblasts can migrate along blood vessels (Honda et al., 2007) and use vessels to migrate radially into the cortex (Le Magueresse et al., 2012). Following ischemia SVZ neuroblasts migrate to the infarct in close association with blood vessels (Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007; Kojima et al., 2010).

NMDA receptors expressed in neuroblasts along the RMS are crucial to the integration of these neurons in existing OB circuitry (Platel et al., 2010), with glutamate released from astroglial cells in the glial tube that surrounds the migrating neuroblasts. NMDA receptor activation in newly born dentate gyrus granule cells also increases survival. Initial GABA depolarization promotes maturation of neurons in the dentate gyrus and OB (Saghatelyan et al., 2005; Ge et al., 2006) and this depolarization and subsequent Ca^{2+} influx are required for dendrite initiation and elongation (Gascon et al., 2006). Agrin signaling is also necessary for integration and survival of newborn neurons in the OB: a loss of agrin leads to improper synapse formation while overexpression of agrin results in an increase in dendritic spines (Burk et al., 2012).

The migration distance for new neurons from the SGZ is relatively short as they travel into the granular layer above the SGZ, where guidance molecules may control this movement. NMDA receptor signaling is required for the proper migration of newborn granular cells in the dentate gyrus (Namba et al., 2011). This is achieved through the activation of Disrupted-in-schizophrenia (DISC1), as neurons without DISC1 migrate further into granular layer and into the molecular layer (Duan et al., 2007; Namba et al., 2011). DISC1 also controls the dendritic maturation of newborn granule cells through GABA depolarization of NKCC1 and activation of the Akt-mTOR pathway (Duan et al., 2007; Kim et al., 2012), while Reelin regulates migration and dendritic development of adult-generated hippocampal neurons (Teixeira et al., 2012). Whether targeting any of the above factors will enhance NSPC responses following neural injury largely remains to be determined.

CONCLUSIONS

Many factors regulate the biology of NSPCs at different stages along their growth and maturation cycle. To date, the majority of studies which have administered extrinsic growth factors or cytokines have concentrated on the effect of a single factor at a time. However, some studies are now emerging where combinatorial effects of different factors have been examined. It will not be sufficient to only increase the number of NSPCs produced by administration of a mitogen. While this can indeed increase neurogenesis, the number of surviving and functionally integrated neurons (or oligodendrocytes) is still very limited. It would be prudent to combine use of mitogens with other factors that enhance neurogenesis or oligogliogenesis respectively, as well as factors that enhance their subsequent survival and integration. Which factors are chosen to combine will likely depend on the injury or disease model to be examined, whether neurons and/or oligodendrocytes need to be replaced and may also be species dependent to a certain extent, although the combination of a mitogen such as EGF and a survival factor, such as BDNF may be generally beneficial. Some factors, such as Epo, have multiple actions, including NSPC proliferation, neuronal differentiation, and oligodendrocyte maturation, as well as being neuroprotective, which makes them particularly attractive as potential therapeutics for treatment of the damaged brain. Most of the work described above was conducted in rodents and to date little work has been performed following neural injury in brains of gyrencephalic species, such as non-human primates (Tonchev et al., 2003a,b, 2005, 2007). Sheep models are also being developed to allow such questions to be answered in a model

more relevant to human brain damage (e.g., Wells et al., 2012). Human clinical trials are also underway to test therapeutic effectiveness of a range of extrinsic factors, such as Epo, following brain injury. For a complete list see www.clinicaltrials.gov. Two decades since their discovery in mammalian brains, facilitation of the NSPC response to brain damage by administration of extrinsic factors still holds great promise of therapeutic potential, although much ground still remains to be covered.

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Characterization of forebrain neurons derived from late-onset Huntington's disease human embryonic stem cell lines

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Huntington's disease (HD) is an incurable neurodegenerative disorder caused by a CAG repeat expansion in exon 1 of the Huntingtin (HTT) gene. Recently, induced pluripotent stem cell (iPSC) lines carrying atypical and aggressive (CAG60+) HD variants have been generated and exhibit disparate molecular pathologies. Here we investigate two human embryonic stem cell (hESC) lines carrying CAG_{37} and CAG_{51} typical late-onset repeat expansions in comparison to wildtype control lines during undifferentiated states and throughout forebrain neuronal differentiation. Pluripotent HD lines demonstrate growth, viability, pluripotent gene expression, mitochondrial activity and forebrain specification that is indistinguishable from control lines. Expression profiles of crucial genes known to be dysregulated in HD remain unperturbed in the presence of mutant protein and throughout differentiation; however, elevated glutamate-evoked responses were observed in HD CAG_{51} neurons. These findings suggest typical late-onset HD mutations do not alter pluripotent parameters or the capacity to generate forebrain neurons, but that such progeny may recapitulate hallmarks observed in established HD model systems. Such HD models will help further our understanding of the cascade of pathological events leading to disease onset and progression, while simultaneously facilitating the identification of candidate HD therapeutics.

Keywords: Huntington's disease, human embryonic stem cells, neuronal differentiation, GABAergic neurons

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expanded CAG repeat tract in exon 1 of the *HTT* gene (Group, 1993). Disease alleles ($CAG_{>35}$) exhibit an age-dependent penetrance, with the lowest disease range (CAG_{36-39}) associated with a later onset (McNeil et al., 1997). CAG_{40+} alleles are associated with full penetrance, while larger expansions (CAG_{60+}) result in juvenile or infantile onset (Andrew et al., 1993; Squitieri et al., 2006). Substantial correlation of ∼50% exists between CAG repeat length and age of onset, with secondary contributions from known and unknown genetic and environmental factors (Andrew et al., 1993; Wexler et al., 2004). HD affects approximately 1 in 10,000 individuals worldwide, with onset of disease usually in the fourth or fifth decade of life. HD culminates in death 15–20 years after persistent, irreversible and debilitating clinical symptoms (Naarding et al., 2001). To date, there is no cure for HD and consequently, there is an unmet clinical need for more effective therapeutics.

The *HTT* gene comprises 67 exons and encodes a ubiquitously expressed protein of approximately 350 kDa called Huntingtin (HTT). HTT plays a critical role in early embryogenesis with homozygous *HTT* null mouse embryos exhibiting incomplete neural development and lethality at embryonic day 8.5 and 10.5 of gestation (Zeitlin et al., 1995). HTT has been extensively studied in relation to its interacting partners, sub-cellular localization and effects on gene expression, and acts in a variety of cellular systems (Zuccato et al., 2010). The clinical symptoms of HD seen in patients can largely be classified as either neural, such as behavior and cognitive alterations, or motor, such as involuntary movements and abnormalities of voluntary movements. Underlying neurodegeneration is most prominent within the GABAergic medium spiny neurons of the striatum although widespread neuronal loss occurs with disease progression (Graveland et al., 1985; Vonsattel et al., 1985).

Understanding the molecular nature of HD is critical to the development of novel and efficacious therapies. Numerous animal models such as the R6/2 transgenic mouse (Mangiarini et al., 1996) together with post mortem patient tissues have proven valuable resources for identifying and elucidating major CNS cellular mechanisms and hallmarks that contribute to HD pathology; these are comprehensively reviewed in Zuccato et al. (2010) and include transcriptional dysregulation, CAG repeat expansion, excitotoxic stress, autophagy-lysosomal, and proteasome-ubiquitin system perturbation, anterograde and

retrograde transport interference, mitochondrial dysfunction and cholesterol biosynthesis alteration.

The hierarchical relationship between the disparate mechanisms/hallmarks and their degree of individual contribution to the overall HD phenotype still remains uncertain. New human HD models such as pluripotent stem cells (PSCs) may provide an alternative system to shed light on this etiological question. Studies investigating hPSC lines, specifically human embryonic stem cell (hESC) lines, carrying pathogenic CAG repeat mutations have provided limited data in relation to the consequences of disease allele expression (Niclis et al., 2009; Bradley et al., 2011; Seriola et al., 2011). More recently, mouse and human induced pluripotent stem cell (iPSC) lines have been analysed for the appearance of HD hallmarks (Camnasio et al., 2012; Castiglioni et al., 2012; HDIPSCC, 2012; Jeon et al., 2012) and several phenotypes have been observed, including transcriptional dysregulation, CAG repeat instability, mutant HTT aggregates, cholesterol biosynthesis perturbation, lysosomal dysfunction and neuronal vulnerability. However, phenotypes reported from the various HD iPSC studies differ significantly and rarely correlate, for example CAG repeat instability is seen in human but not R6/2 iPSCs. A further caveat is that the HD iPSC lines studied to date either carry rare homozygous HD mutations or alleles with egregious repeat tracts (CAG_{60-180}) indicative of juvenile and infantile onset and may represent atypical disease scenarios.

Limited studies assess the appearance of HD phenotypes in hPSC lines with clinically relevant trinucleotide expansions (CAG_{35-60}) that correlate with late ages of onset. To this end, we have extended an earlier study by Niclis et al. (2009) using two HD hESC lines SI-187 ($CAG_{51/19}$) and SI-186 ($CAG_{37/15}$) that were generated from embryos identified with pre-implantation genetic detection (PGD). Here we show that the HD hESC lines are indistinguishable from control hESCs with respect to pluripotent characteristics, mitochondrial function, forebrain neural differentiation capabilities, relevant cellular phenotypes and with no evidence of a dysregulated transcriptome in key HTT associated genes. However, the larger of the two CAG repeat cell lines showed an altered responsiveness to the neurotransmitter glutamate. Overall, these results are informative for establishing a human neuronal cellular model of HD.

MATERIALS AND METHODS

CULTURE AND NEURAL DIFFERENTIATION OF hESC LINES

Two HD hESC lines SI-186 ($CAG_{37/15}$) and SI-187 ($CAG_{51/19}$) (Niclis et al., 2009) as well as two wildtype control hESC lines, HES3 and H9 (Thomson et al., 1998; Reubinoff et al., 2000) were used. All hESC lines were screened for karyotypic abnormalities using Geisma staining and found to maintain euploid 46 XX karyotypes (data not shown). All four hESC lines were grown as bulk cultures on γ-irradiated mouse embryonic fibroblasts (MEFs) according to previously described conditions (Costa et al., 2008); briefly, cells were grown in DMEM/ F-12, supplemented with 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 1% Glutamax, 25 U/ml penicillin, 25 U/ml streptomycin, and 20% knockout serum replacement (all Invitrogen).

hESC neural differentiation was performed as previously described (Song et al., 2011a). Briefly, ∼3000 hESCs were distributed to each well of a round-bottom ultra-low attachment 96-well plate (Corning) containing 100 µl of Differentiation Medium (Neurobasal A, 5% ITS-X, 2.5% Penicillin/Streptomycin, 5% Glutamax, 5% B27 and 5% N2; Invitrogen). From d0–d21 cells were grown as neurospheres at 37°C in 5% CO_2 in air, with 0.125% polyvinyl alcohol and 10 µM Roh-Associated Coil Kinase (ROCK) inhibitor Y-27632 at d0–4. From d0–d21 media was supplemented with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml fibroblast growth factor (FGFb) (R&D Systems), and 100 ng/ml of Noggin (R&D Systems) from d0–8. Neurospheres were passaged at d14 by manual sectioning in half. To promote neuronal differentiation neurospheres were plated at d21 in Differentiation Medium onto 24-well plates (BD Biosciences) coated with 20 µg/ml poly-D-lysine and 20 µg/ml laminin (Invitrogen) in the absence of growth factors and media changed every 4 days for up to 24 days.

FLOW CYTOMETRY

Cells were harvested using TrypLE Select (Invitrogen), passed through a 70 µm filter and 1×10^5 cells stained with various antibodies listed in **Table 1**. Samples were analysed using a BD Canto II flow cytometer with data analyses performed using GateLogic. All samples were gated to assess only single cells as determined by forward scatter area vs. height channels and live cells as determined by negative DAPI selection. Background fluorescence was subtracted using unlabeled cells. A total of 50,000 events (∼40,000 live cells) were recorded, compensations were performed using single label antibody controls; positive gates were designated on concentration-matched isotype control antibodies.

GENOTYPING

Genotyping was performed as previously described (Niclis et al., 2009). Briefly, HU3 and HU4 primers (Sermon et al., 1998) were used to amplify the CAG repeat expansion region in exon 1 of *HTT*. The HU4 sense primer was labeled with 6-carboxyfluorescein (6-FAM) and sizing of amplicons performed with an ABI Prism 3100 DNA sequencer coupled with GeneScan Software according to previously described methods (Cram et al., 2000). The number of CAG repeats was accurately calculated to ±1 bp from GeneScan sized F-PCR products, using

Table 1 | Antibodies and dilutions utilized for flow cytometry and immunocytochemistry.

Antibody target	Species	Conjugate	Dilution	Company
CD9	Mouse	FITC	1:30	BD
TRA-1-60	Mouse	APC	1:25	BD
FORSE-1	Mouse	N/A	1:1000	DSHB
CD56	Mouse	PerCPcy5.5	1:50	BD
β-III-TUBULIN	Mouse	N/A	1:500	Millipore
Huntingtin	Rabbit	N/A	1:100	Millipore
SOX2	Goat	N/A	1:200	ABcam
OTX2	Rabbit	N/A	1:1000	Millipore
GABA	Rabbit	N/A	1:500	Millipore
FOXG1	Rabbit	N/A	1:50	ABCam

the formula: CAG repeat number = (size of PCR product – size of non-CAG repeat region)/3.

IMMUNOCYTOCHEMISTRY

Neurospheres were collected at d10 or 21 and fixed for 15 min at room temperature with 4% paraformaldehyde, cryoprotected in sucrose (20% w/v in 0.1 M PBS) and embedded in OCT compound (Tissue–Tek) before serial sectioning ($6 \times 10\,\mu$m thick sections) on a cryostat (Leica). Neuronal cultures were fixed at d35 or 45 for 15 min at room temperature with 4% paraformaldehyde, permeabilized with 1% Triton X-100 (Ameresco), blocked using 10% normal donkey serum (Invitrogen) and incubated with primary antibodies described in **Table 1** at 4°C overnight. Species-specific AlexaFluor (Invitrogen) or Dyelight (Jackson Laboratories) 488 and 555 secondary antibodies (all 1:200) were added for 2 h at room temperature and then counterstained with 0.1 μg/ml DAPI (SIGMA). Coverslips were mounted onto glass slides using Fluorescent Mounting Media (DAKO). Bound fluorescence was detected using an Olympus BX51 microscope coupled to the ULH100HG fluorescence system. Images were captured using a DP70 camera.

CELL GROWTH AND VIABILITY ASSAYS

Duplicate MEF coated T25 culture flasks were seeded with 1×10^6 hESCs for each line. Cells were harvested at 24 and 72 h. FACS analysis of DAPI, CD9, and TRA-1-60 staining was used to distinguish live hESCs (DAPI–/CD9+/TRA1–60+) and MEFs (DAPI–/CD9–/TRA1–60–). Doubling rates were calculated based on hESC and live cells only, according to the following formulae: $[\mathrm{Log}_2(\beta/\alpha)]/2$; $\alpha = 24$ h count and $\beta = 72$ h count. Viability assays were performed similarly on hESCs 24 h after passage but with DAPI live and dead cell gating performed by FACS after CD9 and TRA-1–60 gating. Neurosphere growth rates were determined by measuring area sizes using Adobe Photoshop CS5, the average value for 12 neurospheres equated a single replicate, with three averaged values analysed at each time point for each line. Data values at d21 were doubled to account for passaging in half of spheres at d14. Data was statistically analysed with PRISM, using a One-Way ANOVA with a Kruskal–Wallis Test and Dunn's post test. Means were graphed with the SEM and a *p*-value (*) of < 0.05 considered significantly different.

NEURONAL FUNCTIONAL ANALYSIS

Neurons were cultured as described above. For intracellular Ca^{2+} $[(Ca^{2+})_i]$ imaging, neurons were loaded with Fluo4-AM (2 μM, for 45 min at 37°C, as previously described (Watmuff et al., 2012). Media was then replaced with HEPES buffer (of composition, mM, NaCl 145; $MgSO_4$ 1; KCl 5; glucose 10; $CaCl_2$ 2.5; HEPES 10; pH 7.4) containing bovine serum albumin (0.3% w:v) and placed on a heated (37°C) stage. Neurons were viewed using a Nikon A1R confocal microscope and excited with a 488 nm laser, emission was recorded at 525/50 nm at two image frames per second. After 2 min of baseline recording glutamate (30 μM) or vehicle was added to each well and imaging continued for 90 s as previously described (Raye et al., 2007; Khaira et al., 2011; Watmuff et al., 2012). After this time KCl (30 mM) was added to each well and imaging continued for a further 60 s. For analysis, background emission light was subtracted from analysis regions defined within each cell body and emission intensity within each region calculated using Nikon Elements software. The maximal elevation of intracellular $[Ca^{2+}]_i$ in response to vehicle or glutamate is expressed as the net fraction of the KCl-induced maximal increase in $[Ca^{2+}]_i$.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from $\sim 1 \times 10^6$ cells using the commercially available RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions and treated with RNase-Free DNase (Qiagen). RNA was quantitated using a ND-1000 Spectrophotometer (NanoDrop Technologies). Purified RNA (0.4–2 μg) was reverse transcribed to single stranded cDNA with SuperScript III reverse transcriptase using the First Strand Synthesis System Kit (Invitrogen) and treated with RNase H to remove contaminating RNA.

cDNA products were subjected to qRT-PCR of selected genes (**Table 2**) using TaqMan® Universal PCR Master Mix and the inventoried TaqMan® Gene Expression Assay Kit (Applied Biosystems) according to manufacturer's recommendations. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used for normalization of RNA input, two independent reactions were prepared for each sample. Reaction plates were run on the 7500 Real Time PCR System (Applied Biosystems) at the following thermocycler conditions: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min and stage 3, 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantification (RQ) of gene expression in each sample was analysed using SDS Software version 1.3 (Applied Biosystems). The Ct value for each sample was measured in duplicate and the average normalized against the endogenous control *GAPDH* to determine the ΔCt value. The ΔCt values were then standardized against the calibrator's ΔCt (wildtype HES3 line) to yield the ΔΔCt. The RQ was then calculated as $2^{-\Delta\Delta Ct}$. Statistical analysis for qRT-PCR data was performed using GraphPad Prism with a one-way ANOVA analysis of each gene at each specific time point. A Kruskal–Wallis test was performed on the non-matched, non-parametric data. The *p*-value was set to <0.05 and a Dunn's post-test performed with RQ data from three culture replicates plotted as a mean and scale bars denoting the mean ± SEM.

Table 2 | TaqMan gene expression assay details used for qRT-PCR.

Gene symbol	Amplicon length (bp)	Gene bank number	Assay ID
GAPDH	93	NM_002046.3	Hs99999905_m1
HTT	66	NM_002111.6	Hs00918134_m1
PGC1a	74	NM_013261.3	Hs01016719_m1
DRP1	88	NM_005690.3	Hs00247147_m1
BDNF	116	NM_170733.3	Hs00380947_m1
DRD2	64	NM_000795.3	Hs00241436_m1
PENK	56	NM_001135690.1	Hs00175049_m1
SREBP1	90	NM_001005291.2	Hs01088691_m1
β-III-TUBULIN	82	N/A	Hs00964967_g1

All primers were purchased from Applied Biosystems.

WESTERN BLOTTING

1×10^6 cells were lysed in 500 µl of Cell Lysis Buffer (Cell Signaling) and protein concentrations determined using a BCA assay (Pierce, Thermo Scientific). Protein samples (10 µg) in 1× SDS sample buffer containing 10% β-mercaptoethanol were electrophoresed on 4–12% NuPAGE gels (Invitrogen). Proteins were transferred to immobilon PVDF transfer membranes (Millipore), blocked in TBS-T containing 5% non-fat dry milk powder and then immunoblotted with either monoclonal antibody 1HU-4C8 (Millipore, 1:2000) specific for epitopes within amino acids 181–810 on wildtype and mutant HTT or monoclonal antibody 5TF1-1C2 (Millipore, 1:5000) which has a high avidity for long polyglutamine epitopes (>15 polyglutamine residues). A secondary anti-mouse HRP antibody (Calbiochem) in combination with an ECL kit was finally used to illuminate HTT protein.

JC-1 MITOCHONDRIAL STAINING

JC-1 a cationic dye that undergoes a fluorescence emission shift from green (529 nm) to red (590 nm) upon accumulation in the mitochondrial matrix (Simeonova et al., 2004), was employed to measure changes in the mitochondrial membrane potential (MMP). Undifferentiated hESCs (1×10^6) prepared from bulk cultures were incubated in media containing 2 µM of JC-1 for 30 min, washed twice in PBS and then analysed using a BD FACS Canto II flow cytometer 488 nm excitation and emission detection dually from both FITC and PE channels. Compensations were performed with hESC specific CD9 and EpCAM antibodies, conjugated to FITC and PE respectively. Data analysis was conducted using FACS Diva software (BD).

RESULTS

CHARACTERIZATION OF PLURIPOTENT HD hESC LINES

HD and control hESC lines were originally derived as colony cultures that intrinsically comprise significant heterogeneity with differential degrees of pluripotency and differentiation capacities across a gradient of isolatable sub-fractions (Laslett et al., 2007; Hough et al., 2009; Kolle et al., 2009). To eliminate this stochasticity, HD hESC lines were adapted to an enzymatically passaged bulk culture system to provide homogenous cultures amenable to stringent molecular and functional comparative evaluations. Of note, adaptation rates of HD lines were commensurate with controls, requiring ∼3–5 passages, and produced morphologically identical bulk culture monolayers of hESCs with low cytoplasmic-to-nuclear ratios (**Figures 1A,B**). Robust homogeneity was achieved with hESC bulk culture conditions, evidenced by a tightly clustered population of TRA-1-60+/CD9+ pluripotent cells and an absence of single positive cells indicating that the hESCs were maintained in a pluripotent state (**Figure 1C**). Both HD hESC lines exhibited FACS profiles with pluripotent markers similar to control lines verifying stable undifferentiated states (**Figure 1C**). Further, no significant differences in cell growth rates (doublings/48 h) were observed between HD and control hESC lines (SI-187 2.24, SI-186 2.8, HES3 2.36, and H9 2.52, $p > 0.05$; **Figure 1H**) or cell viability during enzymatic

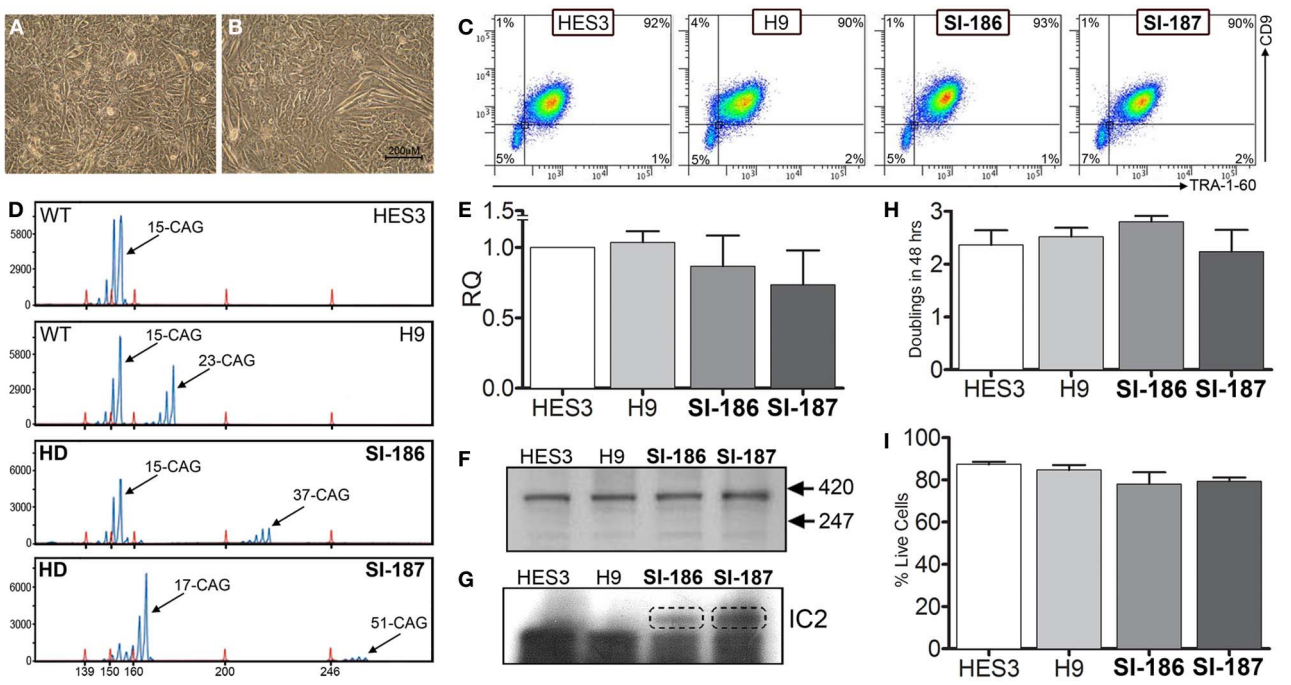

FIGURE 1 | Characterization of pluripotent HD hESC lines demonstrates identical characteristics to control lines. (A,B) Brightfield morphology of hESC bulk cultures. **(C)** Flow cytometry analysis of hESCs seen as a TRA-1-60+/CD9+ population; feeder MEFs are a distinct CD9–/TRA-1-60- sub-population. **(D)** FL-PCR representation of HD genotypes. **(E)** qRT-PCR of HTT mRNA expression levels. **(F)** Western blot of an N-terminal epitope antibody on the 350 kDa HTT protein. **(G)** Western blot of hESC lines immunoprobed with the IC2 monoclonal antibody. **(H)** Growth rates of hESC lines (One-Way ANOVA, $n = 3$, $p \geq 0.05$) and **(I)** cell viability comparisons (One-Way ANOVA, $n = 3$, $p \geq 0.05$).

passaging (SI-187 79.3%, SI-186 78.0%, HES3 84.7%, and H9 87.3%, $p > 0.05$; **Figure 1I**).

Analysis of FL-PCR products confirmed correct CAG repeat genotypes for HD lines SI-187 ($CAG_{51/19}$) and SI-186 ($CAG_{37/15}$) and control lines HES3 ($CAG_{15/15}$) and H9 ($CAG_{23/15}$) (**Figure 1D**). qRT-PCR using primers specific for conserved sequences on both wildtype and mutant alleles confirmed *HTT* mRNA was expressed at similar levels in HD and control hESC lines (**Figure 1E**). Western blot analysis using a polyclonal antibody reactive to an N-terminal epitope on the 350 kDa HTT protein (**Figure 1F**) confirmed HTT protein expression in all four hESC lines. Further immunoprobing using the IC2 monoclonal antibody that specifically reacts with long polyglutamine epitopes >15–20 residues (Trottier et al., 1995) detected a ∼350 kDa band exclusively within SI-187 and SI-186 cells confirming mutant HTT expression in both HD lines (**Figure 1G**).

NEURAL DIFFERENTIATION IS UNPERTURBED BY AN EXPANDED CAG TRACT

HD neurodegeneration emerges within the forebrain and progresses to whole-brain atrophy. Differentiation of HD hESC lines to a regionally relevant forebrain phenotype was achieved using the spin aggregation neurosphere system we have previously reported (Song et al., 2011a). Seeding 3000 hESCs per well resulted in morphologically similar neurospheres within 24 h and throughout the neurosphere differentiation stage between HD and control lines (**Figure 2A**). Neural growth rates were equivalent between HD and control lines indicating the presence of mHTT does not interfere with neural precursor division and differentiation ($p > 0.05$; **Figure 2B**).

Quantitative flow cytometric assessment throughout neurosphere stages demonstrated highly analogous expression profiles across HD and control lines. All lines progress early to a neural fate and near total expression (>90%) of the pan-neural marker CD56 is observed by d21 (**Figure 2C**). Neural differentiation exhibited an anterior identity with >50% of cells expressing the forebrain surface antigen FORSE-1 (**Figure 2C**). The forebrain population was confirmed as neural in character with >90% of the FORSE-1+ population co-staining with CD56 by d21 (**Figure 2D**), and all FORSE-1+ cells separating from minor CD9+ fractions (**Figure 2E**). All hESCs rapidly exit a pluripotent state with low levels of the hESC marker CD9 d10 which falls to ∼2% by d21 (**Figure 2E**). Intriguingly, a delay in CD9 downregulation is observed in the fully penetrant SI-187 line at d10, although by d21 CD9 expression is consistent across all lines (**Figure 2E**). Overall, these findings suggest that pathogenic HD mutations do not perturb neural progenitor generation, expansion or specification to an anterior identity.

Control and HD neurospheres exhibited equivalent progenitor phenotypes at d10 (**Figures 3A–H**). Strong expression of the multipotent neural stem cell transcription factor SOX2 was observed in control (85.8%) and HD (88.2%) cells equally (**Figure 3D**, data not significant $p = 0.05$). This was concomitant with a rostral

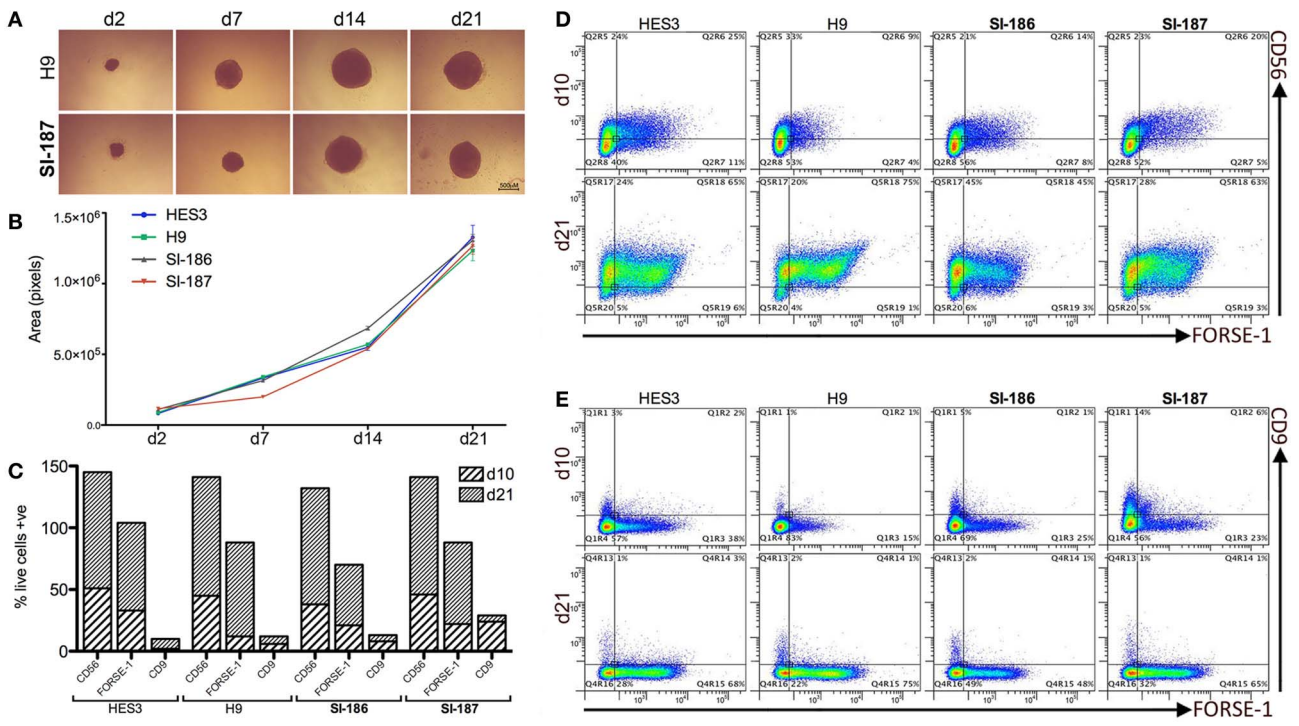

FIGURE 2 | Neural progenitor lineage specification and expansion is consistent between HD and control neurospheres. (A) Brightfield time course representations throughout neurosphere differentiation stages **(B)**. Neurosphere growth rate analysis across all HD and control lines (One-Way ANOVA, $n = 3$, $p \geq 0.05$) from an initial 3000 cell seeding density. Flow cytometry quantification of antigens CD9, CD56, and FORSE-1 at differentiation days, d10 and d21 showing **(C)** absolute single antibody expression levels and **(D)** co-expression scatter plots for CD56 and FORSE-1 and **(E)** CD9 and FORSE-1.

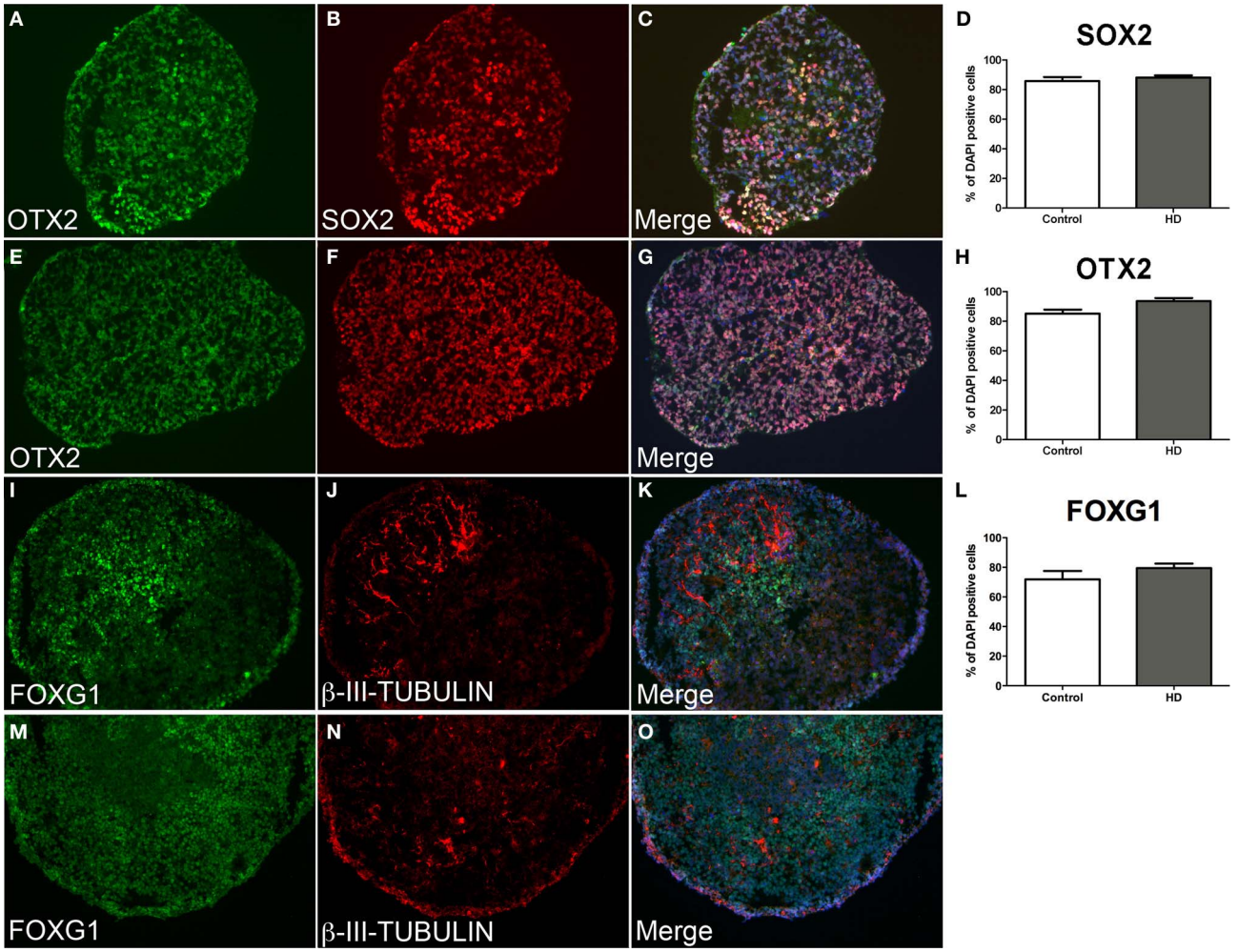

FIGURE 3 | Equivalent neurosphere neural progenitor gene expression and forebrain specification from HD and control cryosectioned immunostained neurospheres. Neurospheres at d10 of differentiation from HD line SI-187 **(A–C)** and control line H9 **(E–G)**, demonstrate robust expression of the telencephalic marker OTX2 (green) and similarly the neural progenitor marker SOX2 (red). Merged images demonstrate co-expression of OTX2 and SOX2 and quantitative evaluation shows no significant difference between control and HD neurospheres (**D, H**, paired *t*-test, $n = 4$ $p \geq 0.05$). Neurospheres at d21 of differentiation from HD line SI-187 **(I–K)** and control line H9 **(M–O)** show extensive expression of the forebrain transcription factor FOXG1 (green) and nascent expression of the cytoskeletal marker β-III-TUBULIN (red) which predominates within FOXG1 expressing regions, with no quantitative differences between control and HD neurospheres (**L**, paired *t*-test, $n = 8$ $p \geq 0.05$).

fate seen by the robust expression of the telencephalic marker OTX2 in comparable proportions between control (88.0%) and HD (92.4%) cells (**Figure 3H**, data not significant $p = 0.05$), and by co-localization of both proteins (**Figures 3C,G**). Importantly, continued differentiation to d21 facilitated robust expression of the forebrain specific transcription factor FOXG1 within the majority of neurosphere cells in both control and HD sections (**Figures 3I–O**). Expression levels of FOXG1 were not significantly different between control (71.9%) and HD (79.4%) neurospheres (**Figure 3L**, $p = 0.05$). Limited numbers of neuronal cells were seen at the end of neurosphere culture, detected by β-III-TUBULIN expression (**Figures 3J,N**). FOXG1 expressing regions preferentially co-localize with β-III-TUBULIN expression indicating maturation of anterior cells to a post-mitotic neuronal fate (**Figures 3K,O**).

Subsequent to neurosphere plating on extracellular matrices, neuronal differentiation disseminated throughout cultures in control (**Figure 4A**) and HD neurons (**Figure 4D**) as shown by cytoskeletal β-III-TUBULIN immunostaining. Quantitative validation was made of these observations during late neurospheres (d21) and neuronal (d35) differentiation stages by β-*III-TUBULIN* transcript expression comparisons (**Figure 4G**). Antibody staining against an N-terminal HTT epitope revealed cytoplasmic and nuclear protein localization (**Figures 4B,E**) and specifically preferential nuclear reactivity within the nuclei of neurons with no discernable differences between control (**Figure 4C**) and HD neurons (**Figure 4F**). Further, quantitative *HTT* transcript expression levels were equal across all HD and control lines during neural precursor and neuronal differentiation stages (**Figure 4H**). Crucially, neuronal differentiation

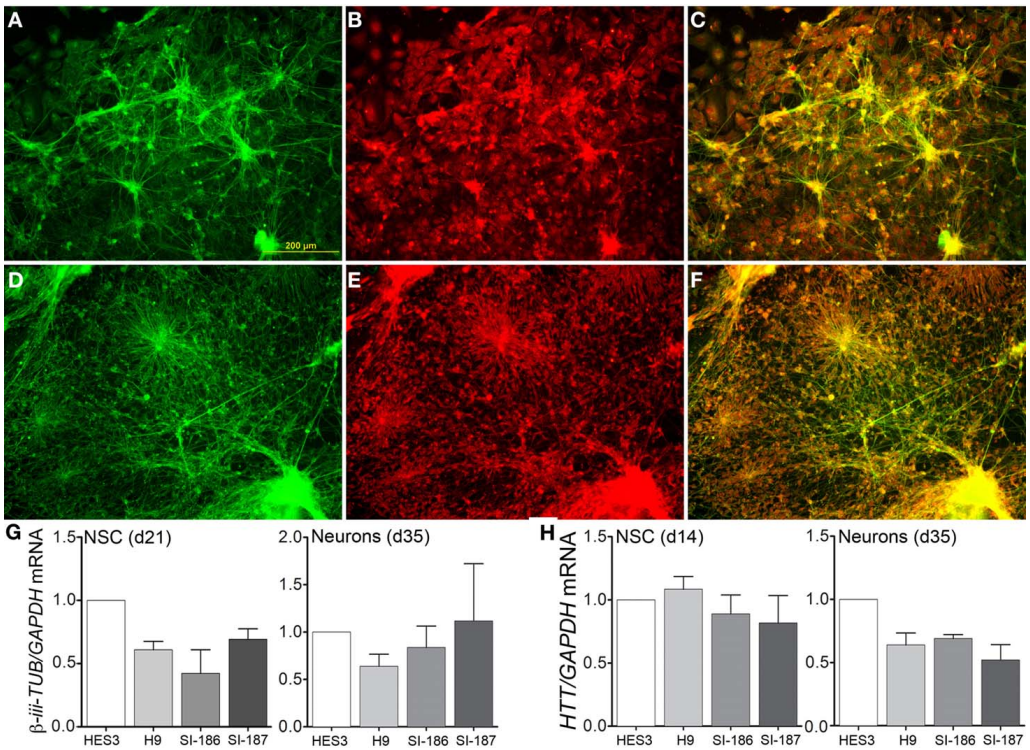

FIGURE 4 | Unperturbed neuronal differentiation in HD lines. Neuronal differentiation of **(A–C)** control HES3 and **(D–F)** HD SI-187 cells at d35, with immunocytochemical staining demonstrating **(A,D)** β-III-TUBULIN expression (green) and **(B,E)** HTT expression (red). **(C,F)** Merged images reveal HTT localization within neuronal cells. **(G)** Neuronal differentiation qRT-PCR comparisons of β-iii-TUBULIN expression during late neurosphere (d21) and neuronal (d35) stages. **(H)** HTT transcript expression across HD and control lines during mid-neurosphere (d14) and neuronal (d35) stages.

of anterior neurosphere cells culminated in robust detection of the neurotransmitter GABA, which was observed in equal proportions throughout the elaborate neuronal networks produced from control HES3 (**Figures 5A–C**) and H9 (**Figures 5D–F**) as well as HD SI-186 (**Figures 5G–I**) and SI-187 (**Figures 5J–L**) cell lines. Co-staining for β-III-TUBULIN and GABA demonstrate the majority of neuronal cells possess a GABAergic identity (**Figures 5C,F,I,L**).

FUNCTIONAL RESPONSES OF NEURONAL CULTURES

Neurons derived from this culture protocol were compared to their respective vehicle control wells with all four hESC derived neuronal cultures responding to glutamate (30 μM) with a significant elevations of $[Ca^{2+}]_i$, **Figure 5M** for a typical raw trace after background subtraction and **Figure 5N** for a typical response profile (One-Way ANOVA with *post-hoc* Tukey test). Interestingly, neurons derived from the fully penetrant SI-187 cell line responded to glutamate with a significantly greater elevation of $[Ca^{2+}]_i$ than neurons from the wildtype HES3 and H9 lines, or the partially penetrant SI-186 line ($p < 0.05$, One-Way ANOVA with *post-hoc* Tukey test, **Figure 5O**).

TRANSCRIPTIONAL ANALYSIS OF MUTANT HTT MODULATED GENES

Recent studies of HD human embryonic carcinoma cells (hECCs) and iPSC lines demonstrate *in vitro* gene expression alterations (Gaughwin et al., 2011; Castiglioni et al., 2012). Whether similar transcriptome disruptions are evident in late-onset CAG length hESC lines remains uncertain. Expression levels of critical transcripts identified in previous studies and those with direct paths from mHTT expression to gene disruption were assessed by qRT-PCR in undifferentiated HD and control lines (day 0), within neurospheres derivatives at day 14 and at neuronal promoting stages (day 35) (**Figure 6**). Selected for assessment were neural-specific genes Brain-Derived Neurotrophic Factor (*BDNF;* Zuccato and Cattaneo, 2007, 2009), dopamine receptor D2 (*DRD2*) and proenkephalin (*PENK*) (Luthi-Carter et al., 2000; Dunah et al., 2002; Hodges et al., 2006). Also assessed were the cholesterol biosynthesis transcripts 7-Dehydrocholesterol reductase (*7-DHCR*) and sterol regulatory element-binding protein 1 (*SREBP1*) (Valenza et al., 2005; Valenza and Cattaneo, 2011), as well as transcription factor EB (*TFEB*) a master regulator of lysosomal biogenesis (Sardiello et al., 2009; Settembre et al., 2011; Camnasio et al., 2012). At day 0 and 14 significant downregulation of *PENK* mRNA expression was observed in SI-187 cells compared to HES3 cells ($p < 0.05$; **Figure 6C**) and *SREBP1* was significantly up-regulated in SI-187 compared to HES3 at day 0 ($p < 0.05$; **Figure 6D**). Nevertheless, significant gene expression changes in *PENK* and *SREBP1* mRNA of HD SI-187 cells were not corroborated when compared to the second control line H9 ($p > 0.05$), suggesting these discrepancies are a consequence of inter-line variation as opposed to CAG repeat length. mRNA expression levels of the remaining genes *BDNF, DRD2, 7-DHCR7*, and *TFEB*

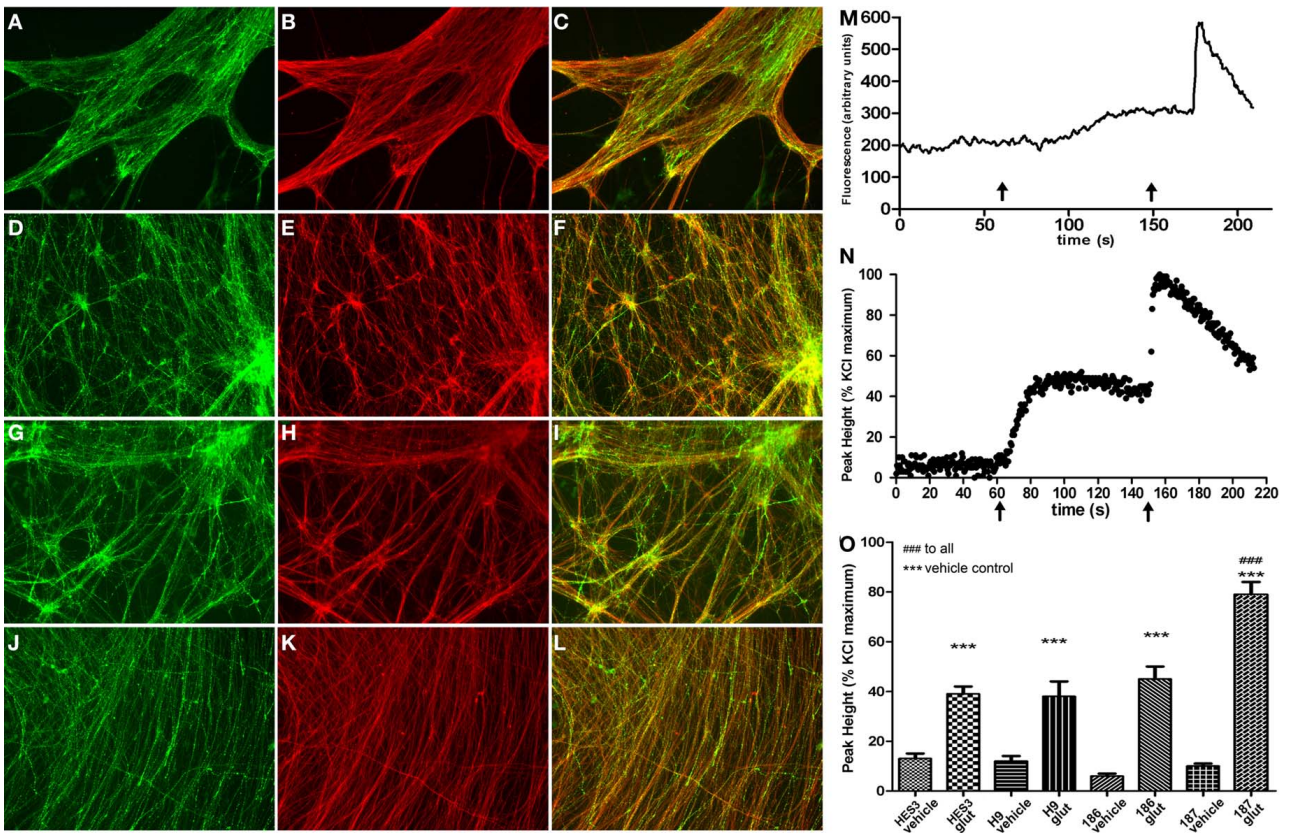

FIGURE 5 | Efficient differentiation to GABAergic neurotransmitter subtypes and glutamate response perturbations in HD neurons. **(B,E,H,K)** Neuronal differentiation extended to d45 generated elaborate networks shown by β-III-TUBULIN (red) immunostaining. GABAergic neurons detected by GABA immunostaining (green) were equivalently detected throughout neuronal cultures from control lines **(A)** HES3 and **(D)** H9, as well as HD lines **(G)** SI-186 and **(J)** SI-187. **(C,F,I,L)** Merged images illustrating co-localization between GABA and β-III-TUBULIN. **(M)** Example of a typical raw trace after background subtraction measured in arbitrary units of fluorescence intensity and **(N)** a typical neuronal Ca^{2+} response profile, left *x*-axis arrows indicate glutamate (30 μM) addition and right *x*-axis arrows KCl (30 mM) addition. **(O)** Intracellular $[Ca^{2+}]_i$ levels in response to vehicle and glutamate (30 μM) addition for all control and HD neuronal cultures.

were similar across HD and control lines at days 0, 14, and 35 ($p > 0.05$) (**Figures 6A,B,E,F**).

ASSESSMENT OF MITOCHONDRIAL GENE EXPRESSION AND FUNCTION

Mitochondrial dysfunction comprises a robust hallmark of HD pathology, measurable by cellular dysregulation of key transcripts, deficits in mitochondrial energy capacities, and fusion/fission events (Cantuti-Castelvetri et al., 2005; Cui et al., 2006; Knott et al., 2008; Song et al., 2011b). qRT-PCR analysis of mitochondrial genes *PGC-1α*, a master regulator of mitochondrial biogenesis and energy metabolism and *DRP1*, a regulator of mitochondrial fusion and fission showed no consistent differences in transcript levels between both control and HD hESCs or neural differentiated derivatives (**Figures 7A,B** respectively). Intriguingly, significant ($p < 0.05$) downregulation of SI-187 *PGC-1α* mRNA levels compared to H9 samples were observed at d14 and a similar yet non-significant trend was seen with SI-186 at d14 and both HD lines at d21 compared to wildtype controls (**Figure 6B**). Mitochondrial functional JC-1 assays that directly measure MMP found all hESC lines demonstrated similar populations of cells exhibiting dual red and green fluorescence (HES3 95%, H9 68%, SI-186 86%, and SI-187 81%; **Figure 7C**), indicative of an active MMP. Remaining green fluorescent cells from each line represented a sub-population of cells with less active mitochondria.

DISCUSSION

Human PSCs provide a unique window to investigate the ramifications of mutant HTT within the intracellular milieu from the earliest stages of human development until the specification of relevant neuronal subtypes. This study assesses two HD hESCs carrying expanded CAG repeat lengths representative of those harbored by individuals with archetypal late clinical onset. For the first time, we report detailed quantitative evaluations of the pluripotent properties and neural differentiation capabilities of these HD hESC lines using two wildtype control hESC lines as biological comparisons. Our studies of undifferentiated hESCs show expanded CAG repeat tracts have no adverse effects on stem cell parameters that were assessed, such as viability, mitochondrial

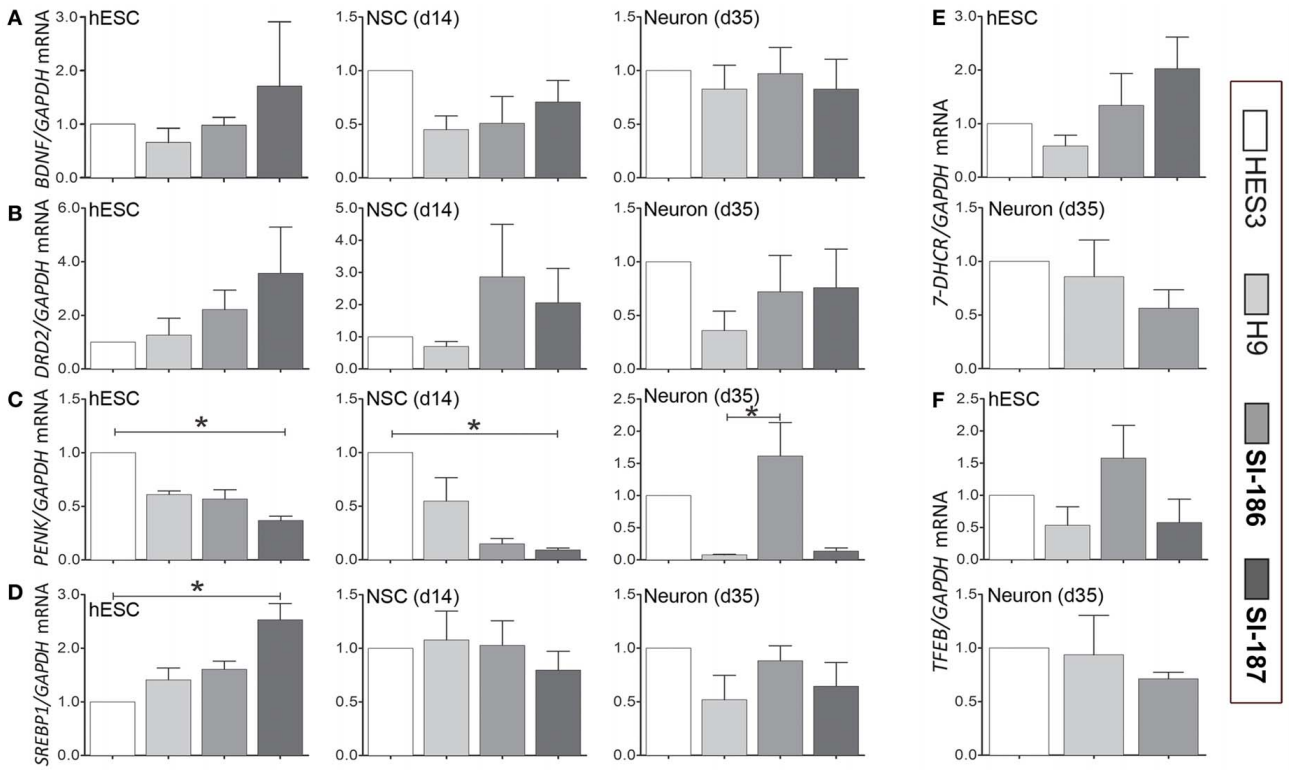

FIGURE 6 | qRT-PCR transcriptional analysis of relevant HD related transcripts modulated by mutant HTT. Relative transcript quantification for all four lines are normalized to the control HES3 line and were evaluated across a differentiation time course at the undifferentiated stage (d0), mid-neurosphere stage (d14) and neuronal differentiation stage (d35). Transcripts are assessed for **(A)** *BDNF*, **(B)** *DRD2*, **(C)** *PENK*, **(D)** *SREPB1*, **(E)** *7-DHCR*, and **(F)** *TFEB*. One-Way ANOVA, $^*p < 0.05$.

function, proliferation, and pluripotency markers. These findings are in agreement with recent pluripotent HD studies that observe no disturbance to similar parameters (Gaughwin et al., 2011; Camnasio et al., 2012; Castiglioni et al., 2012; HDIPSCC, 2012; Jeon et al., 2012).

Further, we demonstrate for the first time that neural differentiation of hESCs with HD mutations is equivalent to wildtype controls. Expression of neural markers CD56 and SOX2 and equivalent growth rates of neurospheres show unaltered differentiation in the presence of mutant HTT. Transient lag in the downregulation of the pluripotency marker CD9 was however, observed in one HD line, and corroborates findings of HD hECCs (Gaughwin et al., 2011). Crucially anterior specification assessed by key markers OTX2, FOXG1, and FORSE-1 were equivalent across all lines irrespective of CAG repeat length, and further extended differentiation produced consistent neuronal networks with similar acquisition of GABAergic identities upon *in vitro* maturation. These findings are consistent with mouse and human HD iPSC studies (Camnasio et al., 2012; Castiglioni et al., 2012; HDIPSCC, 2012).

HD patients and animal models are characterized by corticostriatal synaptic dysfunction and profligate excitotoxicity (Raymond, 2003; Estrada Sanchez et al., 2008). A major component of this pathological mechanism is glutamate receptor hypersensitivity on postsynaptic GABAergic striatal neurons. Striatal neurons receive essential glutamate signaling from cortical axons and mutant HTT is directly responsible for excessive Ca^{2+} influx downstream of glutamate stimulation, as seen in YAC128 (Tang et al., 2005) and R6/2 (Cepeda et al., 2001) transgenic models. Mutant HTT aberrantly binds endoplasmic reticulum $InsP_3R1$ receptors that are critical for Ca^{2+} release. This in turn increases the responsiveness of $InsP_3R1$ receptors to upstream glutamate receptor agonists and induces abnormal Ca^{2+} release (Tang et al., 2003, 2005).

In our studies we identified increased neuronal Ca^{2+} elevation in the fully penetrant HD line SI-187 upon glutamate stimulation that suggests late-onset HD hESCs are be capable of recapitulating some neuronal pathologies that characterize established HD model systems. Detectable alterations in Ca^{2+} glutamate responses are not unexpected with evidence of this phenotype in GABAergic neurons from pre-onset HD mice (Cepeda et al., 2001; Laforet et al., 2001) and even within neurons isolated as early as post-natal day 0-1 (Zeron et al., 2004). Together, these findings suggest increased Ca^{2+} responses may occur in early human stages of HD and thus represent a potentially valuable therapeutic target. The absence of such observations in the partially penetrant SI-186 line may correspond with the lesser pathological intensity of mutant proteins comprising 35–39 polyglutamine tracts and prolonged neuronal culture may be necessary to manifest this functional state.

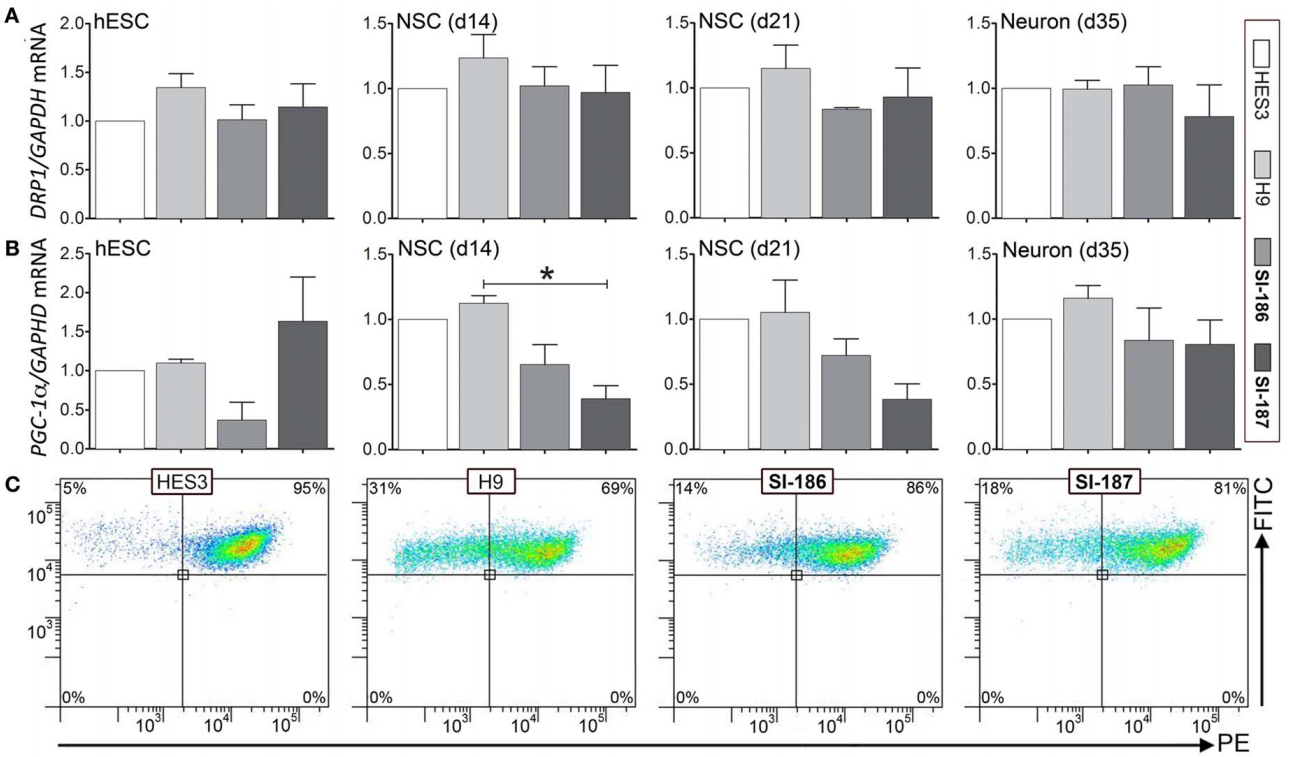

FIGURE 7 | Assessment of mitochondrial gene expression and function. Relative transcript quantification for all four lines are normalized to the control HES3 line for mitochondrial genes **(A)** *DRP1* and **(B)** *PGC-1α* at the undifferentiated (d0), neurosphere (d14) and neuronal (d35) stages of differentiation. **(C)** Mitochondrial functional JC-1 assay quantifying activity of the mitochondrial membrane potential (MMP) between control and HD hESCs. One-Way ANOVA, $^{*}p < 0.05$.

Extensive mutant HTT dysregulation of the cellular transcriptomes is a dominant hallmark seen in post-mortem brain tissue from HD patients and across animal models, reportedly affecting ∼1–2% of total cellular transcripts (Cha, 2000; Luthi-Carter et al., 2002a,b; Hodges et al., 2006). Transcript alteration has emerged as a focal point across HD pluripotent cell studies of aggressive HD subclasses, though little consensus appears to date. Exemplifying this are contrary observations of *BDNF* transcript stability and downregulation compared to controls in generated neural cultures (Gaughwin et al., 2011; Castiglioni et al., 2012; HDIPSCC, 2012). This contradiction is surprising considering clear evidence from model systems of the process by which mutant HTT instigates reduced BDNF expression via nuclear translocation of the transcriptional repressor REST (Zuccato and Cattaneo, 2007; Zuccato et al., 2007). Our study found BDNF is not altered in neural and neuronal derivatives of HD hESCs carrying typical late-onset ranges, suggesting BDNF may not be dysregulated unless significant pathology has progressed.

None of the remaining transcripts analysed in the HD hESC lines were significantly changed at either pluripotent or neural differentiated time points when compared to both control hESC lines. *SREBP1*, a cholesterol biosynthesis regulator, was significantly upregulated in pluripotent SI-187 cells compared to one control and approached significance to the second control line; a finding consistent with overt cholesterol biosynthesis disruption in HD models (Valenza et al., 2007a,b), patients (Markianos et al., 2008) and undifferentiated HD iPSC lines (Castiglioni et al., 2012). Further, there was a transient downregulation of the mitochondrial gene *PGC-1α* at d14 in SI-187 neural derivatives which continued to d21 although the trend was not statistically significant at the latter time-point. To corroborate this finding, we undertook functional studies of MMP activity, which is reduced when *PGC-1α* is down-regulated. In both undifferentiated cells and in d14 neurospheres from SI-186 and SI-187 (data not shown) we found no evidence of altered MMP. Together these findings in conjunction with those of other pluripotent systems imply transcript disturbances in this class of *in vitro* models may be a phenomenon that predominates in lines equivalent to juvenile and infantile disease subclasses, warranting array based studies to scrutinize cellular transcriptomes across a range of lines carrying different CAG repeat lengths.

In summary we have provided detailed characterization of HD hESCs and also those that harbor typical CAG repeat expansions. We demonstrate for the first time that pluripotent dynamics and forebrain neuronal differentiation are unhindered but that glutamate signaling perturbations indicative of Huntington's pathology are observable subsequent to extended *in vitro* culture and cellular acquisition of a forebrain GABAergic neuron identity. These late-onset hESC lines demonstrate the potential of such model systems to provide a platform to interrogate disease mechanisms and hallmarks. Advances describing the efficient generation of DARPP32+ medium spiny neurons provide an

opportunity for probing HD etiology within a more relevant cell type (Ma et al., 2012) and could be coupled with proteasomal inhibition or oxidative stress to exacerbate disease phenotypes as demonstrated recently (HDIPSCC, 2012; Jeon et al., 2012).

Emergent studies of HD iPSCs describe lines that possess atypical alleles representative of rare juvenile/infantile (CAG_{60+}) or homozygous subclasses that are poorly studied in human patients (Camnasio et al., 2012; Castiglioni et al., 2012; HDIPSCC, 2012; Jeon et al., 2012). The observation of some HD specific phenotypes in these lines demonstrate their potential value as *in vitro* human models, however, such phenotypes rarely correlate across lines and some are seen as contradictory. Existing HD iPSC lines may not faithfully represent the molecular perturbations of late-onset HD since they potentially exhibit unknown consequences by the reprogramming somatic cells exposed to mutant HTT proteins for considerable durations, and hence PGD isolated hESCs described in this study provide a more "natural" cellular alternative.

Late-onset HD alleles appear to provide a unique system to probe pre-onset changes at the molecular level and within a neurological context, as attested by observations of elevated Ca^{2+} glutamate responses. Understanding pre-onset HD alterations could elucidate the hierarchical relationship between various HD hallmarks, yet is presently restricted primarily to non-invasive imaging methodologies (Tabrizi et al., 2009; Nopoulos et al., 2011) and peripheral blood analysis (Borovecki et al., 2005; Runne et al., 2007; Bjorkqvist et al., 2008). The application of late-onset hESC to long-term *in vitro* neuronal cultures or cellular challenges that mimic *in vivo* conditions (i.e., oxidative or proteasomal stressors) may provide a system to delineate the thresholds that mark clinical onset that is otherwise difficult in extreme CAG repeat variants. Nevertheless, future work must simultaneously evaluate differing model systems to identify those which most faithfully recapitulate human HD, to steer prospective *in vitro* therapeutic activity toward models with the highest potential.

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Patient-derived stem cells: pathways to drug discovery for brain diseases

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The concept of drug discovery through stem cell biology is based on technological developments whose genesis is now coincident. The first is automated cell microscopy with concurrent advances in image acquisition and analysis, known as high content screening (HCS). The second is patient-derived stem cells for modeling the cell biology of brain diseases. HCS has developed from the requirements of the pharmaceutical industry for high throughput assays to screen thousands of chemical compounds in the search for new drugs. HCS combines new fluorescent probes with automated microscopy and computational power to quantify the effects of compounds on cell functions. Stem cell biology has advanced greatly since the discovery of genetic reprograming of somatic cells into induced pluripotent stem cells (iPSCs). There is now a rush of papers describing their generation from patients with various diseases of the nervous system. Although the majority of these have been genetic diseases, iPSCs have been generated from patients with complex diseases (schizophrenia and sporadic Parkinson's disease). Some genetic diseases are also modeled in embryonic stem cells (ESCs) generated from blastocysts rejected during *in vitro* fertilization. Neural stem cells have been isolated from post-mortem brain of Alzheimer's patients and neural stem cells generated from biopsies of the olfactory organ of patients is another approach. These "olfactory neurosphere-derived" cells demonstrate robust disease-specific phenotypes in patients with schizophrenia and Parkinson's disease. HCS is already in use to find small molecules for the generation and differentiation of ESCs and iPSCs. The challenges for using stem cells for drug discovery are to develop robust stem cell culture methods that meet the rigorous requirements for repeatable, consistent quantities of defined cell types at the industrial scale necessary for HCS.

Keywords: embryonic stem cells, induced pluripotent stem cells, olfactory stem cells, olfactory neurosphere-derived cells, high content screening

INTRODUCTION

The last decades have seen development and applications in cellular neuroscience of DNA, RNA, and protein analysis technologies that provide large volumes of information, getting away from the traditional methods that follow the activities of single genes or single cell types. The application of DNA sequencing, RNA microarrays and higher throughput protein mass spectrometry is allowing examination of biological systems in all their complexity. What has been missing for cell biologists is the ability to interrogate cell functions at the same scale and level of complexity. This is being addressed by the developments in automated technologies developed by pharmaceutical companies to screen massive compound libraries for the discovery of new drug leads. Until recently this high throughput screening, with robotic control and automated data capture and analysis of experiments in 96-, 384-, and 1,536-well plates, centered on enzyme and receptor assays but increasingly, interest is turning to cell-based assays that capture the complexity of the environment in which drugs will actually operate. There has been a move to drug screening based on cellular outcomes (e.g., cancer cell apoptosis, inhibition of growth) rather than predicted mechanism (e.g., enzyme inhibition, receptor antagonism). This led to the concept and development of "high content screening" (HCS) that combines advances in fluorescence labeling of cells, robotic and automated microscopy, and automated image analysis that brings the analysis of cell functions to the high throughput formats of multiwell plates (Zanella et al., 2010). For the neuroscientist this technology opens up new frontiers in the ability to manipulate many experimental variables simultaneously in highly controlled experiments. For example, the effects of multiple drugs at multiple doses could be tested simultaneously on several cell types. With the many fluorescent reporter methods now available it is possible to follow three or four cellular events in the same experiment in hundreds of cells per well. With many well established platforms available, HCS opens the door to neuroscience for large scale, high throughput cell function analyses for understanding cell functions in health and disease.

In neuroscience, the discovery of the genetic causes of familial diseases has driven understanding of the functions of individual genes and proteins in cell function in the nervous system and the effects of mutations on brain function. Identification of a candidate gene is followed by genetically modified cell and

mouse models to identify the functions of the identified gene at cell and systems level. Mouse models have been important for elucidating protein and gene functions but they often do not recapitulate human disease because the mice lack the human genetic background and the introduced human genes are acting in a non-human cellular context. Immortalized human cell lines have a more relevant genetic background but, being derived from tumors, they may not reflect a normal cellular context. Patient-derived primary cells might be a solution for both drawbacks but they cannot usually be maintained for very long in culture and finding an accessible cell type for brain diseases is problematic. Stem cell technologies may have the solution to these drawbacks.

There is an emerging interest in using stem cells to understand the cellular bases of human diseases. There is an imperative here, especially for neurological diseases and conditions. Large pharmaceutical companies are withdrawing from investing in neuroscience research because of the failure of the current paradigms to convert findings in animal models to drugs for human disease (Schnabel, 2008). There are obviously many reasons why animal models, genetic or otherwise, are not proving useful for predicting human responses to drugs. On the other hand, there are good reasons to expect that human stem cells might be useful, if they can be derived from patients with a disease and, if they are the cell types that are affected by the disease. This is clearly a niche that stem cells have the developmental abilities to occupy. Patient-derived stem cells could be used to identify cell functions that are altered by disease and thereby provide a target for drug discovery. Assays can then be developed for HCS to use the patient-derived stem cells to screen large drug libraries for therapeutic activity.

HIGH THROUGHPUT SCREENING

Increasingly in the last 20 years pharmaceutical companies have developed high throughput technologies to screen large chemical libraries of natural products and synthetic compounds for activities against selected enzymes and receptors, candidate targets for diseases of interest (Macarron et al., 2011; **Figure 1**). These high throughput technologies arose from the desire of pharmaceutical companies to test all theoretical chemical compounds (∼100 million) against all theoretical human biological targets, estimated from the number genes (∼20,000) or proteins (∼200,000), indicating that as many as 10^{12} assays would be needed to identify all interactions between chemicals and targets (Sundberg, 2000). These technologies are pharmacology on a large scale, initially using 96-well plates and now routinely 384- and 1,536-well plates. The imperative is to provide a means to screen very large compound libraries in order to find the very small percentage of lead compounds that are active in a selected assay. High throughput screening is historically based on solution based enzyme and receptor assays using scintillation, absorbance, fluorescence, and chemiluminescence with the aim of finding highly specific chemical interactions with individual biological targets (Sundberg, 2000). High throughput screening is also used in cell-based assays, typically to monitor activation of cell surface receptors through subsequent transduction pathways, transcription events, cell proliferation, or cell death. These assays use multiwell plates and monitor colorimetric, fluorescence, luminance, or absorbance within each well. With developments in genetic technologies and the understanding provided by the Human Genome Project, intracellular events are available for high throughput screening through the development genetic fluorescent reporter assays. Cell lines have genes for luminescent or fluorescent proteins, like luciferase or green fluorescent protein (GFP), spliced into reporter systems to read out activation or inhibition of specific genes or proteins of interest (Sundberg, 2000). High throughput technologies have advanced the development of robotic automation for cell culture, assays, and compound library storage; automated and multipurpose plate readers; fluorescence dyes and reporter systems; computational power, and automated data storage and analysis. It currently takes about 1 week to screen 10,000 compounds against a target and 1–3 months to screen a library of one million compounds (Macarron et al., 2011). High throughput screening has been successful in delivering numerous drugs from discovery through to clinical use and the market starting from chemical libraries of 200,000–500,000 compounds (Macarron et al., 2011).

HIGH CONTENT SCREENING

High content screening is a further development in which the principles of high throughput screening are applied to the analysis of individual cells through the use of automated microscopy and image analysis (Zanella et al., 2010; **Figure 2**). This allows quantitative analyses of components of cells such as spatio-temporal distributions of individual proteins, cytoskeletal structures, vesicles, and organelles when challenged with chemical compounds. HCS can be used to monitor activation or inhibition of individual proteins and protein–protein interactions as well as allowing analysis of broader changes in biological processes and cell functions. Recent advances in the range of fluorescent probes for biological processes, functions, and cell components have combined with developments in fluorescence microscopy to give the cell biologists many new ways to understand cell functions in health and disease. These cell-centric developments have converged with high throughput concepts and with developments in automated microscopy and image analysis to evolve into the new technological synthesis of high throughput screening in which cell-based assays are conducted in multiwell plate formats. High content technologies are now used to screen chemical libraries for drug discovery as well as genome-wide RNA interference libraries to probe gene functions (Zanella et al., 2010). One of the advantages of HCS is the ability to apply different criteria to selected cells in the population to account for the heterogeneity of the cell population. For example, a chemical compound of interfering RNA may only act on cells in a particular stage of the cell cycle. Well-based assays provide a readout from the whole cell population, a mixture of cells in different phases of the cell cycle, whereas with cell-based HCS and appropriate markers, cells at identified phases of the cell cycle could be assessed independently. Thus HCS allows quantitative analysis of complex and heterogeneous cell cultures containing multiple cell types. Another advantage of HCS technologies is the ability to make multiple independent and quantitative measurements from single cells of interest. For example, transmitted light might be used to assess morphology; three or more fluorophores

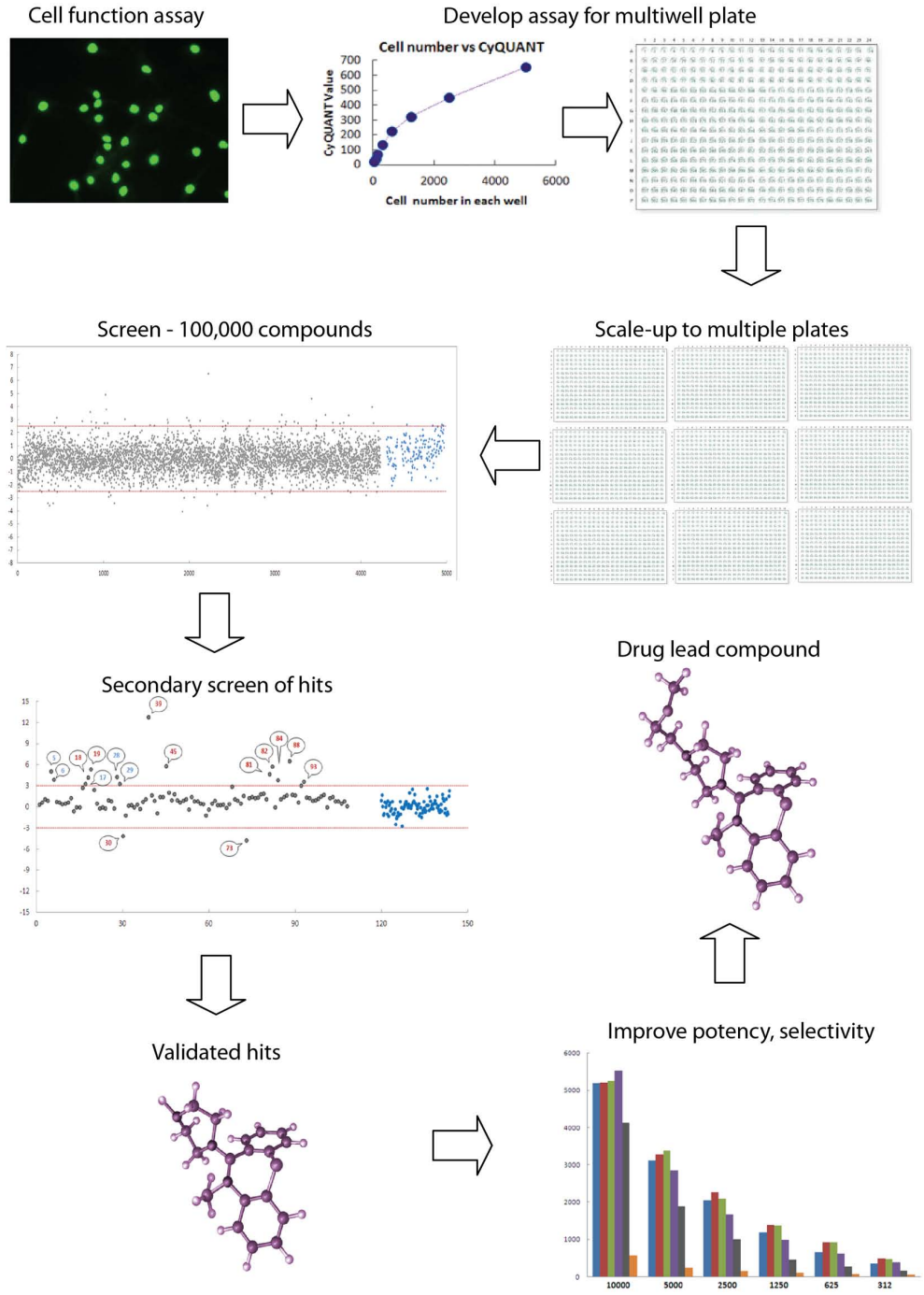

FIGURE 1 | High throughput screening: industrial scale biology. High throughput screening is an automated process for conducting thousands of assays to identify active molecules modulating a biomolecular activity. Assays are performed in multiwell plates with robotics for cell and liquid handling and other aspects such as moving plates from incubator to drug delivery to plate reader. An assay for a cell function of interest must be validated for multiwell plate readers, such as fluorescence, absorbance, or luminescence. Here the assay is a fluorescence assay (Cyquant) for cell number. High levels of quality assurance and quality control are required to reduce the variance of the measurement so that the assay can be reliably scaled to multiple plates. Typically, thousands of compounds are screened for activity, with "hits" being defined as those compounds whose activity on the assay falls outside three standard deviations (red lines) around the mean of the controls on the plate (blue dots). Hits are the gray dots above and below the red lines. Secondary screens are then used to validate the initial hits. These screens may include dose–response curves or assays of related functions of interest, resulting in a selection of "validated hits," which may comprise fewer than 1% of a typical library of compounds of varied structure. Here the validated hits are the numbered dots above the red line. Validated hits are then further assayed and modified chemically in an iterative process to improve potency, selectivity, pharmacological properties, and toxicological profile to produce a drug lead compound that will go into "preclinical" testing in animal models. High throughput screening is applied to large libraries of any potentially bioactive molecules including natural products, small molecule drugs, RNAs, and DNAs.

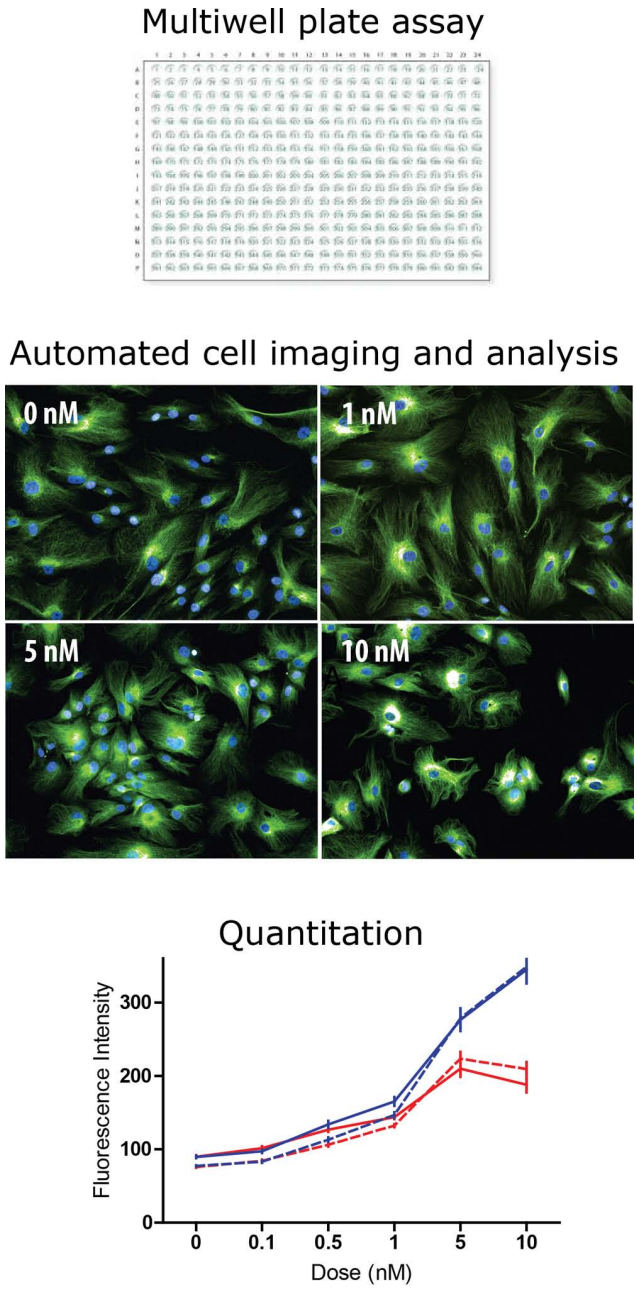

FIGURE 2 | High content screening: industrial scale microscopy. High content screening is the automated microscopic analysis of fluorescence in cells of a multiwell plate. Most high throughput screening assays read activity per well (e.g., fluorescence, absorbance, and luminescence). In high content screening fluorescence in individual cells is quantified with automated image analysis. Typically, algorithms find the cells in the image and then measure fluorescence intensity and spatial distribution. The example shows tubulin fluorescence in cells (green) and cell nuclei (blue) at different concentrations of taxol, a tubulin-binding drug. The drug effect is difficult to see by eye but the analysis algorithm identifies each cell and quantifies tubulin fluorescence intensity, plotted here as a dose–response curve to two drugs, taxol (blue) and vinblastine (red) in two cell types: control-derived ONS cells (solid lines) and HSP patient-derived ONS cells (dashed lines; Abrahamsen et al., 2013). With high content screening thousands of cells can be analyzed to reveal small but significant differences between drugs, doses, and cell types.

might be used to identify molecular components, structures, or organelles; and image analysis might be used to quantify spatial relationships between the fluorescent reporters under control conditions or when challenged with chemical compounds. With assays performed in multiwell plates there is opportunity to scale up experiments to include replicates, concentration–response curves, and parallel assessments of different cells or compounds. Modern instruments equipped with incubators and confocal optics have the ability to investigate cells over time in three spatial dimensions. These technologies will advance further and become cheaper allowing HCS principles to be applied increasingly by academic labs and not restricted to large pharmaceutical companies.

In the neurosciences, there is now an opportunity and challenge to combine patient-derived, disease-specific stem cells with HCS technologies with the aim of finding new drugs for brain diseases and conditions. This is not a simple aspiration because the majority of brain diseases are a result of complex genetic and environmental risk factors. Furthermore, brain diseases are usually just that, "brain" diseases and not "cell" diseases in the sense that cancers are. Nonetheless, it is possible that most brain diseases result from identifiable cellular dysfunctions such as those identified in monogenic disease. Such mutations tell us that specific brain dysfunctions can be manifest in specific cell types and pathways, despite universal genetic mutation. This gives hope that cellular models will shed light on the molecular and cellular mechanisms of emergent properties (e.g., cognition, emotion) evident when the brain functions as a whole.

DISEASE-SPECIFIC PLURIPOTENT STEM CELL MODELS OF NEUROLOGICAL DISEASE

The analysis of gene function through gain- or loss-of-function in cell, fly, and mouse models has been very instructive in elucidating functions of genes and proteins but less successful in providing models that predict drug efficacy in human diseases. An example is the failure of the superoxide dismutase transgenic mouse model for amyotrophic lateral sclerosis that has yielded multiple compounds that are therapeutic in mouse but not humans (e.g., creatine; Klivenyi et al., 1999; Groeneveld et al., 2003). These problems arise because of species and model differences which do not mimic gene dosage, gene mutation variability or genetic background, or the complex of other contributing genetic factors that will be present in individuals with a disease. Patient-derived cells provide a potential solution to some of these problems because they reflect the genetic background and variation of the human population, arising from individuals with natural mutations. Sources of patient-derived cells have included easily accessible fibroblasts and lymphocytes and lymphoblastoid cell lines but there is concern that these cells will not manifest the same disease-associated properties as the cells specifically dysfunctional in the disease of interest, for example dopaminergic neurons of the substantia nigra in Parkinson's disease or oligodendrocytes in multiple sclerosis. For such cell type-specificity the field holds high hopes of pluripotent stem cells which can theoretically be induced to differentiate into any cell type of interest.

Disease-specific pluripotent stem cells include human embryonic stem cells (ESCs) with genetic or chromosomal disorders

derived from surplus blastocysts during *in vitro* fertilization and pre-implantation genetic diagnosis (Stefanova et al., 2012). Although these are not strictly "patient-derived" they carry specific genetic mutations or deletions that would normally lead to disease. Induced pluripotent stem cells (iPSCs) from patients have become the dominant choice for patient-derived pluripotent stem cells. A recent review lists 18 diseases for which ESCs have been derived, compared with 40 for which iPSCs have been derived (Grskovic et al., 2011). At this time many of the publications in this field are mainly demonstrations that pluripotent stem cells have been derived, often without demonstrating a disease-phenotype. Some show that the pluripotent cells can be differentiated into specific cell types of interest and some demonstrate deficits in cellular functions compared to control cells, as proof-of-principle for disease modeling (Grskovic et al., 2011; Maury et al., 2012). No doubt the numbers of diseases for which iPSCs are available will increase greatly in the next few years and deeper analyses of their functions will be forthcoming. It is a major challenge for the field to move beyond the proof-of-principle stage to discovery of new aspects of disease biology and new targets for therapeutic intervention.

The list of neurological diseases and conditions for which ESCs or iPSCs have been derived is largely limited to monogenic diseases including Charcot–Marie–Tooth disease type 1A, Down syndrome-trisomy 21, familial amyotrophic lateral sclerosis, familial dysautonomia, familial Parkinson's disease, Fragile X syndrome, Friedreich ataxia, Gaucher's disease, Huntington's disease, Rett syndrome, Spinal muscular atrophy, spinocerebellar ataxia types 2 and 7, and X-linked adrenoleukodystrophy (Grskovic et al., 2011; Maury et al., 2012; Rajamohan et al., 2013). It is thought that diseases of complex genetics and environmental risk factors may be harder to model with pluripotent stem cells but patient-derived iPSCs have been generated from patients with Parkinson's disease (Soldner et al., 2009) and schizophrenia (Brennand et al., 2011; Pedrosa et al., 2011). Patient-derived iPSCs from people with sporadic Parkinson's disease were differentiated into dopaminergic neurons but failed to show an obvious difference in phenotype compared to control cells (Soldner et al., 2009). Similarly, a disease-associated phenotype could not be demonstrated in iPSCs from two cases of sporadic Alzheimer's disease (Israel et al., 2012). In one study, iPSCs from schizophrenia patients were differentiated into neurons and gene expression profiling identified a cluster of differentially expressed genes involved in neurogenesis, neuronal differentiation, axon guidance, and adhesion with another cluster of differentially expressed genes involved in cell cycle regulation (Pedrosa et al., 2011). A second study in schizophrenia showed that neurons differentiated from patient-derived iPSCs had reduced neurite number and reduced connectivity with other neurons *in vitro* and reduced glutamate receptor expression (Brennand et al., 2011). These studies of patient-derived iPSCs from schizophrenia patients demonstrate that such models can reveal disease-associated cellular deficits in a disease of complex genetics, although the patients were all from families with psychosis, rather than sporadic cases.

It is a challenge to translate pluripotent cells into robust disease models (Maury et al., 2012). For example, ESCs are limited by the availability of genetic testing and pre-implantation genetic diagnosis. For their part, iPSCs are potentially compromised by the methods of their generation; most cell lines published to date were produced by integrating vectors, although this will change as the efficiency and predictability of non-integrating methods improves. There are other technical challenges for disease modeling such as the current lack of robust and efficient protocols for differentiating ESCs and iPSCs into disease-associated cells of interest. Attention is drawn to the importance of selecting appropriate matched controls because case–control cell differences could arise from sampling bias and "disease-associated" differences may result from particular comparator control cells (Zhu et al., 2011; Maury et al., 2012). This difficulty is compounded by the large cost of generation and maintenance of ESCs and iPSCs that will limit sample sizes for most laboratories. One preferred method would be to use each patient-derived cell line as its own control by correcting its genetic defect (Zhu et al., 2011; Maury et al., 2012) but this is possible only in the monogenic disease cases of known mutations that can be selectively corrected. It is expected that many of these challenges will be overcome as new methodologies develop for efficient production of patient-derived iPSCs that are generated with non-integrating reprograming methods (Grskovic et al., 2011) and efficient methods are devised for differentiation into desired cell types: specific neurons, astrocytes, and oligodendrocytes.

PATIENT-DERIVED OLFACTORY STEM CELLS AS MODELS FOR NEUROLOGICAL DISEASES

Published studies of ESCs and iPSCs as disease models are all confined to small numbers of cell lines from patients and controls. This makes it difficult to generalize from these case–control studies to the general population. Variability in the reprograming process and epigenetic status makes it essential that several clones from several individuals are compared to confirm that a "disease-phenotype" is not confounded by individual differences among case or control cell lines (Vitale et al., 2012). Ideally, patient- and control-derived cells should be sampled from large populations to be certain that differences between the samples are representative of the population. In the case of genetic diseases with high penetrance of the clinical phenotype, the case–control design may be robust to small sample size but this may not be true of lower penetrance phenotypes, or of sporadic diseases of complex genetics. This sampling problem can be overcome with accessible cells that can be derived easily from larger numbers of individuals, such as patient-derived fibroblasts and lymphocytes, which have been used to identify potential cellular mechanisms or biomarkers of neurological diseases including Alzheimer's disease (Takahashi et al., 1999; Moreira et al., 2007), Parkinson's disease (Martin et al., 1996; Hoepken et al., 2008), and schizophrenia (Ilani et al., 2001; Wang et al., 2010). Although these cell types have identified cellular and molecular differences between patients and controls, their relevance to diseases of the nervous system is moot and the patient–control differences can be non-existent (Matigian et al., 2008).

Another approach to modeling diseases is to sample patient-derived adult stem cells. Neural progenitor cells were isolated from post-mortem brain from Alzheimer's patients and healthy controls (Lovell et al., 2006). Mesenchymal stem cells were isolated from

controls and patients with amyotrophic lateral sclerosis (Ferrero et al., 2008) and Parkinson's disease (Zhang et al., 2008). None of these studies reported disease-associated differences, on the contrary all stated that patient and control cells were of similar phenotype.

There is a multipotent adult stem cell resident in the olfactory mucosa, the organ of smell in the nose (Murrell et al., 2005). A stem cell in the olfactory epithelium maintains a continual neurogenesis throughout life that regenerates the sensory neurons (Mackay-Sim and Kittel, 1991a,b; Murrell et al., 1996). Olfactory epithelium is one of the few places in the nervous system that contains a neural stem cell but, unlike the other sites in the brain, it is accessible under local anesthetic (Feron et al., 1998) and several studies have shown disease-associated differences in cell biology in this tissue. Olfactory neuroblasts isolated from post-mortem epithelial biopsies from controls and individuals with Alzheimer's disease demonstrated differences in amyloid precursor protein processing (Wolozin et al., 1993) and oxidative stress (Ghanbari et al., 2004). Histological analysis of olfactory epithelium indicates disturbed neural differentiation in schizophrenia (Arnold et al., 2001) and Rett syndrome (Ronnett et al., 2003). Cultures of olfactory mucosa show increased cell proliferation and reduced tissue adhesion in schizophrenia compared to healthy controls (Feron et al., 1999; McCurdy et al., 2006). Clearly the olfactory organ demonstrates disease-associated differences in cell biology in several brain diseases including a monogenic disease (Rett syndrome) and complex genetic diseases of no known genetic cause (Alzheimer's disease and schizophrenia). This led to the development of an olfactory stem cell-based system for investigating brain diseases based on olfactory neurosphere-derived cells (ONS cells; Matigian et al., 2010).

The olfactory mucosa comprises the superficial epithelium and the underlying lamina propria separated by a basement membrane. Within the epithelium are basal cells among which are the multipotent stem cells that can regenerate all the cell types of the epithelium including the sensory neurons as well as other non-neural supporting and gland cells (Leung et al., 2007; Packard et al., 2011). Within the human lamina propria is a multipotent stem cell with characteristics both of neural and mesenchymal stem cells, an ectomesenchymal stem cell (Delorme et al., 2010). The lineage relation between these stem cells, if any, is not known. When biopsies of human olfactory mucosa are dissociated and grown in a serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor 2 (FGF2), neurospheres form that can differentiate into neurons and glia and cells of non-ectodermal lineage, including developing cardiac and skeletal muscle, kidney and liver, and blood (Murrell et al., 2005). Neurospheres are dissociated and when the cells are grown as adherent cultures in a serum-containing medium, these "neurosphere-derived" cells (ONS cells) have a flattened, undifferentiated appearance with a marker phenotype similar to the ectomesenchymal cells derived from primary cultures of olfactory mucosa (Delorme et al., 2010; Matigian et al., 2010), that is, they express markers of both neural and mesenchymal lineages. Patient-derived ONS cells show robust disease-specific differences compared to ONS cells derived from healthy controls (Mackay-Sim, 2012). Gene expression profiling of ONS cells from healthy controls and patients with Parkinson's disease or schizophrenia showed disease-specific differences in gene expression, protein expression, and cell functions (Matigian et al., 2010). Disease-associated gene expression differences were quite different in schizophrenia and Parkinson's disease, with alterations in neurodevelopmental pathways in schizophrenia and in oxidative stress and metabolic function in Parkinson's disease (Matigian et al., 2010). Deeper analysis of the gene expression showed disease-specific differences in the variance of gene expression in the major signaling pathways. Overall schizophrenia patient-derived ONS cells were less variant in their gene expression compared to controls whereas Parkinson's patient-derived ONS cells were more variant than controls, indicating another dimension along which diseases can affect cell functions (Mar et al., 2011).

Parkinson's patient-derived ONS cells showed gene expression and functional differences indicating dysfunctions in pathways involved mitochondrial metabolism and oxidative stress (Matigian et al., 2010). Pathway analysis of the gene expression differences found that the antioxidative nuclear factor (erythroid-derived 2)-like 2 (NRF2) signaling pathway was overrepresented among the dysregulated genes (Matigian et al., 2010). NRF2 is a transcription factor that ameliorates the effects of oxidative stress. Deeper analysis of the ONS cells demonstrated that the NRF2 signaling pathway was dysfunctional: although the NRF2 protein was equally expressed in patient and control cells the downstream targets of NRF2 were reduced, suggesting lesser activation of NRF2 signaling in the patient cells (Cook et al., 2011). In contrast, schizophrenia patient-derived ONS cells showed gene expression and functional differences indicating dysfunctions in pathways involved neurodevelopment (Matigian et al., 2010). Pathway analysis of the gene expression differences identified aspects of the cell cycle that were dysregulated, in particular G1/S phase transition, the check point in cells that controls the beginning of DNA synthesis (Matigian et al., 2010). Functional analysis demonstrated that patient-derived cells proliferated faster than control-derived cells in accord with previous observations in olfactory biopsy cultures (Feron et al., 1999; McCurdy et al., 2006). This faster rate of cell proliferation was due to a 2-h shorter cell cycle period in patient cells (Fan et al., 2012). G1/S phase transition is dependent on the intracellular levels of cyclin D1, a cell cycle control protein; cyclin D1 levels were elevated in patient cells and reached higher levels more quickly than control cells (Fan et al., 2012). In schizophrenia, ONS cells gene expression was significantly dysregulated in the focal adhesion kinase signaling pathway, which is involved in regulating attachment to the extracellular matrix through cell surface integrin receptors (Fan et al., 2013). Functional analyses of the patient-derived cells showed they were less adhesive and more motile than control-derived cells with smaller and fewer sites of adhesion that disassembled more quickly than in control-derived cells (Fan et al., 2013). These studies demonstrate that patient-derived ONS cells can show robust disease-specific differences in cell biology even in sporadic diseases of complex genetics.

Olfactory neurosphere-derived cells are also proving useful for understanding monogenic diseases. Hereditary spastic paraplegia (HSP) is an autosomal dominant disease affecting the long spinal axons from the motor cortex to the lower motor neurons

in the spinal cord. ONS cells from patients with HSP were similar in many basic cell functions to ONS cells from healthy controls despite dysregulation of expression of 60% of the genome, indicating a high level of homeostatic regulation in response to dominant mutations in *SPAST*, which codes for a microtubule severing protein (Abrahamsen et al., 2013). Closer inspection of cell functions using HCS revealed significant reductions in stable microtubules and in the intracellular distributions of peroxisomes and mitochondria (Abrahamsen et al., 2013). Live-cell time-lapse imaging revealed that peroxisomes traveled more slowly in HSP patient-derived cells, consistent with the corticospinal axon pathology in HSP (Abrahamsen et al., 2013). Ataxia telangiectasia (AT) is a fatal autosomal recessive disease characterized by radiation sensitivity, cancer, and cerebellar dysfunction. ONS cells from children with AT had radiation sensitivity and DNA-repair deficits that were corrected by insertion of the full-length gene (Stewart et al., 2013). Immature neurons from AT patient-derived ONS cells showed evidence of impaired differentiation (Stewart et al., 2013). Olfactory ectomesenchymal stem cells, similar to ONS cells, demonstrated a disease-associated effect of mis-splicing of the *IKBKAP* gene leading to reduced levels of IKBKAP protein and altered cell migration in patient-derived cells in familial dysautonomia (Boone et al., 2010).

PATIENT-DERIVED OLFACTORY STEM CELLS FOR DRUG DISCOVERY

Patient-derived ONS cells have several advantages for HCS for drug lead identification. They are cheap to grow and maintain, growing in standard cell culture conditions with no expensive growth factors after the neurosphere-forming stage. As ONS cells they can be grown for at least 16 passages without significant change in gene expression thus demonstrating minimal phenotypic change and without change in karyotype (unpublished observations). ONS cells are derived from neural tissue and can obviously show disease-specific phenotypes relevant to the neurological diseases from which the donors suffer. Proof-of-principle analyses have shown that brain diseases "in a dish" can be ameliorated by drug treatment. For example, Parkinson's patient-derived ONS cell functions were restored to control-derived cell levels by treatment with L-sulforaphane, an agonist of NRF2 (Cook et al., 2011). Similarly, a small molecule drug kinetin was able to reverse the mis-splicing of the *IKBKAP* gene in familial dysautonomia patient-derived ectomesenchymal cells (Boone et al., 2010) as it has in iPSCs from these patients (Lee et al., 2009). HCS was used to test the differential sensitivity of HSP patient-derived and control-derived ONS cells to the tubulin-binding drugs, taxol and vinblastine (Abrahamsen et al., 2013). HSP patient-derived cells had 50% the control level of spastin, a tubulin-severing enzyme, 150% of the control level of stathmin, a tubulin depolymerizing enzyme, and 50% of the control level of acetylated a-tubulin, an indicator of stabilized microtubules (Abrahamsen et al., 2013). HCS showed that patient-derived and control-derived ONS cells were highly sensitive to the drugs, with effects at 0.1 nM (**Figure 2**). Both drugs increased acetylated a-tubulin, but with different dose–response curves, and low doses of both drugs (~0.3 nM) restored patient cell acetylated a-tubulin to the control cell level (Abrahamsen et al., 2013).

These experiments show that disease-associated dysfunctions in olfactory cells can be ameliorated by candidate chemical compounds acting on targets known to be disrupted in the patient-derived cells compared to controls. The next challenge is to see whether ONS cells are useful for screening libraries of compounds. They have some of the necessary characteristics such as ease of generation, low cost, robust and repeatable growth characteristics and predictable phenotype. These properties make them useful for building up banks of cells that will allow assessment of variability of cell biology across a wider population of patients and controls, to discriminate disease-specific differences from individual differences in complex diseases like Parkinson's disease and schizophrenia.

FUTURE PROSPECTS

One of the challenges for the field is to develop robust and repeatable protocols for producing the large quantities of specified neurons or glia that are required for high throughput screening. For ESCs and iPSCs differentiating protocols exist for making different types of neurons, such as dopaminergic neurons, cortical neurons, and motor neurons (Chambers et al., 2009) but the yield is generally low and variable. This is not necessarily limiting for low-throughput investigations of disease-associated cell phenotypes and lead validation but is certainly limiting for primary drug screens. ONS cell production is not limiting for drug discovery but they currently also lack robust and repeatable protocols for differentiating them on demand into neurons and glia *in vitro*. In growth factor-free medium 50% of the cells were "astrocytes" [glial fibrillary acidic protein (GFAP)-positive cells], a proportion elevated by ciliary neurotrophic factor (Murrell et al., 2005). "Neurons" (β-tubulin-III-positive cells) were less frequent but rose to 25% of the population with nerve growth factor in the medium. (Murrell et al., 2005). Retinoic acid induced a majority (50%) of O4-positive "oligodendrocytes" (Murrell et al., 2005). ONS cell differentiation was defined in terms of morphology and immunofluorescence and so lacks the definitive demonstration of differentiation shown repeatedly for ESCs and IPSCs.

The concept of drug discovery through patient-derived stem cell models of brain diseases is attractive but has many other challenges apart from the practical issues of cost and reliable production. Concerns are raised about the epigenetic status of iPSCs and ESCs – epigenetic status is variably altered by reprograming and by culture methods (Stadtfeld and Hochedlinger, 2010) hence diseases in which epigenetics plays a role may not be modeled well by iPSCs and ESCs (Zhu et al., 2011; Maury et al., 2012). There is not yet a consensus about which cell type is the best for reprograming (Stadtfeld and Hochedlinger, 2010) or which method of reprograming is the most robust and reliable (Grskovic et al., 2011). Differentiation of human iPSCs is not yet routine and predictable and can take many weeks (Grskovic et al., 2011). There is discussion about the applicability of iPSCs to model late-onset diseases like Parkinson's disease or Alzheimer's disease, with the inference that iPSCs may be better suited to neurodevelopmental disease modeling (Juopperi et al., 2011). Olfactory stem cell models appear to be suited to late-onset and sporadic diseases perhaps because they are not subject to the genetic and epigenetic changes due to reprograming. Olfactory stem cells are relatively

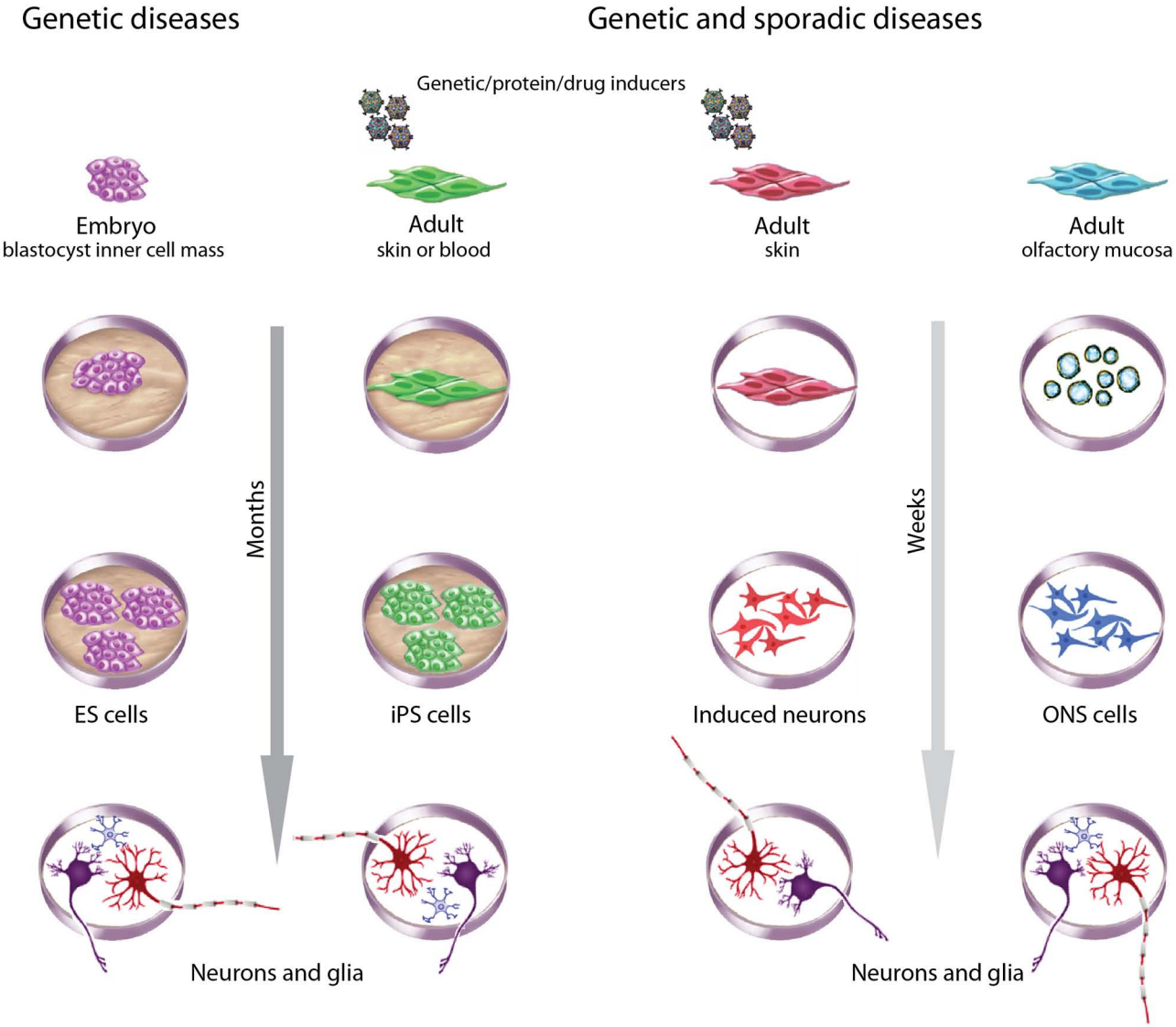

FIGURE 3 | Choices of stem cells to model brain diseases. Different criteria guide choices of cells to model brain diseases. ESCs derived during pre-implantation genetic diagnosis are useful for monogenic diseases. Patient-derived adult cells are useful for genetic and sporadic diseases, with the advantage of an associated clinical history. ESCs and iPSCs take many months to generate, validate, and then to produce neurons and glia but have the advantage of being highly proliferative and pluripotent. iPSCs and induced neurons require reprograming with genes, proteins or drugs, whereas ESCs and ONS cells do not. ONS cells and induced neurons may retain the methylation status of differentiated cells whereas ESCs and iPSCs do not. All methods introduce variability associated with cell culture but iPSCs and induced neurons may be more variable because of clonal selection due to the low efficiencies of the induction processes. ESC and ONS cell production average inter-clonal variation across large populations.

inexpensive and can be reliably grown in large quantities but they also suffer from difficulties in robust and routine differentiation protocols. As more diseases get modeled by iPSCs and ONS cells the strengths and weaknesses of each model will be determined for specific applications and the relevance of each model to the disease of interest will be determined. The use of particular cell models for drug discovery will depend on many variables including relevance to the disease, ease and cost of use, and stage of the drug discovery process (**Figure 3**). These questions and challenges are all signs of a field at the very beginning of its genesis and many will undoubtedly be resolved in the coming years.

Other developments in reprograming will affect this future. It is now possible to generate neurons directly from skin fibroblasts (Vierbuchen et al., 2010). This may greatly reduce the cost of producing patient-derived neurons for drug discovery because fibroblasts, like ONS cells are cheaper to generate, expand, and maintain than iPSCs. Through similar direct reprograming it may be possible to on demand specific classes of neurons, astrocytes, and oligodendrocytes. Ultimately whether any of these new technologies reach routine application in drug discovery will depend on cost, robustness, and industrial scalability as well as the biological validity of the cells as disease models.

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Generating regionalized neuronal cells from pluripotency, a step-by-step protocol

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Human pluripotent stem cells possess the potential to generate cells for regenerative therapies in patients with neurodegenerative diseases, and constitute an excellent cell source for studying human neural development and disease modeling. Protocols for neural differentiation of human pluripotent stem cells have undergone significant progress during recent years, allowing for rapid and synchronized neural conversion. Differentiation procedures can further be combined with accurate and efficient positional patterning to yield regionalized neural progenitors and subtype-specific neurons corresponding to different parts of the developing human brain. Here, we present a step-by-step protocol for neuralization and regionalization of human pluripotent cells for transplantation studies or *in vitro* analysis.

Keywords: human embryonic stem cells, pluripotent stem cells, differentiation, protocol, neuronal subtypes, regionalization, GSK3, brain development

INTRODUCTION

Given that the human brain is far more complex than brains of rodents, chicks, or invertebrate model organisms, it is clear that knowledge of human brain development cannot be fully extrapolated only from animal models. Therefore, studies on human fetal tissue in combination with a reliable human *in vitro* modeling system are required. Due to very limited access to fetal tissue at different developmental time points, human cell sources such as pluripotent cells are necessary to allow for comprehensive, dynamic analysis of human neural development, and to generate sufficient amounts of cells for future regenerative therapies (Kirkeby and Parmar, 2012).

Based on previously published methods for neural differentiation (Zhang et al., 2001; Perrier et al., 2004; Nat et al., 2007; Chambers et al., 2009; Fasano et al., 2010), we have developed and refined a standardized protocol for rapid neural conversion of pluripotent cells using defined culture settings free of feeder cells, matrigel, knockout serum replacement (KSR), and manual picking steps (Kirkeby et al., 2012). By mimicking developmental cues important for neural tube patterning in mammals, the cells can be efficiently patterned to regionalized cultures of a high purity. The protocol presented here is based on rapid neural conversion through dual SMAD inhibition (Chambers et al., 2009) and regionalization of the cells in this protocol constitutes a flexible system controlled by dose-dependent chemical inhibition of glycogen synthase kinase 3 (GSK3) for rostro-caudal patterning and dose-dependent activation of sonic hedgehog (SHH) and bone morphogenetic protein (BMP) signaling for dorso-ventral patterning (Kirkeby et al., 2012).

DIFFERENTIATION PROCEDURE

The protocol presented here will produce neural cells of a telencephalic fate in the absence of any patterning factors. To control rostro-caudal and dorso-ventral patterning, see separate section below. This protocol is preferably started on a Monday to avoid medium changes during the weekends (see overview of differentiation procedure in **Figure 1**). It is important to start the differentiation with healthy, pluripotent cells. All differentiated colonies should be removed from the culture before initiating differentiation.

Optional: If pluripotent cells are cultured on mouse embryonic fibroblasts (MEFs), colonies can be passaged onto matrigel in conditioned media or onto vitronectin in Essential 8 medium for 1 passage (3–7 days) before starting differentiation. This step will remove MEFs from the culture. Differentiation may also be initiated from colonies on MEFs, but if too many MEFs are present in the culture, this may result in attachment of embryoid bodies (EBs) to the bottom of the culture dish.

1. **Day 0**: Check that the colonies appear pluripotent by visual criteria, and if needed, remove differentiated colonies from the culture. Aspirate hESC media and wash cells once in PBS. Add minimal volume of EDTA (0.5 mM) to the cells (i.e., 0.5 ml for a 6-well) and incubate at 37°C for 5–8 min until colonies are easily washed off the dish with a pipette.
2. Gently pipette colonies off the dish and spin down in 10 ml culture medium at 200× g for 5 min.

NOTE: When dissociating cells with EDTA, a washing step is not necessary. Here, cells are spun down to avoid dilution of the differentiation medium with EDTA solution. Alternatively, colonies can be enzymatically dissociated with dispase, but this requires additional washing steps and may result in a lower cell yield.

3. To start differentiation, re-suspend the cells in differentiation medium: NIM + Y-27632 (10 μM) + SB431542 (10 μM) + noggin (100 ng/ml). The volume of differentiation medium will depend on the amount of starting material. In general, the volume of differentiation medium should be equivalent

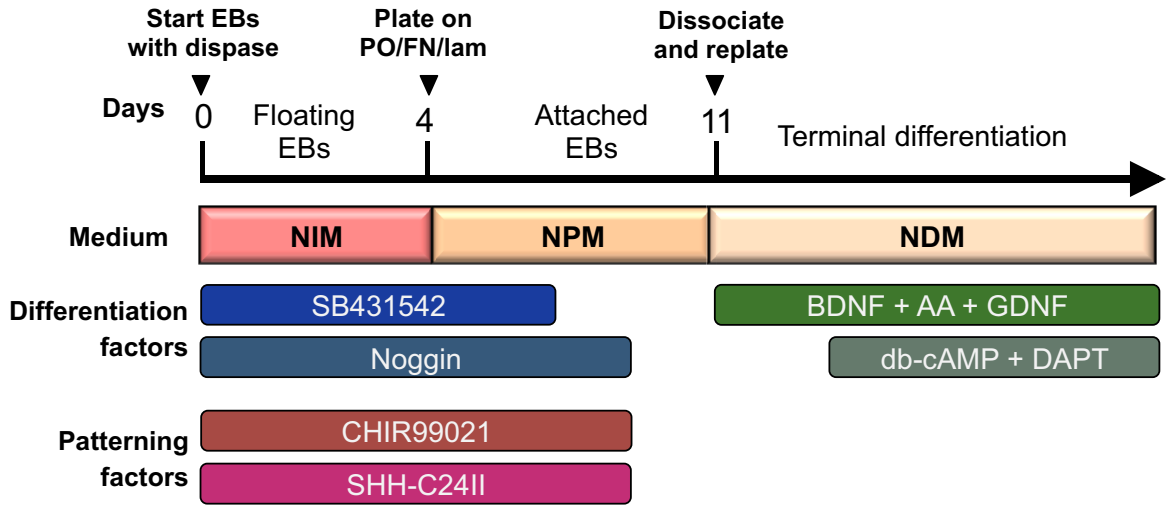

FIGURE 1 | Overview of differentiation protocol. Cells are differentiated as EBs for the first 4 days, and then cultured as attached colonies on coated plates with subsequent dissociation and replating at day 11. Neuralisation and patterning factors are present between day 0–9 (only SB431542 is withdrawn at day 7), and terminal differentiation is initiated from day 14 and onward with db-cAMP and DAPT.

to 1–1.5× the volume of growth medium used for the undifferentiated cells. That is, if starting with cells from 1 well of a 6-well plate, the differentiation volume should be 1.5–2.5 ml depending on the density of the cells. Plate cell suspension in non-treated cell culture plates for EB formation (i.e., 0.5 ml/well in a 24-well plate or 1 ml/well in a 12-well plate). Optional patterning factors can be added to the medium as needed (see section "Rostro-caudal and Dorso-ventral patterning of neural progenitors").

NOTE: ROCK inhibitor is added to the differentiation medium from day 0–2 to increase survival of EBs. If Fc-conjugated noggin is used, add 200 ng/ml noggin instead of 100 ng/ml. For some cell lines, 10 μM SB431542 may appear toxic, and the concentration should then be lowered to 2–5 μM. Start coating with poly-ornithine (PO) on this day.

4. **Day 2**: Transfer EB suspensions to a tube and spin down gently at 80× g for 5 min. Aspirate the medium and re-suspend the EBs in new NIM medium + SB431542 (10 μM) + noggin (100 ng/ml) + optional patterning factors using the same volume as on day 0. Return EBs to the wells they came from.

NOTE: If necessary, wash wells with medium to retrieve any EBs that have attached to the well bottom. If big aggregates of EBs are present, these should be broken up with a pipette. If medium is very yellow by day 2, increase the volume of differentiation medium. Coat plates with fibronectin and laminin (FN/lam) on this day.

5. **Day 4**: Transfer EB suspensions to a tube and spin down gently at 80× g for 5 min. Resuspend EBs in NPM medium + SB431542 (10 μM) + noggin (100 ng/ml) + optional patterning factors. The volume of differentiation medium should now be 2× the previous volume. Transfer EBs to wells coated with PO/lam/FN (see section "Reagent Setup") corresponding to a 1:2 split. If big aggregates of EBs are present, these should be broken up with a pipette before plating.
6. **Day 7**: Replace medium with NPM medium + noggin (100 ng/ml) + optional patterning factors. Coat with PO on this day.
7. **Day 9**: Replace medium with plain NPM medium without growth factors or patterning factors. Coat with FN/lam on this day.
8. **Day 11**: Replating: Wash cells with PBS, and add minimal volume of accutase to the wells. Leave cells in the incubator for 10 min or longer, until cells are easily suspended from the dish with a pipette. Dissociate cells with a pipette and spin down at 300× g for 5 min (before spinning, take an aliquot out for cell counting). Resuspend cells to a density of 5–15,000 cells/μl in NDM medium. Wash freshly coated plates with PBS and remove all liquid from the plates with a vacuum aspirator to achieve completely dry wells. Plate cells in droplets of 5–100 μl depending on future use. If many small wells are needed for immunocytochemistry, droplets of 5 μl can be plated in individual wells of 48-well plates to yield multiple replicates. If large amounts of cells are needed for transplantation or sorting, all cells can be plated in a single large droplet. Leave dishes in the incubator for 15–20 min to allow cells to attach to the well bottom. When attachment is verified in the microscope, add medium to the wells: NDM + BDNF (20 ng/ml) + GDNF (10 ng/ml) + AA (0.2 mM).

NOTE: The cell density at this point is critical for the survival and proper differentiation of the cells, and optimal density should be adapted for each individual purpose. The cells need to be maintained at a very high density to avoid cell death and selective loss of neural subtypes. On the other hand, if the cell density is too high, neuronal maturation will be attenuated.

9. **Day 14**: Change medium to NDM + BDNF (20 ng/ml) + GDNF (10 ng/ml) + ascorbic acid (0.2 mM) + db-cAMP (500 μM) + DAPT (1 μM). Maintain the cells in this medium from now and onward to induce terminal neuronal maturation.

NOTE: Cells are at an optimal stage for transplantation between days 14–20 of differentiation. If cells are to be used for transplantation, avoid adding DAPT to the medium, as this will result in premature neuronal maturation causing increased cell death upon dissociation.

Optional: If cells are needed for long-term studies of mature neuronal phenotype, it may be necessary to replate cells again between days 16 and 20 to avoid too high density of the cultures. In that case, perform replating using the same procedure as on day 11. Replating at later timepoints should be avoided due to stress on mature neuronal cells. At late stages of differentiation, neurons may begin to detach from the plate. This can be attenuated by adding lam + FN to the cell culture medium.

ROSTRO-CAUDAL PATTERNING OF NEURAL PROGENITORS

Rostro-caudal patterning of the cells can be controlled by dose-dependent addition of the GSK3 inhibitor CHIR99021/CT99021. The compound should be added to the cells from day 0 to 9 of differentiation at an appropriate concentration for the purpose needed. Use 0.2–0.4 μM for diencephalic fates; 0.6–0.8 μM for mesencephalic fates, 1–2 μM for anterior rhomencephalic fates and ≥4 μM for posterior rhomencephalic fates. Since different cell lines may show variations in the response to GSK3 inhibition, we recommend that each lab performs a dose-response curve of the CHIR99021 to determine the optimal concentration needed for generating the wanted cell type in their settings. Read section "Reagent Setup" for instructions on CHIR99021 preparation.

DORSO-VENTRAL PATTERNING OF NEURAL PROGENITORS

Ventralization of the cells can be obtained by adding Shh-C24II to the medium from day 0 to 9 of differentiation at any given concentration of CHIR99021. If no Shh is added to the culture, the cells will be enriched for alar plate fates. To enrich for basal plate, add 50–150 ng/ml, and to enrich for floor plate, add ≥200 ng/ml Shh-C24II. If more potent ventralization is wanted, purmorphamine (0.5 μM) can be added together with Shh from day 2 to 9. To achieve dorsalization of cultures toward roof plate fates, remove SB431542 and noggin from the cultures at day 4 to allow for BMP activation.

NOTE: Chemical compounds are generally used at lower concentrations in this protocol compared to protocols using knockout serum replacement (KSR) in the differentiation medium (Kriks et al., 2011). This may be due to a higher protein content and chemical buffering capacity in media containing KSR, necessitating higher concentrations of chemical patterning factors. Higher concentrations of chemical factors in this protocol may result in toxicity (purmorphamine shows beginning toxicity at 1 μM, CHIR99021 at 8 μM and DAPT at 3 μM in this protocol). Different batches of Shh-C24II may have different potencies, thus requiring adjustments in concentration.

MATERIALS (EUROPEAN CATALOG NUMBERS)

- Human embryonic stem cells or induced pluripotent stem cells of good quality with minimal spontaneous differentiation. Grown on mouse embryonic feeder cells or in defined medium on matrigel or vitronectin.
- EDTA 0.5 M pH 8.0 (15575–020). Dilute to 0.5 mM in PBS and store at RT.
- DMEM/F-12 (Invitrogen, cat. no. 21331–020).
- Neurobasal (Invitrogen, cat no. 21103–049).
- L-Glutamine (200 mM, Invitrogen, cat. no. 25030–081), aliquot and store at −20°C.
- Accutase (Invitrogen, cat. no. A11105–01), aliquot and store at −20°C.
- B27 supplement without vitamin A (Invitrogen, cat no 12587–010), aliquot and store at −20°C.
- N2 supplement (Invitrogen, cat no 17502–048), aliquot and store at −20°C.
- SB431542 (TGFβ inhibitor, Tocris Bioscience, cat. no. 1614 or Axon Medchem, cat. No. 1661). Make stock aliquots of 20 mM in DMSO and store at −20°C.
- Y-27632 dihydrochloride (ROCK inhibitor; Tocris Bioscience, cat. no. 1254 or Axon Medchem 1683). Make stock aliquots of 10 mM in H_2O and store at −20°C.
- CHIR99021/CT99021 (GSK3 inhibitor; Axon Medchem, cat no 1386). Make stock aliquots of 10 mM in DMSO and store at −20°C.
- Recombinant human Noggin (R&D Systems, cat. no. 6057-NG)*.
- Recombinant human BDNF (R&D Systems, cat. no. 248-BD)*.
- Recombinant human GDNF (R&D Systems, cat. no. 212-GD)*.
- Recombinant modified human Sonic Hedgehog C24II (R&D Systems; cat. no.1845-SH-025)*.
- Purmorphamine (Smoothened agonist; Stemgent 04-0009). Make stock aliquots of 10 mM in DMSO and store at −20°C.
- Ascorbic acid (Sigma, cat. no. A5960). Make stock aliquots of 200 mM in H_2O and store at −20°C.
- Dibutyryl-cAMP (Sigma cat no. D0627). Make stock aliquots of 50 mM in H_2O and store at −20°C.
- DAPT (γ-secretase inhibitor; Tocris Bioscience, cat no. 2634). Make stock aliquots of 10 mM in DMSO and store at −20°C.
- Polyornithine (Sigma cat no. P3655). Make sterile filtered stock aliquots of 1.5 mg/ml in H_2O and store at −20°C.
- Laminin (Invitrogen cat no. 23017-015). Aliquot and store at −80°C.
- Fibronectin (Invitrogen cat no. cat no 33010-018). Make stock aliquots of 0.5 mg/ml in PBS + 5 mM NaOH and store at −20°C.
- Non tissue-culture treated multiwell plastic plates (12- or 24-wells).
- Tissue-culture treated multiwell plastic plates (12-, 24-, or 48-well).
- PBS.
- Sterile water.

*For protein growth factors, make stock solutions in PBS + 0.1% BSA and store at −20°C.

REAGENT SETUP

Neural induction medium (NIM):

DMEM/F-12: Neurobasal (1:1)
1× N2 (1:100)
1× B27 (1:50)
2 mM L-Glutamine (1:100)

Neural proliferation medium (NPM):

DMEM/F-12: Neurobasal (1:1)
0.5× N2 (1:200)
0.5× B27 (1:100)
2 mM L-Glutamine (1:100)

Neural differentiation medium (NDM):

Neurobasal
1× B27 (1:50)
2 mM L-Glutamine (1:100)

POLYORNITHIN/LAMININ/FIBRONECTIN (PO/lam/FN) COATED DISHES

For coating, dilute PO stock solutions 1:100 in H_2O to yield a final concentration of 15 μg/ml. Add the solution to wells and incubate at 37°C for 48 h (i.e., 0.2 ml/cm^2 = 350 ul in a 24-well dish and 700 in a 12-well dish). After 48 h, aspirate the PO solution and wash three times in sterile H_2O. Prepare FN + lam solution by adding 1:100 of FN (0.5 mg/ml) to PBS in a 50 ml tube. Swirl the tube well to ensure that FN is in solution (ensure that is does not form a precipitate or sticks to the tube wall). Then place the tube at 4°C for 15–30 min to cool down the solution. In the meantime, thaw an aliquot of laminin at 4°C. When the PBS-FN solution is cold, add laminin to the tube to yield a final concentration of 5 μg/ml for each. Swirl the tube. Immediately add the FN/lam solution to the washed PO-coated wells at 0.2 ml/cm^2 and incubate at 37°C for 48–72 h. Wash the plates twice in PBS prior to plating the cells. Plates incubating with FN/lam solution can be left in the incubator for up to 1 week before use.

DILUTION OF CHIR99021/CT99021

Since patterning with CHIR99021 is extremely concentration dependent, it is important that the compound is diluted in exactly the same way for every experiment. The compound should be kept frozen and exposure to light should be minimized to avoid degradation.

- Keep CHIR99021 stock aliquots (10 mM in DMSO) at −20°C.
- These aliquots can be thawed and used up to three times, and in between they should be stored at −20°C.
- For use in experiments, thaw the frozen aliquot and dilute 3 μl in 297 μl medium (1:100) to yield a 100 μM solution. Aim at always using the same pipettes for dilution to avoid drifts in concentration between experiments. Always use freshly prepared dilutions for experiments.
- Mix well and add the 100 μM solution to the cells to yield the desired concentration (i.e., for 0.8 μM final concentration, add 8 μl solution to 1 ml of medium).

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Cortical interneurons from human pluripotent stem cells: prospects for neurological and psychiatric disease

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Cortical interneurons represent 20% of the cells in the cortex. These cells are local inhibitory neurons whose function is to modulate the firing activities of the excitatory projection neurons. Cortical interneuron dysfunction is believed to lead to runaway excitation underlying (or implicated in) seizure-based diseases, such as epilepsy, autism, and schizophrenia. The complex development of this cell type and the intricacies involved in defining the relative subtypes are being increasingly well defined. This has led to exciting experimental cell therapy in model organisms, whereby fetal-derived interneuron precursors can reverse seizure severity and reduce mortality in adult epileptic rodents. These proof-of-principle studies raise hope for potential interneuron-based transplantation therapies for treating epilepsy. On the other hand, cortical neurons generated from patient iPSCs serve as a valuable tool to explore genetic influences of interneuron development and function. This is a fundamental step in enhancing our understanding of the molecular basis of neuropsychiatric illnesses and the development of targeted treatments. Protocols are currently being developed for inducing cortical interneuron subtypes from mouse and human pluripotent stem cells. This review sets out to summarize the progress made in cortical interneuron development, fetal tissue transplantation and the recent advance in stem cell differentiation toward interneurons.

Keywords: cortical interneurons, embryonic stem cells, cell therapy, proof-of-principle fetal transplant, seizure

INTRODUCTION

The complex circuitries of the cerebral cortex comprise networks produced by two major neuronal cell types: the excitatory glutamatergic projection neurons (Pyramidal cells) and gamma-aminobutyric containing (GABAergic) interneurons. Pyramidal neurons are the primary neural cells that specialize in transmitting information between different cortical regions and to other regions of the brain. Interneurons on the other hand represent a minority (∼20%) of the entire neocortical neuronal population (Whittington and Traub, 2003; Wang et al., 2004; Hensch, 2005). GABAergic interneurons are highly heterogeneous and had been overlooked for many years due to their vast complexities. However, it is thought that they play a vital role in the function of the cerebral cortex. Interneurons provide inhibitory inputs that shape the responses of pyramidal cells and prevent runaway excitation. They regulate the timing and synchronization of population rhythms expressed as cortical oscillations (Haider et al., 2006; Klausberger and Somogyi, 2008). Consequently, reduction in tonic inhibition through interneuron hypoplasia in the cortex has been implicated in major neurological and psychiatric illness, including epilepsy, mental retardation, autism, and schizophrenia (Powell et al., 2003b; Lewis et al., 2005; Akbarian and Huang, 2006; Yizhar et al., 2011).

Interneurons exhibit a remarkable intrinsic ability to migrate and therefore offer a potential neuronal source for cell-based therapies for treating the above mentioned interneuron deficiencies. Recently, several elegant works have shown that transplanting fetal interneuron precursors can create a new critical period of plasticity in the recipient brain and reduce seizures in animal models of epilepsy (Wichterle et al., 1999; Alvarez-Dolado et al., 2006; Baraban et al., 2009). Interneuron transplants can also reduce movement deficits in a rat model of Parkinson's disease (Martinez-Cerdeno et al., 2010). When transplanted into the spinal cord, interneurons also help decrease pain sensation (Braz et al., 2012). Together, these proof of principal studies raise hope for the possibility of using neuronal transplantation to treat diseases like epilepsy and Parkinson's.

In this review, we provide a brief overview of the development and function of cortical interneurons. We then evaluate the current progress of interneuron production from pluripotent stem cells. Finally, we discuss the potential and the challenges of exploiting stem cell technologies for modeling neurological and neuropsychiatric disorders, drug discovery and cell therapy.

CLASSIFICATIONS OF CORTICAL INTERNEURONS

Cortical interneurons are cells that connect only with nearby neurons, to distinguish them from "projection" neurons, whose axons span to more distant regions of the brain. Interneurons typically express the inhibitory neurotransmitter gamma-amino butyric (GABA) and have aspiny dendrites (Markram et al., 2004). Despite these common features, interneurons display huge diversity in morphology, physiology, and marker expression. Here we

provide a brief summary of the complex classification of cortical interneurons. For a thorough evaluation of the subject, refer to the excellent reviews by Ascoli et al. (2008) and Batista-Brito and Fishell (2009).

Efforts to distinguish interneurons based on morphology have led to complex conclusions. Classifications based on synaptic targets of interneurons, i.e., axon, soma, proximal dendrite, or distal dendrite, provide a useful starting point (DeFelipe, 1997). This classification defines the role of the interneuron in the microcircuit. Interneurons that form synapses on pyramidal cell axons have the potential to eradicate action potential transmission; conveying power to this subtype after sensing the global excitatory environment. This is in contrast to interneurons that contact distal dendrites of pyramidal cells that may influence signal formation in more subtle ways, e.g., necessitating summation of input signals (Markram et al., 2004). Interneuron groups that have dendritic connections are more numerous and diverse than those on the soma and axons due to the intricacies involved in these roles.

It should be noted that not all GABAergic neurons in the cortex are local interneurons with short-range associations. Approximately 0.5% of cortical GABAergic cells are "projection" neurons with long-range axons. The function of these cells in the mature cortex remains uncertain (Tamamaki and Tomioka, 2010).

The branching morphology of an interneuron and its cortical layer position can enable classifications into interneuron type. Some of these cells were first described by the monumental work by Cajal y Ramon over a century ago (DeFelipe, 2002; Sotelo, 2003). The groups include large basket cells, small basket cells, nest basket cells, chandelier cells, Martinotti cells, bitufted cells, bipolar cells, and double bouquet cells. This information is also relevant for the synaptic targets of the cells discussed above (Ascoli et al., 2008).

Interneurons can be classified based on their electrophysiological properties, explored in slice culture studies. Classes such as fast-spiking, non-adapting non-fast-spiking, adapting, irregular spiking, intrinsic burst firing, and accelerating have been described (Ascoli et al., 2008). It is important to note that the morphology and electrophysiological properties of the cells do not correlate directly, adding further complexity to the classification problem. In an elegant study, Toledo-Rodriguez and colleagues performed electrophysiological investigations followed by single cell expression analysis (Toledo-Rodriguez et al., 2004). Interestingly, they found individual genes that code ion channels which are predictive of electrophysiological class, for example *Kv1.1*, *HCN1,* and *Caα1A*; potassium, sodium and calcium channel proteins respectively. This may become critical for grouping interneurons into discrete subtypes.

Finally, interneurons can be distinguished based on expression of calcium binding proteins and neuropeptides. It has become convenient to distil the cells into three groups based on expression of Parvalbumin, Somatostatin, and Calretinin, which largely do not overlap (Kubota et al., 1994; Kawaguchi and Kubota, 1997). There are a number of other markers that may be used to distinguish interneuron subclasses. These include Calbindin, a calcium binding protein often used to describe cortical interneurons. Calbindin is coexpressed with Parvalbumin or Somatostatin in up to 80% of interneurons, making Calbindin an inappropriate marker (Kawaguchi and Kubota, 1997). Additionally, Neuropeptide Y (NPY) and Vasoactive Intestinal Polypeptide (VIP) are useful markers proteins, although these can display some colabelling with Calretinin positive cells. All of these expression markers may have little functional relevance; however, it has become clear from developmental biology that these classes originate in distinct locations, producing a useful fate map for developmental biologists. Again, there is no complete overlap between morphology, physiology and these expression-based classes.

The extensive variability of interneurons and the complexity in defining subclasses has led to the theory of an interneuron continuum, negating the presence of distinct subclasses (Parra et al., 1998). The study by Toledo-Rodriguez, demonstrating clustering of subgroups dependent upon voltage gate channels, argues against this continuum and encourages new techniques to classify subgroups. The field of stem cell biology utilizes information from developmental biology to mirror development *in vitro*. For this reason, it is convenient to employ developmental origin-based division in this review, i.e., Parvalbumin, Somatostatin, and Calretinin.

DEVELOPMENT OF CORTICAL INTERNEURONS

Although distributed dorsally in the cerebral cortex of the mature brain, cortical interneurons are derived from neural precursors generated in the ventral forebrain (telencephalon) and undergo major tangential migration to their dorsal target tissues. Fate mapping studies via isochronic, homotopic transplantation of labeled ventral forebrain tissues *in utero* and *in vitro* have demonstrated the vast migratory capacity of ventral progenitors and their ability to form GABAergic interneuron subtypes (Anderson et al., 1997; Lavdas et al., 1999; Nery et al., 2002).

The ventral telencephalon (also referred to as the subpallium) is divided into three neurogenic domains, the lateral- medial- and caudal-ganglionic eminences (LGE, MGE, and CGE respectively), see **Figure 1**. The LGE is the birthplace of the striatal projection neurons and a small population of olfactory bulb interneurons that migrate rostrally (Waclaw et al., 2009). The MGE and the CGE are the major sites of interneurogenesis, shown via the transplantation of labeled tissue (Xu et al., 2004; Butt et al., 2005). The CGE has been described as a caudal extension of the LGE and the MGE and as such the three tissues share many common gene expression profiles (Flames et al., 2007). For example *Gsx2*, *Dlx2,* and *Mash1* are three transcription factors involved in neurogenesis, patterning and migration and are expressed throughout the ganglionic eminences. Despite this similarity, there are genetic differences and precise expression domains that are starting to be described (Flames et al., 2007; Willi-Monnerat et al., 2008). Most importantly, the expression of *Nkx2.1*, a Shh responsive gene (Xu et al., 2010), defines the MGE and the ventral CGE, discerning these tissues from the LGE and the dorsal CGE (Sussel et al., 1999).

Nkx2.1 expression is vital for MGE-based interneurogenesis, whereby its knockout reduced GABAergic populations in the cortex by 50% (Sussel et al., 1999). *Nkx2.1* has a critical function to induce *Lhx6*, a gene which is required

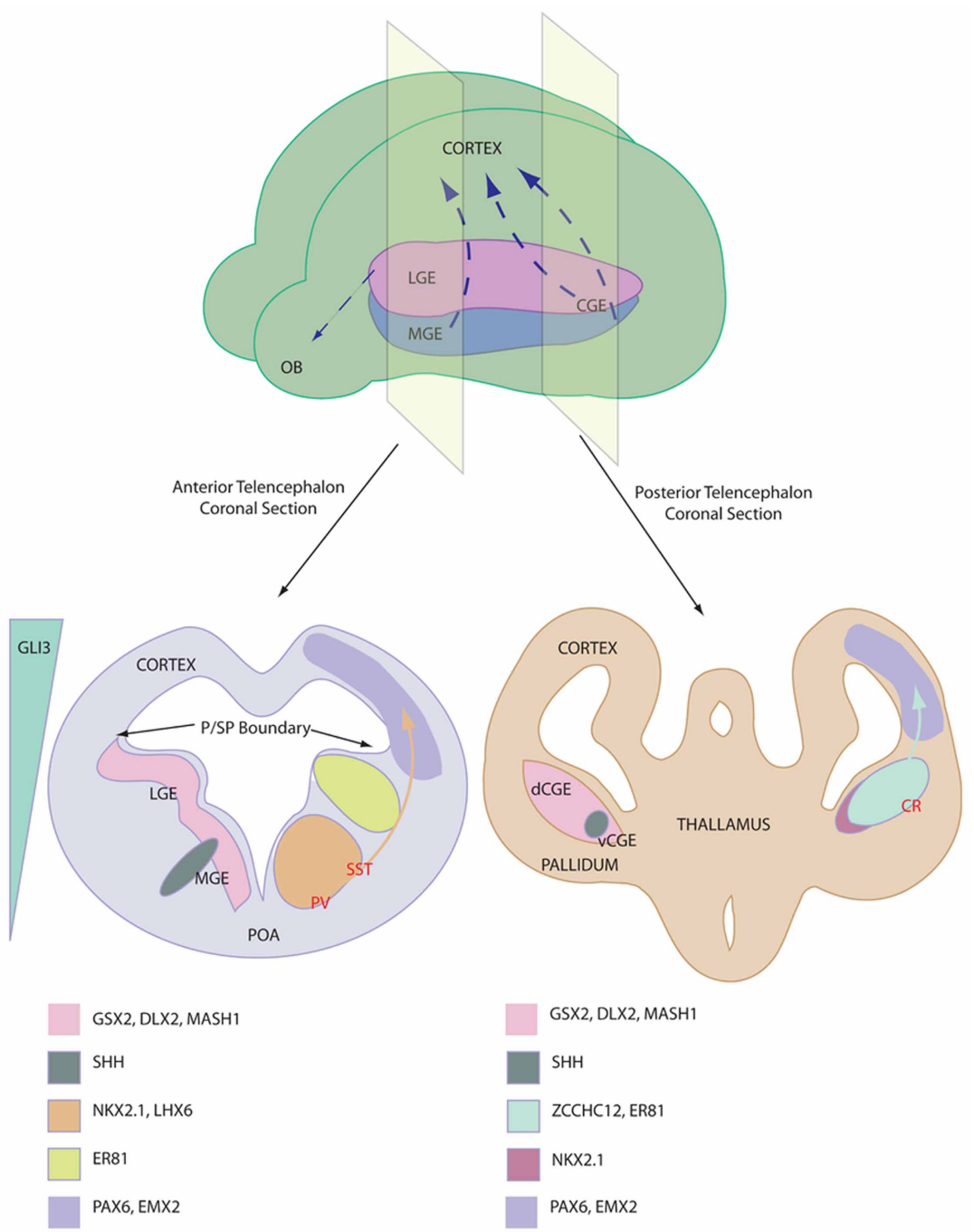

FIGURE 1 | Depiction of interneuron birth place and relevant gene expression profile. Top shows ganglionic eminence distribution in a mouse brain and the major interneurons migration streams, NB CGE is a caudal extension of the more anterior tissues. **Bottom** shows anterior (left) and posterior (right) coronal sections through the telencephalon of an E13 mouse. Pertinent marker gene expression profiles are depicted of the proliferative niches of the ganglionic eminences. Relative origin of interneuron subtypes are shown in RED. Note the Shh expression domains and the reciprocal Gli3 expression. Abbreviations; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence (v ventral and d dorsal); OB, olfactory bulb; P/SP, pallial subpallial; POA, preoptic area; PV, Parvalbumin; SST, Somatostatin; CR, Calretinin.

for specification and migration of MGE-derived GABAergic interneurons (Du et al., 2008). After migrating to the cortex, these *Shh/Nkx2.1/Lhx6*-patterned progenitors mature *in situ* into Parvalbumin and Somatostatin expressing cortical interneurons. Interneuron specification is origin specific (Butt et al., 2005) and different regions of the MGE are thought to give rise to the two cell types. Somatostatin-expressing interneurons originate in the more lateral MGE, where Shh expression is higher, whereas

Parvalbumin-expressing interneurons are derived from the more ventral MGE domain (Wonders et al., 2008; Xu et al., 2010; Inan et al., 2012). FGF signaling has also been implicated in ventral forebrain development, as receptor knockout leads to aberrant development (Storm et al., 2006). It should be noted that the MGE gives rise to other cell types, including striatal interneurons, cholinergic cells and glia, displaying distinct marker profiles.

Calretinin-expressing interneurons are unaffected by *Nkx2.1* knockout (Sussel et al., 1999) and were subsequently demonstrated to be derived in the dorsal CGE (dCGE) (Xu et al., 2004). These interneurons are dependent upon early expression of *Gsx2*, which is expressed higher in the dCGE and LGE compared with the MGE. Recent work has proposed a novel role for Activin signaling in inducing *Gsx2* and Calretinin-expressing interneurons in mouse and human embryonic stem cells (ESCs) (Cambray et al., 2012). Additionally, NPY-expressing cells are specified in the CGE in an undefined manner. Reports of the presence of Parvalbumin and Somatostatin immunoreactivity in the CGE can be attributed to progenitors migrating through the CGE from the MGE. In addition to the cortex, CGE-derived progenitors contribute strongly to interneuron populations in the hippocampus (Nery et al., 2002).

Once specified in the early subpallium, the interneuron progenitors migrate to their target tissues in the upper layers of the cortex or the hippocampus (Miyoshi and Fishell, 2011). This migration is dependent upon *Dlx1/2* and *Mash1* expression, as progenitors accumulate in the ventral forebrain in mouse knockout models of these genes (Long et al., 2009) and cortical tissues exhibit a 75% reduction in GABAergic cell numbers (Anderson et al., 1997). The intricacies of the interneuron migration are not well understood with a multitude of undefined factors directing this complex process (Faux et al., 2012). However, it appears that the cells do not follow corticofugal fibers toward target locations (Nery et al., 2002). Many contact the cortical ventricular zone together with newly-born cortical cells before migrating radially to their target laminae (Nadarajah et al., 2002). Interestingly, the different subtypes of cortical interneuron have differential target tissues, with respect to gross domains in the cortex as well as layer preference (Nery et al., 2002). Temporal differences in migration capacity have been described and cortical interneurons are believed to migrate in an "inside-out" pattern, with deep layer cells migrating earlier in development. This may have important implications on transplantation and is discussed below.

These above observations of cortical interneuron development have been based in rodent models. Recent data has reinforced the conserved nature of interneuron birth and migration in human development. For example, Calretinin progenitors have been shown to originate in the human basal forebrain (Jakovcevski et al., 2011; Zecevic et al., 2011) and loss of the ventral forebrain in the human fetus due to stroke leads to reduced numbers of Somatostatin and NPY-expressing interneurons (Fertuzinhos et al., 2009). These studies also indicated that there are critical differences between human and rodent development. Rodent interneurons do not express *Nkx2.1* during migration, whereas human progenitor cells were shown to maintain NKX2.1 expression into the cortex (Rakic and Zecevic, 2003). Here there is an additional mitotic event before the cells migrate radially to their final positions (Letinic et al., 2002). This may add to the complexity, size and additional cortical layering evident in the higher mammalian cortex and is a feature repeated in simian development (Petanjek et al., 2009). Therefore, generalization and inferences for human development based on murine data must be taken with caution; however, evidence does suggest the presence of many parallels between the two developmental systems.

Thus, interneurons are an extremely diverse population. Some diversity can be attributed to differential developmental origins, different morphogen responsiveness and fate determinant expression. This data becomes useful for stem cell biologists to parallel development *in vitro*.

INTERNEURON DYSFUNCTION AND DISEASES

Deficiencies in cortical interneuron numbers may arise from brain trauma, viral infections or gene mutations effecting function or migration; reviewed in Rossignol (2011). The "GABA hypothesis" states that a reduced level of tonic inhibition in the cortex or hippocampus leads to over-excitability and a spread of seizure (Cossart et al., 2001). Therefore, interneuron dysfunction has been associated with epilepsy, autism (a disease characterized by seizure) (Hussman, 2001), Tourette's syndrome, schizophrenia, and anxiety disorders (Lewis, 2000).

After initial epileptogenesis, there are a number of physiological alterations in the local cellular environment that contribute to chronic disease progression. This includes aberrant neurogenesis, alterations in cellular distributions, and gliogenesis (Maisano et al., 2009). One example is the recruitment of astrocytes to the focal site of seizure that express adenosine kinase; a molecule that degrades the anticonvulsant adenosine and potentiates chronic seizures (Gouder et al., 2004).

There are a number of animal models for seizure-based disease. Overstimulation via the chemoconvulsant substances kainic acid or pilocarpine leads to acute seizure and development of spontaneous seizures over time (Maisano et al., 2009). Additionally, there are a number of genetically altered paradigms that lead to interneuron deficiencies, many of which are neonatal lethal. Loss-of-function mutations of *Shh* or *Nkx2.1* lead to improper patterning of the ventral telencephalon and a lack of interneuron generation (Chiang et al., 1996; Sussel et al., 1999). *Dlx1/2* knockout mice exhibit a defect in migration (Long et al., 2009). Mutations of the homeobox gene *ARX* in human subjects have demonstrated an interneuron migration defect and patients display autism and epilepsy. *ARX* is regulated by *DLX2* (Friocourt and Parnavelas, 2010). Mutations in *uPAR*, the receptor for hepatocyte growth factor/scatter factor (HGF/SF), lead to aberrant migration of Parvalbumin cell precursors specifically and the adult mice display increased anxiety compared to littermates (Powell et al., 2003a). Finally, DISC1 (Disrupted-in-Schizophrenia-1) represents a factor crucial for neurite outgrowth and mutations in this gene can lead to schizophrenia in patients (Kamiya et al., 2005; Ayhan et al., 2011).

In addition to seizure-based diseases, interneuron deficiencies have been implicated in schizophrenia; whereby a reduction in GABAergic synapses by 40% have been noted in the post mortem schizophrenic prefrontal cortex (Lewis, 2000).

Also, similar studies revealed reductions in most interneuron markers in developing schizophrenic brains (Fung et al., 2010). Interneurons have also been implicated in bipolar disorder, anxiety disorders and Tourette's syndrome (Jetty et al., 2001; Kalanithi et al., 2005).

PROOF-OF-CONCEPT TRANSPLANTATION STUDIES

One third of epileptics are resistant to current drug treatments and all antiepileptics have major side-effects (Loring et al., 2007; Loescher et al., 2008). Surgical resection of the seizure focal point can be beneficial but depends on the function of the brain location and is therefore only possible in a subset of patients. Therefore, novel therapeutic avenues are of great interest.

The potential for cell replacement therapeutic strategies for treating interneuronopathies has been demonstrated in the literature and is summarized in **Table 1**. The first critical evidence that promoted ventral forebrain-derived GABAergic cells was the extensive migrational capacity of grafted interneurons after cortical transplantation. Compared with LGE and cortically-derived cells that show little migration, MGE derivatives migrate several millimeters into the cortex (Wichterle et al., 1999). These grafted cells have the ability to differentiate into interneurons that express the correct markers, display characteristic physiological features and integrate to provide inhibitory synaptic activity (Alvarez-Dolado et al., 2006).

To investigate the potential of MGE-derived tissue to reverse the diseased state, several studies have grafted fetal GABAergic precursors into seizure model rodents. Typically MGE tissue from E12.5–E14.5 mouse embryos (a time of major interneurogenesis in the mouse embryo) is dissociated and grafted into the cortex (Xu et al., 2004). Baraban and colleagues demonstrated that wild-type MGE-tissue grafted into the cortex of a Shaker-like model of epilepsy (*Kv1.1* knockout mice) reduced the number and duration of spontaneous seizures 30 days after transplantation (Baraban et al., 2009).

The convulsive drug 4-aminopyridine (4-AP) is able to elicit focal ictal-like events due to evoked neurotransmitter release. Transplantation of E13.5 MGE cells are able to reduce the power of the seizure 2.5–8 weeks post grafting (De la Cruz et al., 2011). Interestingly, there was no relationship between graft effectiveness and the number of MGE cells transplanted in this study.

Maximum electroconvulsive shock (MES)-induced epileptic rodents have unaltered brain circuitry and so represent a good model for chronic epilepsy. Rodents that received MGE grafts in this model displayed a higher threshold to MES and a lower mortality rate 2 months after grafting (Calcagnotto et al., 2010a). Kainic acid-induced mouse models of chronic temporal lobe epilepsy have been grafted with neural stem cells (NSCs) expanded from E14 MGE tissues. Cells were grown *in vitro* using EGF and FGF2 before being transplanted into rodent hippocampus. The authors observed reduced frequency and severity of seizures three months after transplantation, although cognitive function (learning and memory) was not improved (Waldau et al., 2010).

Injection of neurotoxin-conjugated Substance P (SSP-Sap), that targets interneurons expressing the Substance P receptor NK-1, has been used to selectively degrade interneurons in the hippocampus (Martin and Sloviter, 2001). This leads to reduced numbers of Somatostatin-, NPY- and NK-1-expressing interneurons while the Calretinin and Parvalbumin

Table 1 | Transplantation of fetal-derived interneuron precursors.

Author	Transplanted tissue	Model	Results
Baraban et al., 2009	E13.5 MGE	Kv1.1 mutant mouse Epilepsy model	GABAergic cells, increased GABAergic synapses. PV, SST, CR, NPY Reduced episodes of seizure and duration 30 days post-transplant
Calcagnotto et al., 2010b	E12.5 MGE tissue	SSP-Sap injected cortex	Restored inhibition within cortex
Calcagnotto et al., 2010a	MGE tissue	Maximum electroconvulsive shock model	Reduced seizure and decreased mortality PV, CR, NPY, 2 months after graft
De la Cruz et al., 2011	E13.5 MGE tissue (ventral vs. dorsal)	Ictal discharges induced by 4-AP	Attenuated propagation of ictal discharges. 2.5–8 weeks after transplant
Waldau et al., 2010	NSCs expanded from E14 MGE	Kainic acid induced epilepsy	Reduced frequency, duration and severity of seizure 3 months after graft
Zipancic et al., 2010	E12.5 MGE	SSP-Sap	Repopulate hippocampus after 2 months, Reduced seizure severity and mortality
Alvarez-Dolado et al., 2006	E12.5–E13.5		Evident interneuron morphology, expression, physiology. Increased GABAergic synaptic activity on pyramidal neurons Up to after 60 days

Details of tissue source and preparation, animal model and a summary of the results are described. Abbreviations: MGE, medial ganglionic eminence; NSC, neural stem cell; 4-AP 4-aminopyridine; PV, Parvalbumin; SST, Somatostatin; CR, calretinin; NPY, neuropeptide Y.

subpopulations unaffected or slightly reduced, respectively. The group of Alvarez-Dolado demonstrated that E12.5 MGE-derived cells were able to repopulate the SSP-Sap treated hippocampus two months after grafting and increase inhibitory synaptic events on hippocampal pyramidal cells. This was associated with reduced mortality rate and seizure severity (Calcagnotto et al., 2010b; Zipancic et al., 2010).

Other cell sources have been shown to have potential benefit in models of interneuron disease. Firstly, NSCs derived from 15 week human fetal tissue were injected into the rat tail vein of a pilocarpine model. GAD67 expressing cells were detected in the recipient hippocampus which was accompanied by reduced pyramidal cell excitability (Chu et al., 2004). Neonatal hippocampal-derived NSCs have displayed an ability to differentiate into neurons and glia (Shetty et al., 2008). However, these cells have limited GABAergic potential, especially compared to ventral-forebrain-derived NSCs (He et al., 2001).

Other strategies tested to reverse epilepsy include transplanting cells constitutively expressing GABA to replace the inhibitory deficiency (Gernert et al., 2002; Thompson and Suchomelova, 2004; Thompson, 2005), or to inhibit the action of adenosine kinase which degrades the anticonvulsive drug adenosine in the injured brain (Huber et al., 2001; Guttinger et al., 2005; Ren et al., 2007; Li et al., 2008). These strategies showed limited benefits as the cells do not integrate into the cortical circuits and are not activity dependent; features that may be required to reduce seizures. Finally, ventral telencephalic tissue grafted into the ventral midbrain [substantia nigra (SN) pars reticulata] also demonstrated a reduction in seizure propagation (Loscher et al., 1998). This tissue has been implicated seizure propagation in various epilepsy models.

Although interneuron dysfunction has been implicated in neuropsychiatric diseased states such as anxiety disorders and attention deficit disorder, the potential use of cellular replacement therapy has not been addressed (Jetty et al., 2001; Boy et al., 2011; Edden et al., 2012).

INTERNEURONS FROM PLURIPOTENT STEM CELLS

Human ESCs and iPSCs can be differentiated into any somatic cell type and offer an unlimited supply of medically important cells (Thomson et al., 1998). These cells may be employed to elucidate disease etiology and develop novel therapies, including putative cell replacement strategies. The goal of stem cell research is to understand lineage and cell fate specification in normal development in order to produce disease-relevant and functional post mitotic cells. Several lines of study have employed morphogenic conditions capable of directing mouse and human ESCs toward a ventral telencephalic fate. However, when compared to some other neuronal cells, such as midbrain dopamine neurons (Jaeger et al., 2011; Kriks et al., 2011), relatively little is known on how to control human pluripotent stem cell differentiation into distinct cortical interneuron subtypes (for current progress see **Table 2**).

Ruschenschmidt et al. described the first ESC-derived neurons to be grafted into pilocarpine-treated chronically epileptic rats (Ruschenschmidt et al., 2005). Mouse ESCs were differentiated in aggregates termed embryoid bodies (EBs) in a neural albeit undirected manner. The resultant neurons, presumably of mixed identity, were transplanted into the hippocampus of epileptic rats. The grafts exhibited neuronal properties but did not produce any functional recovery and there was no extensive migration into the host brain, features that might be expected with interneuron-rich grafts (Wichterle et al., 1999).

Subsequent studies attempted a more directed differentiation scheme, i.e., toward a telencephalic progenitor fate. These studies demonstrate that, in serum-free conditions, neural precursors with a rostral identity can be generated from both human and mouse ESCs using an EB differentiation method (Watanabe et al., 2005, 2007). Telencephalic progenitors can also be generated using adherent monolayer-based differentiation protocols together with SMAD inhibitors in a manner parallel to the Anterior Neuroectoderm Ridge instructed development *in vivo* (Ying et al., 2003; Chambers et al., 2009).

Dorsoventral patterning of ESC-derived forebrain precursors by morphogenic gradients appears to mimic that observed during normal development. Shh is a strong ventralizing factor throughout the neural tube (Ericson et al., 1995) and specifies MGE identity through induction of *Nkx2.1*. Studies that applied graded dosage of Shh to telencephalic progenitor cells showed that a high concentration of Shh (or Shh agonists) is able to induce *Nkx2.1* and results in characteristic interneuron marker expression in ESC-derived neural cultures (Watanabe et al., 2005, 2007; Gaspard et al., 2008; Li et al., 2009; Danjo et al., 2011; Ma et al., 2012). All these studies demonstrated the instructive capabilities of Shh to promote MGE-like identity at high dose and an LGE-like fate at lower doses. Cortical fate was induced when Shh signaling was antagonized. It should be noted that *Nkx2.1* is also expressed in the ventral diencephalon and so is not specific to MGE-derived interneuron progenitors (Ohyama et al., 2005).

A number of studies have investigated the functionality of *in vitro* derived interneurons and performed grafting studies of ESC-derived cultures into rodent model organisms. Maroof et al. utilized an EB based differentiation scheme followed by Shh treatment to produce ventral telencephalic-like cells from mouse ESCs (Maroof et al., 2010). Using an Lhx6-reporter that specifically marks the migrating MGE-derivatives, the authors purified putative interneurons by FACS and grafted the GFP expressing cells into the adult mouse cortex. They observed extensive cellular migration and survival. Graft-derived cells exhibited classical interneuron marker expression (predominantly Parvalbumin and Somatostatin) and electrophysiological properties nine months post-transplantation.

Maisano et al. subsequently produced interneuron-like cells from mouse ESCs via adherent monolayer differentiation (Maisano et al., 2012). To promote ventral telencephalic differentiation, the authors used a Shh agonist and witnessed faithful differentiation *in vitro*. Following transplantation into the hippocampus of a pilocarpine-based mouse model for temporal lobe epilepsy, the neural precursors reliably formed GABAergic neurons, replacing those lost in this model. These cells were physiologically indicative of GABAergic cells and expressed Parvalbumin, Calbindin, and Calretinin but not Somatostatin. This may be explained by the sensitivity of Somatostatin-expressing cells to pilocarpine treatment. Unfortunately, the

Table 2 | Progress in ESC-derived cortical interneurons and their transplantation *in vivo*.

Author	Species	Differentiation	Strategy	Growth factors	*In vitro*	*In vivo*
Goulburn et al., 2011	Human	Spin EBs	NKX2.1 reporter	FGF2 and RA	Appropriate progenitor markers GABAergic neurons, PV, SST expression. Electrophysiology and migration	Migration capacity and expressed GABA
Cambray et al., 2012	Human and mouse	Adherent monolayer		Activin	CGE markers, CR neurons	Bipolar orientated cells in cortex
Maisano et al., 2012	Mouse	Adherent monolayer	Sox1 Reporter	HH agonist	CR and CB expression	TLE model, PV, CR, CB, small effect on mossy fiber sprouting, electrophysiology
Maroof et al., 2010	Mouse	EBs	Lxh6 reporter	SHH, IGF, FGF2	Interneuron precursor expression,	Migration in cortex, PV, SST Electrophysiology, 9 month survival.
Danjo et al., 2011	Mouse	Spin EBs	Foxg1 reporter	SHH, FGF8 vs. FGF15/19	Nkx2.1 expression, PV, SST, NPY, CR	Slice culture migration
Ma et al., 2012	Human	EBs		Shh	Nkx2.1, Gsx2	
Li et al., 2009	Human	EBs	Gli3 RNAi	Shh, Dkk1	Nkx2.1, Gsh2, Isl1, GAD67	
Watanabe et al., 2007	Human	EBs		Shh	Nkx2.1	
Watanabe et al., 2005	Mouse	EBs		Dkk1, LeftyA, Shh	Nkx2.1, Gsx2, Isl1	
Gaspard et al., 2008	Mouse	Adherent monolayer		Endogenous Shh in basal conditions	Nkx2.1, multipolar cells, inhibitory synaptic activity	

Details of species of ESCs used, differentiation technique and precise strategies and the results both in vivo and in vitro are given. Abbreviations: EB, embryoid body; HH, hedgehog; PV, Parvalbumin; SST, Somatostatin; CGE, caudal ganglionic eminence; CR, calretinin; CB, Calbindin; NPY, neuropeptide Y; TLE, temporal lobe epilepsy.

authors did not observe a statistical reduction in mossy fiber sprouting, a characteristic trait of epilepsy.

Goulburn et al. utilized an NKX2.1-GFP human ESC reporter cell line to purify ventral telencephalic-like cells (Goulburn et al., 2011). The group used an EB-based neural induction protocol with FGF2 and retinoid acid (RA) treatment to promote NKX2.1 promoter-driven GFP expression. Although the use of RA is contentious, the RA signaling pathway has been shown to be relevant for early forebrain development (Schneider et al., 2001; Stavridis et al., 2010; Chatzi et al., 2011). Sorted NKX2.1-GFP^{+} cells gave rise to GABAergic and Somatostatin-expressing neurons *in vitro* that displayed migration capacity both *in vitro* and *in vivo*. These sorted cells also expressed diencephalic marker genes; a second region that is dependent on NKX2.1 expression. Therefore, all the evidence thus far indicates that the MGE is patterned by Shh and that Shh can induce ESCs to generate at least some subtypes of MGE-derived interneurons.

A recent study described the generation of Calretinin-expressing cortical interneurons from mouse and human ESCs/iPSCs (Cambray et al., 2012). During development, the majority of Calretinin interneurons are believed to be generated in the dorsal CGE, which appears to be non-responsive to Shh [see **Figure 1** (Xu et al., 2010)]. Using an adherent monolayer differentiation strategy, Cambray et al. reported that signaling via Activin induces the expression of genes that are associated with the CGE and promote the generation of Calretinin-expressing interneurons with minimal generation of MGE-derived interneuron subtypes (Cambray et al., 2012). Following transplantation, these neurons exhibited bipolar morphology in the adult rodent cortex. Members of fibroblast growth factors (FGFs) have also been reported to specify either an MGE or CGE-like fate (FGF8 vs. FGF15/19 respectively) as well as their neuronal derivatives (Danjo et al., 2011).

Together these studies provide a strong argument for the potential benefit of investigating further ESC-derived interneurons for the treatment of interneuronopathies. It is clear that the vast intricacies of cell specification require a great deal of work to improve our understanding of the developmental events, for example genome-wide investigations. As well as the patterning of interneuron precursors, aspects such as developmental timing

and transplantation site may have a huge impact on the final phenotype of a transplanted cell through exogenous signaling environments. The safety of ESC-derived cells must be carefully considered before transplantation; namely proliferation of undifferentiated cells, side-effects of cell heterogeneity and complications associated with surgery. These factors will need to be investigated fully to maximize the potential of this technology.

FUTURE PERSPECTIVES

The power of ESCs in generating subpallial-derived interneurons is starting to be unlocked. Future work is needed to expand the repertoire of interneuron subtypes generated from hESCs and iPSCs. Precise temporal control of developmental signaling pathways may be critical to direct preferential production of individual interneuron subtypes. For example, within the MGE, progenitors respond to higher doses of Shh preferentially to give rise to Somatostatin neurons while the progenitors that experience lower level of Shh signaling differentiate into Parvalbumin-expressing neurons (Wonders et al., 2008; Xu et al., 2010; Inan et al., 2012). It would be interesting to see how these developmental findings translate to neuronal subtype specification in stem cell systems. The development of novel interneuron differentiation protocols would benefit from better characterization of interneuron diversity in model organisms that establish links between cellular morphology, neurochemical phenotypes, synaptic connection types, and electrophysiological properties of the interneurons.

Neuropsychiatric disorders present a major burden to modern society. The major limitation for the development of new treatments has been the lack of defined etiology and limited knowledge of the disease pathophysiology (Lewis and Gonzalez-Burgos, 2006). Studies of these diseases rely on imaging techniques and post-mortem analysis of patients' brains. It is generally recognized that neuropsychiatric diseases are developmental disorders due to aberrant neuronal wiring. In this regard, human ESCs provide a robust *in vitro* model to gain insight into human cortical development. Much of our knowledge on the human brain and psychiatric diseases stems from rodent models. However, the human cortex is significantly larger than the rodent's with extra cortical germinal layers (Molnar et al., 2011). Particularly relevant to interneuron disease is the fact that the human and rodent brains differ in the ratio of inhibitory/excitatory neurons. This difference may impact on interneuron regulated network function and hence the extent to which rodent models can mimic human conditions. Furthermore, the genetic variants identified in psychiatric patients often involve long range genomic duplications or deletions spanning multiple genes. Such complex genetic variants can be difficult to model in an animal model. Patient-derived iPSCs offer unprecedented opportunity to generate human neurons with identical genetic information to that of patients to unravel the cellular defects and underlying molecular mechanisms caused by disease gene mutations. Therefore, hiPSCs offers a tractable alternative to rodent models.

The success of fetal tissue-based grafts has paved the way for stem cell-based interneuron replacement strategies. However, stem cell-derived interneurons have so far not been shown to produce a functional effect on disease models (Maroof et al., 2010; Goulburn et al., 2011; Cambray et al., 2012; Maisano et al., 2012). Furthermore, certain hurdles should be kept in mind with regard to interneuron transplantation therapy. Firstly, a proportion of pharmacoresistant epileptic patients do not respond to exogenous GABA administration as a treatment. Would grafted GABAergic cells have a beneficial effect in this paradigm? Cortical interneurons also have much interneuron-to-interneuron synaptic contact. For this reason, the subtype of grafted neurons must be carefully monitored to prevent altered activity of existing inhibitory cells within the disease setting. As discussed, the developmental timing and the transplantation site may have extrinsic effects on a transplanted cell's fate and so must be appreciated prior to transplant optimization. Finally, as with all stem cell-derived cell therapy postulations, a number of caveats may limit the safety of such an approach. For example, contaminating undifferentiated cells from the *in vitro* differentiation process may lead to cellular overgrowths and teratomas as well as other, off-target cells leading to adverse effects.

Nevertheless, the field of cortical interneuron dysfunction is set to welcome ESC based strategies that will enable a big step toward disease modeling and cell-based therapies for treating common neurological disorders.

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Systemic administration of valproic acid and zonisamide promotes differentiation of induced pluripotent stem cell–derived dopaminergic neurons

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Cell replacement therapy using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is a promising strategy for the treatment of neurologic diseases such as Parkinson's disease (PD). However, a limiting factor for effective cell transplantation is the low survival rate of grafted cells, especially neurons. In this study, we modified the host environment and investigated whether the simultaneous administration of soluble factors can improve the survival and differentiation of murine iPSC-derived dopaminergic (DA) neurons in host brains. With the goal of applying this technology in clinical settings in the near future, we selected drugs that were already approved for clinical use. The drugs included two commonly used anti-convulsants, valproic acid (VPA) and zonisamide (ZNS), and estradiol (E2), also known as biologically active estrogen. Following neural induction of murine iPSCs, we collected neural progenitor cells (NPCs) by sorting PSA-NCAM$^+$ cells, then treated the PSA-NCAM$^+$ cells with drugs for 4 days. An immunofluorescence study revealed that 0.01 mM and 0.1 mM of VPA and 10 nM of E2 increased the percentage of tyrosine hydroxylase$^+$ (TH: a DA neuron marker) cells *in vitro*. Furthermore, 0.1 mM of VPA increased the percentage of TH$^+$ cells that simultaneously express the midbrain markers FOXA2 and NURR1. Next, in order to determine the effects of the drugs *in vivo*, the iPSC-derived NPCs were transplanted into the striata of intact SD rats. The animals received intraperitoneal injections of one of the drugs for 4 weeks, then were subjected to an immunofluorescence study. VPA administration (150 mg/kg/daily) increased the number of NeuN$^+$ post-mitotic neurons and TH$^+$ DA neurons in the grafts. Furthermore, VPA (150 mg/kg/daily) and ZNS (30 mg/kg/daily) increased the number of TH$^+$FOXA2$^+$ midbrain DA neurons. These results suggest that the systemic administration of VPA and ZNS may improve the efficiency of cell replacement therapy using iPSCs to treat PD.

Keywords: induced pluripotent stem cells, valproic acid, zonisamide, estradiol, transplantation, dopaminergic neurons

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a loss of nigrostriatal dopaminergic (DA) neurons. In several clinical studies, the transplantation of fetal midbrain cells has successfully improved the motor symptoms of many PD patients, thus implying that cell replacement therapy is a promising strategy for the treatment of PD (Freed et al., 2001; Olanow et al., 2003; Mendez et al., 2005). More recently, pluripotent stem cells, especially induced pluripotent stem cells (iPSCs), have attracted much attention as a new source of donor cells due to their potential to supply a large quantity of DA neurons. However, iPSC-derived DA neurons and their precursors survive poorly in host brains (Hargus et al., 2010; Rhee et al., 2011). Furthermore, the procedure requires modification to improve the number as well as the quality of DA neurons in transplants to achieve the maximum efficacy of the therapy.

In the present study, we aimed to determine whether simultaneous administration of soluble factors can improve the survival and differentiation of murine iPSC-derived DA neurons in host brains. With the goal of applying this technology in clinical settings in the near future, we only used factors that were already approved for clinical use. Furthermore, candidate factors must cross the blood-brain barrier and diffuse into the brain parenchyma. Therefore, we selected two commonly used anti-convulsant drugs, valproic acid (VPA) and zonisamide (ZNS). We also selected estradiol (E2), also known as biologically active estrogen, an important female sex hormone that is widely used to treat patients suffering from ovarian deficiency symptoms, menopausal disorders, and osteoporosis.

ZNS has been shown to be neuroprotective in 6-OHDA- (Asanuma et al., 2010) and MPTP-lesioned mice (Yano et al., 2009; Sonsalla et al., 2010; Choudhury et al., 2011).

Neuroprotective effects against MPTP toxicity are also observed with 17β-E2 (Dluzen et al., 1996, 2001; Miller et al., 1998; Callier et al., 2001; Ramirez et al., 2003) and VPA (Kidd and Schneider, 2010, 2011). Furthermore, these compounds are also advantageous in cell transplantation studies using neural progenitor cells (NPCs). A study by Abematsu et al. demonstrated remarkable improvement of the hind limb function in a mouse model of spinal cord injury after NPC transplantation with VPA treatment, which favors NPC differentiation toward neurons rather than glial cells (Abematsu et al., 2010). E2 increases the proportion of human NPC-derived DA neurons *in vitro* and *in vivo*, thus suggesting that it promotes DA differentiation and supports the survival of mature DA neurons (Kishi et al., 2005).

Based on these previous studies, we investigated whether VPA, ZNS, or E2 affects the differentiation of DA neurons derived from murine iPSCs *in vitro*. We then evaluated their effects *in vivo* by grafting the iPSC-derived NPCs into the striata of rats that received daily injections of one of the test compounds.

MATERIALS AND METHODS

DIFFERENTIATION OF DOPAMINERGIC NEURONS FROM MURINE iPS CELLS

A murine iPS line 440A-3 (a gift from Dr. Okita, Kyoto University Center for iPS Cell Research and Application, Kyoto, Japan) was used after 10–25 passages. Generated with a plasmid vector containing three genes, *Oct3/4*, *Klf4*, and *Sox2*, the 440A3 cells carried the green fluorescence protein (GFP) and the puromycin-resistance gene under the *Nanog* enhancer and promoter, which are only active when the cells are in an undifferentiated state (Okita et al., 2008). No integration of the exogene was reported.

Undifferentiated cells were maintained on mitomycin C-treated murine embryonic fibroblast (MEF) feeder cells in DMEM (Wako) supplemented with 1% fetal calf serum, 5% knockout serum replacement (KSR; Invitrogen), 0.1 mM of non-essential amino acids, 1 mM of sodium pyruvate, 0.1 mM of 2-mercaptoethanol (2-ME; Invitrogen), 2000 U/ml of leukemia inhibitory factor (Invitrogen), and 1.5 μg/ml of puromycin (Takara) to eliminate differentiated cells. For neural induction of iPS cells, we used the serum-free culture of embryoid body-like aggregates (SFEB) method (Watanabe et al., 2005). Briefly, 440A3 cells were dissociated with 0.25% trypsin/1 mM EDTA and seeded onto 96-well low-adhesion plates (Lipidure-Coat Plate A-U96, NOF Corporation) at a density of 3000 cells/well to induce re-aggregation on day 0 in differentiation medium containing GMEM with 5% KSR, 0.1 mM of non-essential amino acids, 1 mM of sodium pyruvate, and 0.1 mM of 2-ME. During the differentiation period, various factors were added to induce the midbrain DA phenotype, as indicated in **Figure 1A**: 20 ng/ml of murine FGF-8b (R&D Systems) from days 3 to 7, 10 ng/ml of recombinant murine sonic hedgehog (C25II) N-terminus (R&D Systems) from days 4 to 7, 1% N-2 Supplement (Gibco) and 200 nM of ascorbic acid from day 7 onwards. KSR was withdrawn from the differentiation medium on day 7.

FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

On day 9, 440A3 cells were rinsed twice in PBS(–) and dissociated into single cells using a 5-min incubation with Accumax (Innovate Cell Technologies) at 37°C. The cells were collected with a FACS buffer consisting of PBS(–) with 2% FBS, 20 mM of D-glucose and 1% Penicillin/Streptomycin (P/S, Invitrogen), and mechanically dissociated into a single cell suspension by gentle pipetting. Subsequently, the cells were incubated with murine anti-PSA-NCAM antibodies (1:200, Millipore) for 30 min at 4°C and washed twice by centrifugation, followed by another 30-min incubation with the secondary antibody AlexaFluor 594 donkey anti-mouse IgG (1:400, Invitrogen). Dead cells and debris were excluded using 7-aminoactionomycin-D (7-AAD, BD Pharmingen) staining, and the viable cells were again suspended at a final concentration of 1×10^7 cells/ml. Cell sorting was performed using a FACSAriaII cell sorter (Becton Dickinson) equipped with 488-nm argon and 633-nm Helium-Neon lasers, a 100-μm nozzle, and the FACSDiva software program. PSA-NCAM positivity was determined according to the negative control lacking the primary antibody.

In vitro TREATMENT OF DOPAMINERGIC PROGENITORS WITH TEST COMPOUNDS

After cell sorting, the PSA-NCAM$^+$ population was seeded onto 96-well plates at a density of 20,000 cells/well in DMEM/F12 medium (Wako) supplemented with 1% N-2 Supplement, 200 nM of ascorbic acid, 2% B27 Supplement (Invitrogen), 0.5 mM of L-glutamine, and 1% P/S to induce re-aggregation. The ROCK inhibitor Y-27632 (Wako) was used during the sorting procedure and the following overnight culture at 30 μM to prevent apoptosis (Koyanagi et al., 2008). On day 10, either VPA (Sigma), ZNS sodium salt (provided by Dainippon Sumitomo Pharma, Osaka, Japan), 17β E2 (Sigma), GDNF (R&D Systems), or PBS(–) was added to the culture for 4 days. VPA, ZNS, and E2 were each used at three different concentrations: 0.01 mM, 0.1 mM, and 1 mM for VPA, 1 μM, 10 μM, and 100 μM for ZNS, and 1 nM, 10 nM, and 100 nM for E2. GDNF was added at 20 ng/ml to provide a positive control. To antagonize the effects of VPA and E2, either an adenylate cyclase inhibitor 2,5-dideoxyadenosine (ddA, 100 μM; Santa Cruz Biotechnology) or an estrogen receptor antagonist ICI182780 (ICI, 2 μM; Wako) was added to the culture medium on day 10.

TRANSPLANTATION STUDY

Ten-week-old male Sprague–Dawley rats (Shimizu Laboratory Supplies, Kyoto, Japan) were cared for and handled according to the Guidelines for Animal Experiments of Kyoto University. The animals were anesthetized and injected stereotactically with the donor cells in the bilateral striatum (from the bregma: A +1.0 mm; R or L +3.0 mm; V +4.5 mm). For each tract, two aggregations of the day 9 population containing 3.1×10^5 cells on average were collected in 1 μl PBS(–) supplemented with 30 μM of Y-27632 and used for transplantation. Intraperitoneal injections of VPA (150 mg/kg/day), ZNS sodium salt, (30 mg/kg/day), E2 (80 μg/kg/day), or saline were administered 2 days in advance of the procedure and continued until the day of sacrifice. All animals also received a daily dose of 10 mg/kg of

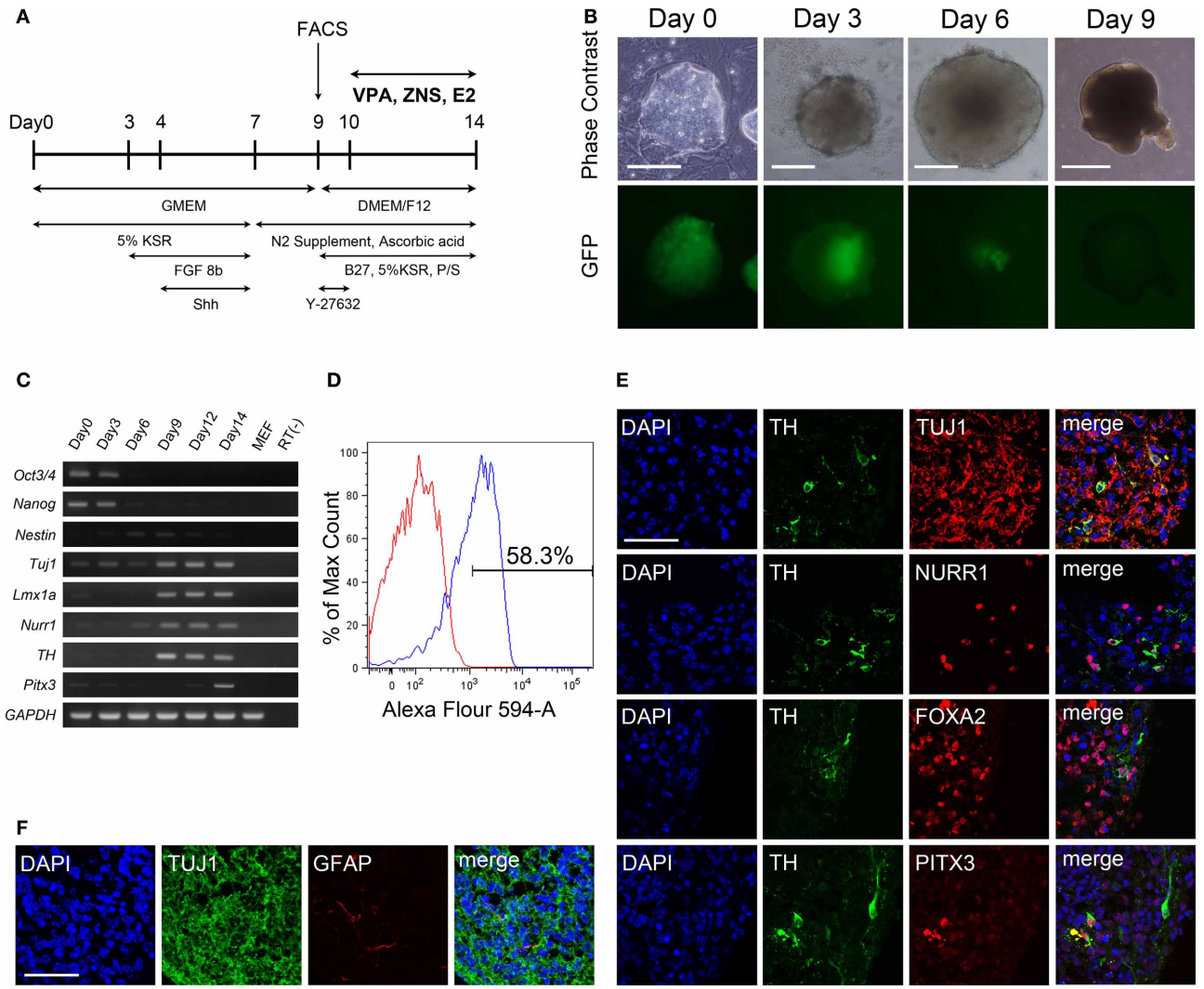

FIGURE 1 | Generation of dopaminergic neurons from murine iPSCs. **(A)** Murine iPSCs (440A3) were induced to differentiate into DA neurons via the SFEBq method with the addition of various factors during 14 days of suspension culture. **(B)** Phase contrast images (upper) and fluorescent images for nanog-GFP (lower) of the suspension culture over time. Scale bars = 100 μm (day 0), 200 μm (days 3, 6), and 500 μm (day 9). **(C)** An RT-PCR analysis illustrating the temporal changes in the expressions of genes associated with pluripotency (Oct3/4, nanog), immature NPCs (nestin), post-mitotic neurons (Tuj1), and DA neurons (Lmx1a, Nurr1, TH, *Pitx3*). GAPDH was used as a control. **(D)** A flow cytometric profile of the population at day 9 labeled by the neural cell adhesion molecule PSA-NCAM. **(E,F)** Immunocytochemistry of a sliced aggregate fixed on day 14 indicating the presence of DA neurons **(E)** and astrocytes **(F)** in the culture. The scale bar applies to all pictures and represents 50 μm.

cyclosporine A (CsA, Wako) for immunosuppression. At 4 weeks post-transplantation, the animals were intracardially perfused with 4% paraformaldehyde under deep anesthesia. On the day of sacrifice, blood samples were collected from each animal 1 hour after the final injections of the test drugs or CsA. These samples were sent to SRL, Inc. (Tokyo, Japan) for measurement of the blood concentrations of these drugs.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen), and reverse transcribed using the Super Script III First-Strand Synthesis System (Invitrogen). PCRs were performed using Hot StarTaq DNA polymerase (Qiagen). For each primer, a control amplification reaction was performed without the addition of reverse transcriptase. MEF was used as the other negative control. The primer sequences and product sizes are shown in **Table 1**.

IMMUNOFLUORESCENCE STUDY

For the *in vitro* experiments, on day 14, drug-treated cell aggregates were fixed in 4% paraformaldehyde, frozen and cut into 10-μm-thick slices using a microtome for immunocytochemistry. Following the *in vivo* experiments, the brains were removed and re-fixed for two days in 4% paraformaldehyde, cryopreserved in 30% sucrose for 3 days, frozen, and cut into 40-μm-thick slices for immunohistochemistry. Frozen sections of the spheres and brains were permeabilized and blocked with 0.3% Triton

Table 1 | Summary of primers for RT-PCR.

Gene name	Forward primer	Reverse primer	Product size
Oct3/4	CCTGGGCGTTCTCTTTGGAAAGG	GTAGGGAGGGCTTCGGGCACTT	263
Nanog	AGCAATGGTCTGATTCAGAAGGGCTC	AAATGCGCATGGCTTTCCCTAGTG	368
Nestin	GGCTTCTCTTGGCTTTCCTGACCC	GGGGGACATCCTGGGCTCTGAC	269
Tuj1	GGGCCAAGTTCTGGGAGGTCATC	GTCCAAAGGCGCCAGACCGA	198
Lmx1a	CCAGAACCAGCGAGCCAAGATGA	AGGCATCTGGGGTGGGGTGAG	238
Nurr1	GCGCTTAGCATACAGGTCCAACCC	CCTTGAGCCCGTGTCTCTCTGTGA	212
TH	TCCGGGCTTCTCTGACCAGGC	GCCAGTCCGTTCCTTCAAGAAGTGAG	280
Pitx3	GGGACGCACTAGACCTCCCTCCAT	AAGCCACCTTTGCACAGCTCC	420
GAPDH	CTCATGACCACAGTCCATGCCATCA	TCATCATACTTGGCAGGTTTCTCCAGG	251

X-100 and 2% donkey serum in PBS(–) for 1 h at room temperature, followed by overnight incubation with primary antibodies at 4°C. The primary antibodies used in this study included rabbit anti-tyrosine hydroxylase (1:400, TH;Millipore), mouse anti-TH (1:200, Millipore), sheep anti-TH (1:400, Millipore), mouse anti-Tubβ3 (1:1000, Tuj1; Covance), rat anti-NURR1 (1:1000, a gift from Dr. Ono, KAN Research Institute, Kobe, Japan), rabbit anti-Ki67 (1:1000, Novocastra: NCL-Ki67p), rabbit anti-Caspase3 (1:500, Santa Cruz Biotechnology), rat anti-M2M6 (1:50, Developmental Studies Hybridoma Bank), mouse anti-Nestin (1:500, Millipore), rabbit anti-Pitx3 (1:500, Chemicon), goat anti-HNF-3β (1:500, Foxa2; Santa Cruz Biotechnology), and mouse anti-NeuN (1:500, Chemicon). After three washes with 0.05% Tween-20 in PBS, the samples were incubated with Alexa Fluor-conjugated secondary antibodies at room temperature for 1 h. Following three additional washes, the samples were incubated with DAPI for nuclear staining and mounted using Permafluor (Dako). The immunoreactive cells were visualized with a confocal laser microscope (Fluoview FV1000D; Olympus, Tokyo, Japan). To determine the percentage of positive cells for each marker, labeled cells were manually counted for at least three independent experiments. The graft volume and number of $Ki67^+$/$Nestin^+$ cells were determined by identifying $M2M6^+$ areas in every sixth 40-μm-thick section using the BZ-II Analyzer software program (Keyence) and totaling the volumes of all 240-μm-tall cylinders according to Cavalieri's principle. To estimate the number of immuno-reactive cells in each graft, the cells were manually counted in every sixth section, and the Abercrombie correction was applied (Abercrombie, 1946).

STATISTICAL ANALYSIS

The statistical analyses were performed using the GraphPad Prism software program Ver. 5.0 b (GraphPad Software). All quantitative data are presented as the mean value ± SD, and One-Way ANOVA and Newman–Keuls *post-hoc* tests were used. Differences were considered to be statistically significant for $P < 0.05$.

RESULTS

GENERATION OF DA NEURONS FROM MURINE iPSCs

Murine iPSCs (440A3) were induced to differentiate into DA neurons via the SFEB method with the addition of FGF 8 b and Shh, as described in **Figure 1A**. On day 0, dissociated iPSCs quickly re-aggregated in each well, and these cells proliferated continuously over 14 days in culture (**Figure 1B**). Along with the differentiation, the expression of Nanog-GFP gradually decreased until day 9, when it almost disappeared. The temporal changes in the gene expression profile shown in **Figure 1C** clearly illustrate the step-wise differentiation of the $Oct3/4^+$ and $Nanog^+$ pluripotent population on day 0 into $Nestin^+$ immature NPCs around days 6–9 then into $Tuj1^+$ neurons as they began to express the markers specific for DA subtype such as *Lmx1a*, *Nurr1,* and *TH*.

Although DA neurons were successfully generated in our suspension culture, undifferentiated iPSCs and non-neural cells possibly remained. In order to obtain a more homogenous population of NPCs, we used FACS to select for cells positive for PSA-NCAM, a cell adhesion molecule that is specifically expressed on the surface of neural cells (Bonfanti, 2006). On day 9, ∼60% of the cells were positive for PSA-NCAM (**Figure 1D**). Following FACS, PSA-$NCAM^+$ cells were made to re-aggregate and allowed to mature for another 5 days, then subjected to immunocytochemical studies on day 14. Immunofluorescent staining of the sliced aggregates revealed that most of the cells were $TUJ1^+$ neurons, and among them were some mesencephalic DA neurons that simultaneously expressed TH, NURR1, FOXA2, and PITX3 (**Figure 1E**). Only a few (<0.1% of the total number of cells) cells expressed GFAP, a marker for astrocytes (**Figure 1F**).

VPA AND E2 INCREASED DOPAMINERGIC NEURONS *in vitro*

First, we examined whether the test drugs, VPA, ZNS, and E2, affected the differentiation of DA neurons *in vitro*. In addition to these three drugs, GDNF was used as a positive control since it has been shown to support DA neurons *in vitro* (Young et al., 2010) and *in vivo* (Sinclair et al., 1996; Yurek, 1998). Re-aggregated PSA-$NCAM^+$ cells were treated with these drugs for 4 days starting on day 10. Immunocytochemistry performed on day 14 revealed that more than 90% of the cells expressed the neuronal marker Tuj1 under all conditions (**Figures 2A**,**B**) and that 5.2 ± 1.1% of the control cells were TH^+ (**Figures 2A**,**C**). The percentage of TH^+ neurons significantly increased, by ∼2-fold, when the cells were treated with 0.01 mM or 0.1 mM VPA, or 10 nM E2 (12.1 ± 1.5, 11.7 ± 0.4, or 12.2 ± 2.3%, respectively). To investigate whether this effect of VPA and E2 is mediated through the cyclic AMP pathway or the estrogen receptor, we used an adenylate cyclase inhibitor ddA (DeCastro et al., 2005), or an estrogen receptor antagonist ICI, respectively. When 100 μM of ddA or 2 μM of ICI were added simultaneously with 0.1 mM of VPA or 10 nM of E2,

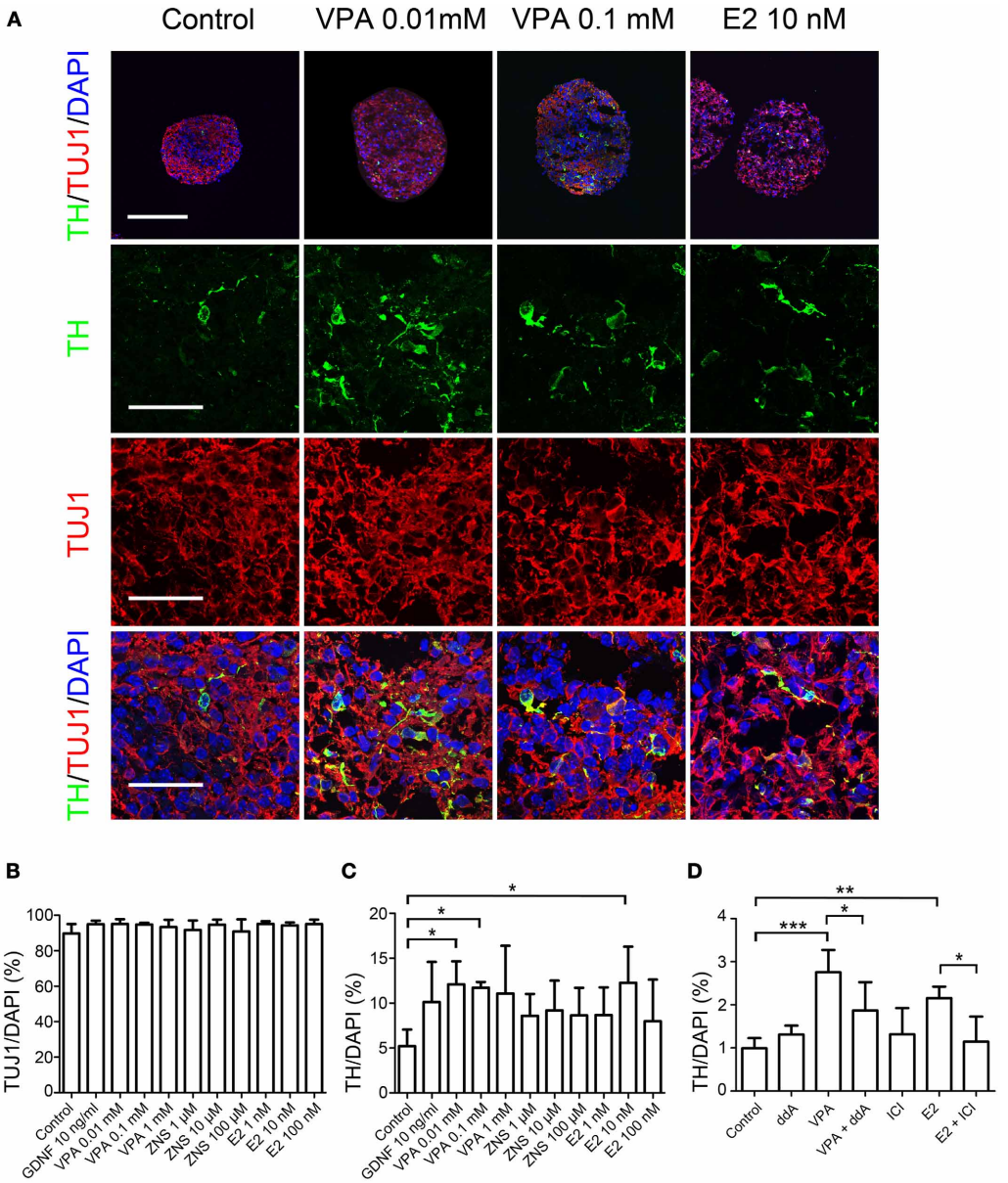

FIGURE 2 | Differentiation of iPSCs under VPA, ZNS, and E2 treatment *in vitro*. Suspension cultures of murine iPSCs (440A3) were selected for PSA-NCAM$^+$ neural progenitor cells on day 9, re-aggregated and then treated for 4 days with GDNF (as a positive control), VPA, ZNS, and E2. On day 14, the aggregates were fixed, sliced into 10-μm-thick sections and subjected to an immunocytochemical analysis for the expressions of neuronal (Tuj1) and dopaminergic (TH) markers. In the experiments for inhibition, ddA (100 μM) or ICI (2 μM) was added to the culture medium between days 10 and 14 either alone or in combination with VPA (0.1 mM) or E2 (10 nM), respectively. **(A)** Representative immunofluorescent images for TH/Tuj1 in the control and drug-treated cultures. The scale bar applies to all pictures and represents 200 μm (top) and 50 μm (lower). **(B–D)** A comparison of the number of cells labeled for Tuj1 **(B)** and TH **(C,D)** per the total number of cells. The data are presented as the mean ± SD. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $n = 3$).

respectively, for 4 days, the increase in TH$^+$ neurons was reduced significantly (**Figure 2D**). Addition of ddA or ICI alone did not change the percentage of TH$^+$ neurons.

In order to further characterize the TH$^+$ neurons, we performed double-labeled immunocytochemistry for the markers of midbrain DA neurons, including FOXA2, NURR1, and PITX3 alongside TH. The percentages of TH$^+$ FOXA2$^+$, and TH$^+$NURR1$^+$ cells significantly increased when the cells were treated with 0.1 mM of VPA compared to that observed under the control conditions (1.00 ± 0.58% vs. 0.25 ± 0.22%, and 1.00 ± 0.70% vs. 0.37 ± 0.32%, respectively; **Figure 3**). There were only a few PITX3$^+$ cells (<0.1% of the total number of cells), most likely because the period for differentiation was too short and there were no supportive cytokines such as GDNF in the culture

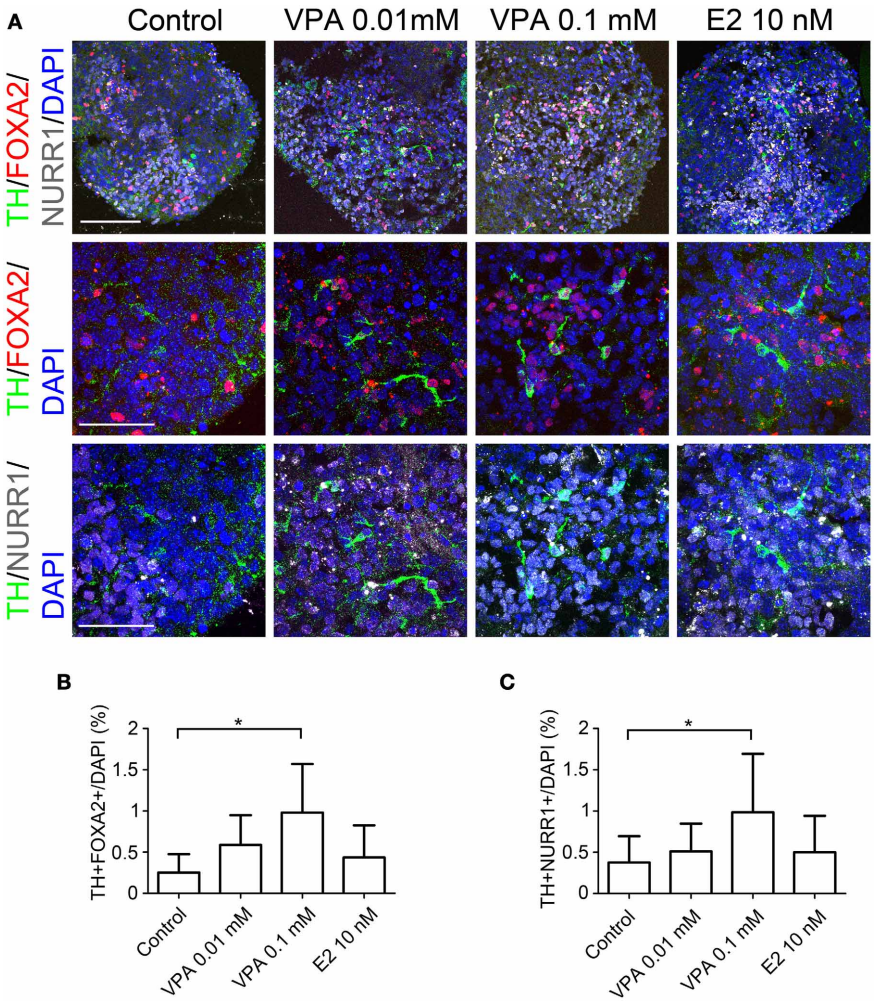

FIGURE 3 | Generation of midbrain DA neurons from iPSCs under VPA and E2 treatment *in vitro*. On day 14, the aggregates subjected to an immunocytochemical analysis for the expressions of midbrain DA neuron markers. **(A)** Representative immunofluorescent images of TH, FOXA2, and NURR1 in the control and drug-treated cultures. The scale bar applies to all pictures and represents 100 μm (top) and 50 μm (lower). **(B,C)** A comparison of the number of cells labeled for both TH and FOXA2 **(B)** or TH and NURR1 **(C)** per the total number of cells. The data are presented as the mean ± SD. (*$P < 0.05$; $n = 6$).

medium. These results suggest that VPA treatment promotes DA differentiation and the acquisition of a midbrain-like DA neuron phenotype.

Next, we evaluated whether the drugs affected the survival of TH^+ neurons by labeling apoptotic cells for caspase 3 expression. In control spheres, 18.0 ± 5.9% of TH^+ neurons were also labeled with caspase 3, indicating that 1/5 of the DA neurons were undergoing apoptosis (**Figure 4**). **Figure 4B** shows that VPA- (8.2 ± 9.0% by 0.01 mM, 11.1 ± 9.7% by 0.1 mM) and E2- (8.9 ± 12.9% by 10 nM)) treatment tended to yield lower percentages of apoptotic DA neurons; however, there were no significant differences in the percentages of apoptotic fractions of DA neurons between the four groups.

VPA PROMOTED NEURONAL DIFFERENTIATION OF GRAFTED NPCs

Next, we investigated whether the systemic administration of VPA, ZNS, or E2 influenced the survival and differentiation of DA neurons in the grafts. In this transplantation study, unsorted cell populations (3.1×10^5 cells in two aggregates, in PBS) were injected into the striata of intact SD rats on day 9. The rats received intraperitoneal injections of one of the drugs and the immunosuppressant CsA every day starting 2 days before the transplantation and continuing until the day of sacrifice at 4 weeks. On the day of sacrifice, the blood concentration of CsA was 3700 ± 898 ng/ml on average. The blood concentrations of VPA, ZNS, and E2 were 158.5 ± 3.9 μg/ml, 2.43 ± 0.13 μg/ml, and 1141 ± 926 pg/ml, respectively.

Double-labeled immunohistochemistry at 4 weeks post-transplantation against Nestin (an NPC marker) and Ki67 (a marker of proliferating cells) revealed that 15–20% of the grafted cells were $Nestin^+$, but again there were no statistically significant differences (**Figures 5A,B**). The percentages of $Ki67^+$ cells per $Nestin^+$ cells were quite low in all grafts (<0.1%), suggesting that the $Nestin^+$ cells were mostly quiescent or becoming post-mitotic

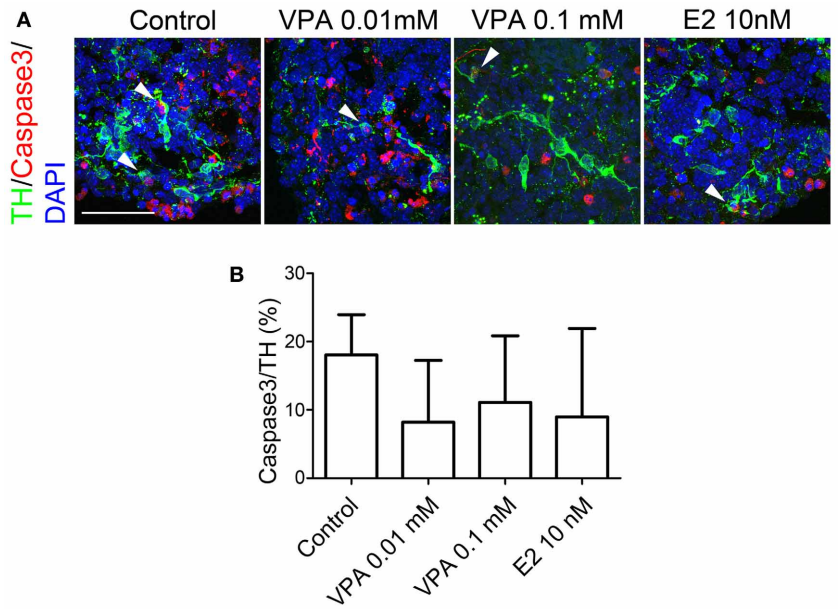

FIGURE 4 | Apoptosis of iPSC-derived TH$^+$ cells under VPA, ZNS, and E2 treatment *in vitro*. On day 14, the aggregates were subjected to an immunocytochemical analysis for dopaminergic (TH) markers as well as caspase 3, which is expressed by apoptotic cells. **(A)** Representative immunofluorescent images of TH (green) and caspase 3 (red) in the control and drug-treated cultures. The arrowheads indicate double-positive cells. The scale bar applies to all pictures and represents 50 μm. **(B)** A comparison of the number of TH$^+$ cells labeled for caspase 3. The data are presented as the mean ± SD (no significant differences; $n = 6$).

at that time point. In contrast, immunohistochemistry against NeuN, a mature neuronal marker, revealed that the percentage of NeuN$^+$ cells per the total number of cells was significantly increased when the animals were treated with VPA (77.9 ± 5.1% vs. 57.7 ± 9.4% in the control group; **Figures 5A**,**C**). These results suggest that VPA promoted the neuronal differentiation of the grafted NPCs.

The grafted cells were identified using immunofluorescent staining for M2M6, which is only expressed by mouse cells and not by the host rat cells. At 4 weeks post-transplantation, labeling with M2M6 revealed that the grafts in all groups survived well, with no signs of tumor formation (**Figure 6A**, $n = 8$ in the control group, $n = 6$ in the VPA, ZNS, and E2 groups). The average estimated graft volume was smallest in the VPA-treated animals (4.33 ± 2.14 mm^3) and was as large as 9.76 ± 3.19 mm^3 in the control group, but there were no statistically significant differences (**Figure 6B**).

ENHANCED SURVIVAL OF MIDBRAIN DA NEURONS IN VPA- AND ZNS-TREATED GRAFTS

Based on our *in vitro* data showing that the test drugs increased the number of TH$^+$ neurons, we compared the number of TH$^+$ neurons in the grafts of each group at 4 weeks post-transplantation. A double-labeled immunofluorescence study revealed that the VPA-treated grafts yielded significantly higher numbers of TH$^+$ cells than the control grafts (1396 ± 864 and 393 ± 311, respectively; **Figures 6A**,**C**). Only a small fraction of total TH$^+$ neurons co-expressed the midbrain marker FOXA2 in the control grafts (24.7 ± 9.3%). In contrast, the majority of TH$^+$ neurons in the VPA- and ZNS-treated grafts were FOXA2$^+$ (81.8 ± 33.6% and 80.4 ± 21.1%, respectively). The statistical analysis demonstrated that the number of midbrain DA neurons (TH$^+$FOXA2$^+$) in the grafts were significantly increased in the animals treated with VPA or ZNS compared to those observed in the controls (984 ± 770, 835 ± 540, and 97 ± 76, respectively; **Figures 6A**,**D**). Overall, these observations indicate that the systemic administration of VPA and ZNS increased the yield of midbrain DA neurons *in vivo* by promoting the DA differentiation of the grafted NPCs.

DISCUSSION

In this study, we investigated whether VPA, ZNS, or E2 affects the survival and differentiation of DA neurons derived from murine iPSCs. We found that treatment with VPA at 0.1 mM significantly increased the percentage of TH$^+$FOXA2$^+$ and TH$^+$NURR1$^+$ DA neurons derived from the iPSC-derived NPCs *in vitro*. In the subsequent transplantation study, the systemic administration of VPA and ZNS significantly improved the yield of TH$^+$FOXA2$^+$ DA neurons in the grafts.

Here, for the first time, we showed that a low dose of VPA (0.01 or 0.1 mM) is advantageous in promoting DA differentiation from murine iPSC-derived NPCs *in vitro*. The positive effects of VPA on neuronal differentiation have been demonstrated in a previous study (Hsieh et al., 2004); however, the effects on the differentiation of DA neurons have not been well-documented. In a study by DeCastro *et al.*, a short chain fatty acid including VPA was shown to induce TH mRNA transcription in the pheochromocytoma cell line PC12 through the cyclic AMP-dependent signaling pathway (DeCastro et al., 2005). Our result that simultaneous addition of an adenylate cyclase inhibitor ddA

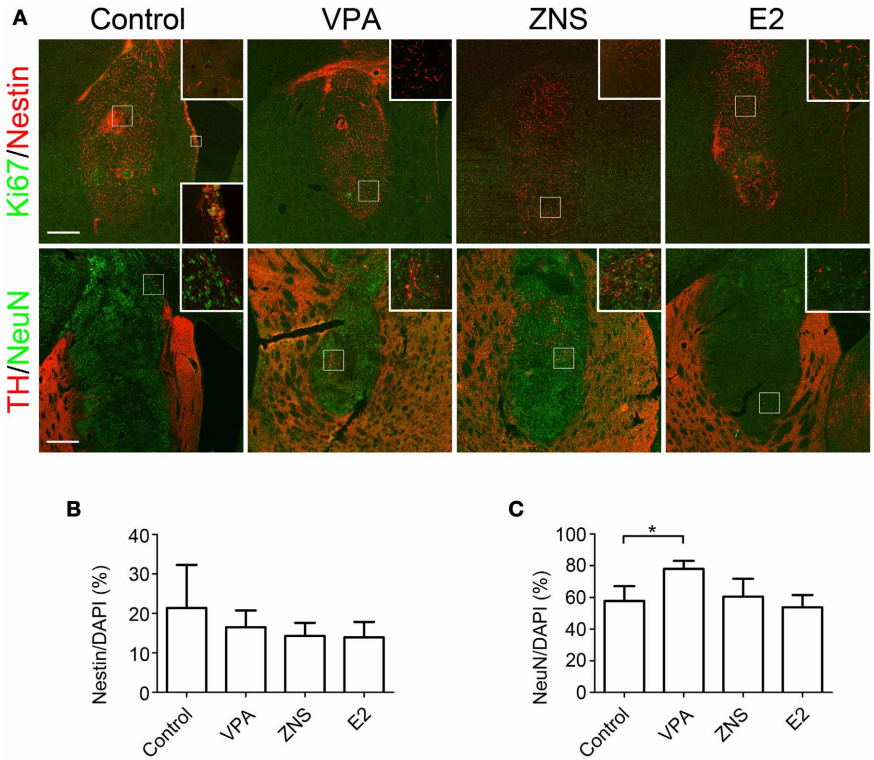

FIGURE 5 | Analyses of iPSC-derived transplants in animals treated with VPA, ZNS, or E2. On day 9, aggregations of differentiated cells were injected into the striata of intact SD rats (3.1×10^5 cells in two aggregates/tract). The rats received intraperitoneal injections of saline, VPA, ZNS, or E2. **(A)** Representative immunohistologic images of nestin (immature NPCs; red) and Ki67 (proliferation marker; green), post-mitotic neurons (NeuN; green), and DA neurons (TH; red). A magnified image of the boxed area is shown in the inlet. The lower inlet displays a high magnification of the proliferating neural precursors lining the ventricular surface as a positive control for Ki67 staining. The scale bar applies to all pictures and represents 500 μm. A comparison between each group for **(B)** the number of Nestin$^+$ cells per the total number of cells in the graft, **(C)** the number of NeuN$^+$ cells per the total number of cells in the graft. The data are presented as the mean ± SD. (*$P < 0.05$; $n = 8$ control, $n = 6$ VPA, ZNS, E2).

with 0.1 mM of VPA suppressed the effects of VPA suggests that the increased DA differentiation induced by VPA is mediated by the cyclic AMP-dependent pathway. On the other hand, 1 mM of VPA, a moderate dose that corresponds to the therapeutic plasma concentration (Blaheta and Cinatl, 2002), seemed to increase the rate of apoptosis of DA neurons. This observation corroborates the study by Hsieh in which VPA increased the apoptosis as well as the neuronal differentiation of NPCs in a dose-dependent manner (Hsieh et al., 2004). Due to the fragile nature of differentiated DA neurons, the cytotoxic effect of 1 mM of VPA might have been more prominent than the TH-inducing effect. VPA is also known to be a histone deacetylase (HDAC) inhibitor, associated with the increased transcription of a variety of factors that may contribute to the protection of midbrain DA neurons, including free radical scavengers, heat-shock proteins, and anti-apoptotic bcl-2 family members (Kidd and Schneider, 2010, 2011). Therefore, the potential mechanisms of VPA may be multiple and remain unclear. Extensive exploration is needed.

Despite the lack of noteworthy effects *in vitro*, ZNS administration resulted in an increase in the yield of TH$^+$FOXA2$^+$ DA neurons in the grafts. A recent study showed that ZNS supported DA neurons *in vitro* via an indirect effect mediated by astrocytes (Asanuma et al., 2010). Asanuma et al. demonstrated that ZNS markedly increases glutathione, a powerful anti-oxidant, in astrocytes and acts as a neuroprotectant against oxidative stress in 6-OHDA-lesioned rats. Therefore, the absence of astrocytes in the culture (>95% were TUJ1$^+$ neurons) likely hindered ZNS from exerting such beneficial effects *in vitro*. On the other hand, the *in vivo* effects of ZNS were likely mediated by astrocytes that are abundant in the host brain. The neuroprotective effects of ZNS in PD model animals and the effects in enhancing TH have been reported in several other studies (Yano et al., 2009; Sonsalla et al., 2010; Choudhury et al., 2011). Therefore, we suggest that the systemic administration of ZNS can also be an effective strategy for increasing the yield of TH$^+$FOXA2$^+$ DA neurons obtained from grafted NPCs.

In addition to VPA at a concentration of 0.01 or 0.1 mM, treatment with E2 at a concentration of 10 nM also resulted in a good yield of TH$^+$ neurons *in vitro*. These effects of increasing the number of DA neurons *in vitro* are consistent with the previous findings of our group and others in which E2 increased the proliferation of DA progenitors through actions mediated by estrogen receptors (Kishi et al., 2005; Díaz et al., 2009). Increased proliferation induced by E2 has also been observed more generally in the NSCs of rats (Brännvall et al., 2002) and humans (Wang et al., 2008). Despite the favorable results obtained *in vitro*, the systemic

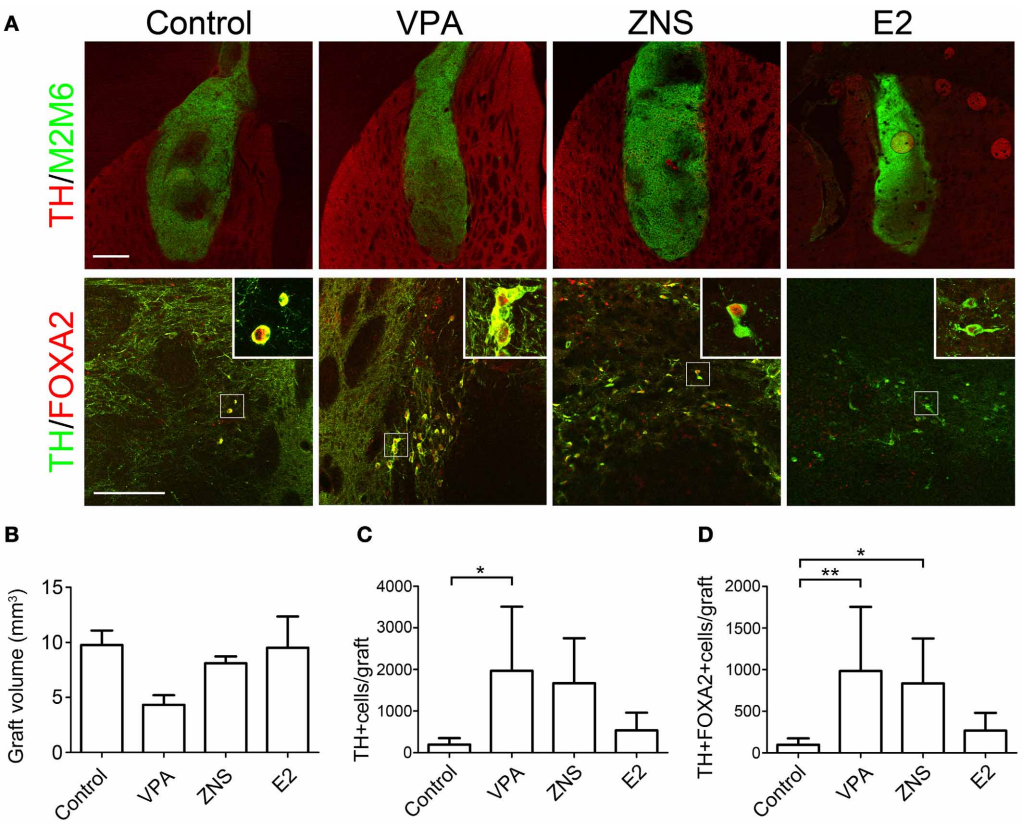

FIGURE 6 | Analyses of iPSC-derived midbrain DA neurons in animals treated with VPA, ZNS, or E2. (A) Representative immunohistologic images of grafts (M2M6; green) containing DA neurons (TH; red), FOXA2 (ventral-midbrain marker; red), and TH (DA neuron marker; green). The scale bar applies to all pictures and represents 500 μm (upper) and 50 μm (lower). A comparison between each group for **(B)** average graft volume, **(C)** the number of TH^+ cells per graft, and **(D)** the number of TH^+FOXA2^+ cells (midbrain DA neurons) per graft. The data are presented as the mean ± SD. ($^*P < 0.05$, $^{**}P < 0.01$; $n = 8$ control, $n = 6$ VPA, ZNS, E2).

administration of E2 resulted in a poor yield of DA neurons in the transplants. We might have observed more TH^+ cells in the grafts if there was a more appropriate concentration for E2 administration or if we had examined the samples at a later time point rather than at 4 weeks post-transplantation.

Approximately 20–75% of the TH^+ cells in the grafts did not express FOXA2, suggesting that they are not of the midbrain but possibly of the forebrain subtype of DA neurons. In addition, ∼95% of the $NeuN^+$ cells in the grafts did not express TH, indicating that they are not DA but rather another type of neuron. Although the protocol used to generate midbrain DA neurons from iPSCs has been developing, the one used in the present study is not yet perfect. To achieve more abundant survival of midbrain DA neurons, further improvements in the protocol and purification of midbrain DA progenitor cells are needed. We attempted to transplant PSA-$NCAM^+$ cells after FACS, which resulted in a low survival rate of the grafted cells, particularly DA neurons (less than 50 TH^+ cells per graft under each condition). Therefore, the method of cell sorting and recovery after sorting also needs to be improved.

In conclusion, we herein demonstrated improved survival and differentiation of iPSC-derived DA neurons showing a midbrain-like phenotype following the systemic administration of well-established anti-epileptic drugs, VPA, and ZNS. Recent efforts in the field of stem cell therapy have primarily focused on deriving more genuine cells of clinically relevant cell types such as midbrain DA neurons from pluripotent stem cells. It is, however, equally important to develop strategies to modify the microenvironment of the host brain in order to achieve the optimal results of such therapies. Because there are so many safety issues to overcome before pluripotent stem cells can be used for the treatment of otherwise incurable diseases, we believe that taking advantage of drugs that are already safely used can save valuable time in the clinical application of stem cell technology.

ACKNOWLEDGMENTS

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Genetic strategies to investigate neuronal circuit properties using stem cell-derived neurons

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The mammalian brain is anatomically and functionally complex, and prone to diverse forms of injury and neuropathology. Scientists have long strived to develop cell replacement therapies to repair damaged and diseased nervous tissue. However, this goal has remained unrealized for various reasons, including nascent knowledge of neuronal development, the inability to track and manipulate transplanted cells within complex neuronal networks, and host graft rejection. Recent advances in embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) technology, alongside novel genetic strategies to mark and manipulate stem cell-derived neurons, now provide unprecedented opportunities to investigate complex neuronal circuits in both healthy and diseased brains. Here, we review current technologies aimed at generating and manipulating neurons derived from ESCs and iPSCs toward investigation and manipulation of complex neuronal circuits, ultimately leading to the design and development of novel cell-based therapeutic approaches.

Keywords: stem cell, synapse, circuit, neuron, optogenetic, transsynaptic, reprogramming

INTRODUCTION

Within the mammalian nervous system, billions of neurons are intricately interconnected via trillions of synapses. From this complex network of synaptic connectivity, specialized neural circuits emerge, forming the foundation for diverse physiological processes and behaviors. Due to the sheer complexity and heterogeneity of the cell and tissue types that contribute to neural circuits, the brain is prone to an array of developmental and neurodegenerative diseases, ranging from epilepsy (Noebels et al., 2012; Pun et al., 2012), mental illness (Lieberman et al., 2012a,b), Alzheimer's disease (Cao et al., 2012; Grienberger et al., 2012), Parkinson's disease (Armstrong et al., 2002; Rochester et al., 2012), and Huntington's disease (Mazzocchi-Jones et al., 2009; Niclis et al., 2009). Although highly heterogeneous in presentation, etiology, and affected cell populations, neurological disorders fundamentally involve the loss of proper circuit function (**Figure 1**) (Miller et al., 2011; Noutel et al., 2011; Ramamoorthi and Lin, 2011; Wang et al., 2011; Wesson et al., 2011; Ghiglieri et al., 2012). In recent times, pharmacological interventions have shown therapeutic promise toward the treatment of many neurological disorders (Kraft et al., 2009; Ballatore et al., 2012; Das et al., 2012; Fridhandler et al., 2012; Pedersen et al., 2012; Weaver et al., 2012). For example, several forms of epilepsy can be well controlled with pharmacology. Yet, in cases of severe neurodegenerative disorders, drug therapy has shown more variable results. This variability is compounded by the fact that medications are often initiated at late stages of disease when symptoms become clinically evident, providing limited, if any, symptomatic relief. Even in cases with promising drug therapy, one major limitation is that patients may become desensitized and thus require increasingly higher doses of medication until they reach the point of tolerance (Gancher et al., 1996). Further, even if a patient's symptoms are controlled with medication, prohibitive side effects may restrict the utility of pharmacological therapy. Finding the right therapeutic approach is therefore a challenge for many patients, especially when such limits of drug therapy have been reached. Neurosurgical options, including deep brain stimulation implants in Parkinson's disease patients, are increasingly offered to those whose symptoms are pharmacologically non-responsive, to promote activity of remaining functional neuronal tissue. However, these approaches can be accompanied by the general risks of invasive procedures, and their mechanisms of action remain to be characterized. In the emerging era of stem cell therapy, much hope lies in developing a less invasive, cell-based therapy that offers the unique ability to replace dysfunctional, damaged, or lost neurons. The promise of curative approaches for such progressive and devastating conditions as Huntington's disease, Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis has lured many patients to undergo alleged stem cell transplants from a range of medical providers, offering these procedures in absence of a critical mass of controlled clinical trials and detailed understanding of the mechanisms of a cell-based therapeutic approach to neurodegenerative diseases.

Recent efforts have focused on developing mechanistic insight into cell-based therapies (Alvarez Dolado and Broccoli, 2011; Dyson and Barker, 2011; Freed et al., 2011; Chen and Blurton-Jones, 2012; Kauhausen et al., 2012; Moviglia et al., 2012).

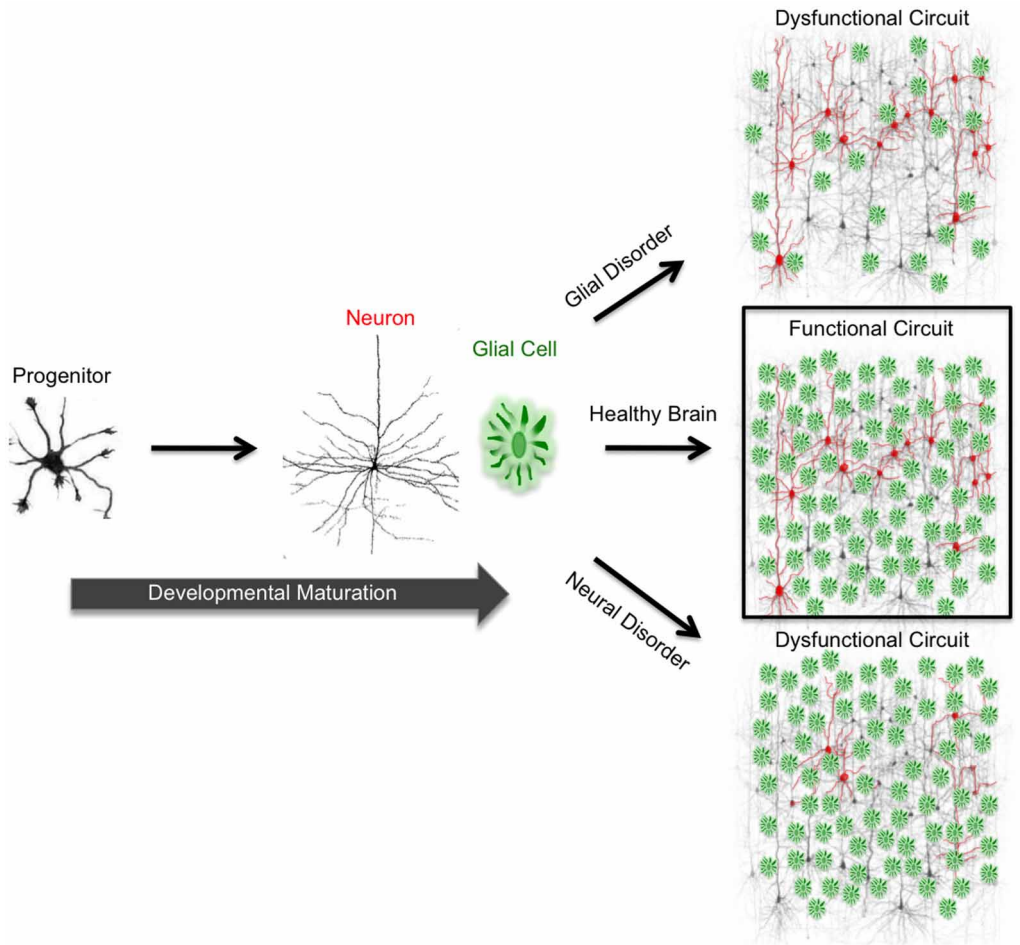

FIGURE 1 | Functional circuit formation stems from precise coordination between diverse neuronal and glial subtypes. In the mammalian brain, neurons are connected to each other via synapses, forming complex neuronal circuits. The development and maturation of these circuits requires precise coordination between heterogeneous cell populations consisting of diverse neuron and glial subtypes. During development, neural progenitor cells give rise to neurons and glia. In neurodevelopmental and neurodegenerative disorders, proper neuron and glial interactions do not occur due to cellular malfunction. This malfunction impairs functional circuit formation and leads to circuit dysfunction. Impaired neuronal circuits are implicated in a wide variety of disorders such as epilepsy, Alzheimer's, Parkinson's, and Huntington's diseases.

Cellular approaches aimed at understanding and treating neurological diseases have ranged from the direct transplantation of embryonic, neuronal, and fetal stem cells (Bjorklund et al., 2002; Erdo et al., 2004; Hattiangady et al., 2007; Kauhausen et al., 2012; Moon et al., 2012) in rodent models, to the *in vitro* generation and investigation of induced pluripotent stem cells (iPSCs) and induced neural stem cells (iNSCs) from affected patients' tissues (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Han et al., 2012; Ring et al., 2012; Thier et al., 2012). Although induced stem cells have yielded valuable *in vitro* models of several neurological disorders (Camnasio et al., 2012; Israel et al., 2012; Ooi et al., 2012; Yagi et al., 2012), numerous studies have identified major challenges that have hindered transplantation efforts. Notable examples include teratoma formation (Bjorklund et al., 2002; Seminatore et al., 2010; Cunningham et al., 2012; Garcia et al., 2012), graft rejection (Krystkowiak et al., 2007), neuronal death (Nolte et al., 2008; Wang et al., 2012), and improper integration into pre-existing brain circuits (Kelly et al., 2007; Wang et al., 2012). In order to begin to harness the potential of stem cell therapy toward the treatment of neurological disorders, these issues must be addressed. Here, we review current literature regarding the generation of neurons from different stem cell populations and discuss their potential use for both *in vitro* studies and *in vivo* transplantation. We further provide an overview of current strategies to mark and manipulate neuronal activity in intact brain tissues, and discuss the interface between these genetic and cellular technologies to investigate circuit formation and function. Finally, we conclude by exploring the future of therapeutic interventions for damaged and diseased nervous systems using genetically modified stem cell-derived neurons.

GENERATING NEURONS FROM EMBRYONIC STEM CELLS

In the hopes of curing or providing therapeutic measures for damaged and diseased nervous tissue, significant interest has

been placed in developing stem cell-based therapies for common neurological disorders (Babaei et al., 2012; Chen and Blurton-Jones, 2012; Lescaudron et al., 2012; Moon et al., 2012). Early attempts in animal models were aimed at transplanting pure populations of ESCs directly into damaged or diseased brain tissue (Deacon et al., 1998; Bjorklund et al., 2002; Erdo et al., 2004), with the intent of providing a source of renewable cells capable of functionally integrating into existing circuits. This notion was supported by optimism that naive stem cells would respond to cues from the surrounding tissue and ultimately differentiate and function as mature neurons with appropriate synaptic connections. However, many obstacles have hindered this approach. First, transplantation of pluripotent stem cells into the brains of animals can lead to restrictively high incidence of teratomas (Bjorklund et al., 2002; Erdo et al., 2004); in some cases, 25% or more of all grafts can result in undifferentiated brain tumors (Garcia et al., 2012). In attempt to avoid teratoma formation, efforts next turned toward transplantation of adult and fetal neural stem cells (NSCs) (Fainstein et al., 2012; Moon et al., 2012; Muneton-Gomez et al., 2012). With putative lineage restriction, NSCs were considered to have potential as a renewable source of neuronal and glial subtypes, without the attendant risk of teratoma formation. However, in lieu of generalized teratoma formation, transplanted NSCs have been observed to produce neural lineage-restricted brain tumors, such as medulloblastomas and gliomas in animal models (Swartling et al., 2012). Alongside these challenges other obstacles have surfaced, including graft rejection (Capetian et al., 2011; Chen et al., 2011). While cell transplantation can be straightforward, the procedure may activate host immune responses, which can result in rejection of transplanted cells prior to circuit integration. To circumvent this, immunosuppressive drugs are required during and after cell transplantation (Leveque et al., 2011; Hovakimyan et al., 2012). Unfortunately, immunosuppressants do not completely eliminate graft rejection, and they invite susceptibility to various types of opportunistic infections (Kasper et al., 2011; Pustavoitau et al., 2011; Dhar et al., 2012).

Significant efforts have since been made to develop *in vitro* differentiation protocols to program stem cells into distinct neuronal lineages, thus avoiding unrestricted clonal expansion and tumor formation prior to terminal differentiation (Caiazzo et al., 2011; Chung et al., 2011; Jing et al., 2011; Juliandi et al., 2012; Kirkeby et al., 2012). It is now possible to routinely generate high numbers of ESC-derived neurons of various subtypes *in vitro* (Bibel et al., 2007; Parsons et al., 2011; Garcia et al., 2012; Kirkeby et al., 2012; Moon et al., 2012; Salti et al., 2012). Molecular marker analysis and electrophysiological characterizations have demonstrated that these neurons appropriately differentiate *in vitro* and exhibit patterns of action potentials and neurochemical profiles characteristic of neurons found in intact brain tissue (Bissonnette et al., 2011; Caiazzo et al., 2011; Cho et al., 2011; Garcia et al., 2012). Using specialized protocols, heterogeneous populations of NSCs, as well as GABAergic, glutamatergic, dopaminergic, and various other neuronal and glial subpopulations can be obtained at high quantities. Importantly, using these *in vitro*-generated neuronal subpopulations, it is possible to specifically target affected cell populations in diverse disease models. Furthermore, these differentiated cellular subtypes provide a valuable resource for *in vitro* studies. With renewable sources of diverse neuronal lineages, numerous studies have implemented transplantation methods to attempt treatment of specific neurological disorders. In some cases, therapeutic effects have been observed (Song et al., 2007; Chung et al., 2011; Kriks et al., 2011). For example, improved behavioral outcomes in animal models of Parkinson's disease and other movement disorders have been observed following stem cell-based therapies (Song et al., 2007; Chung et al., 2011; Kriks et al., 2011), and transplantation of GABAergic neurons have shown modest decreases in seizure activity in epilepsy models (Castillo et al., 2008; Maisano et al., 2009). However, it remains unknown whether observed therapeutic effects are evidence of functional restoration by engrafted cells or are the result of stromal neuroprotective effects, including improved vascularization and secretion of neurotrophic factors. Therefore, it has become a priority to investigate the potential for transplanted progenitor cells to properly differentiate, synaptically mature, and appropriately integrate within functional neural circuits *in vivo*. Toward this effort, numerous methodologies have emerged to differentiate stem cells into neuronal lineages and genetically engineer them to mark and manipulate their patterns of synaptic connectivity (**Figure 2**).

GENERATING NEURAL STEM CELLS FROM SOMATIC CELL TYPES

To circumvent host stem cell graft rejection, emphasis has been placed on developing methods to genetically reprogram adult somatic cells to a state of pluripotency, resembling embryonic stem cells (ESCs). These iPSCs can be differentiated into neuronal subtypes *in vitro* and autologously transplanted back into the donor brain. Because these cells are patient-derived, they are thought less likely to be rejected by the host immune system, leading to improved graft survival. The first successful method of cellular reprogramming published by Takahashi and Yamanaka, demonstrated that somatic cells from both mice (Takahashi and Yamanaka, 2006) and humans (Takahashi et al., 2007) can be manipulated to adopt a pluripotent state. This discovery received the Nobel Prize in Physiology or Medicine in 2012. Cellular reprogramming is accomplished by ectopic expression of transcription factors in somatic cells. When cultured appropriately, these cells further produce lineages derived from any of the three embryonic germ layers (Vierbuchen et al., 2010; Han et al., 2012; Ring et al., 2012; Thier et al., 2012). Notably, these studies demonstrate effective generation of ectodermal-like neuronal precursors with capacity to mature into functional neurons capable of generating specific patterns of action potentials *in vitro*. Techniques have since advanced to facilitate reprogramming from a variety of starting cell types, including fibroblasts (Pfisterer et al., 2011; Ring et al., 2012), keratinocytes (Petit et al., 2012), and blood cells (Ma et al., 2011). Additionally, methods have been devised to bypass the pluripotent state, thereby directly generating iNSCs (Hanna et al., 2007; Denham and Dottori, 2011; Pfisterer et al., 2011; Soldner et al., 2011; Yusa et al., 2011; Han et al., 2012; Ring et al., 2012; Thier et al., 2012). Bypassing the pluripotent state allows for direct generation of neuronal subtypes, minimizing risk of teratoma formation. Reprogramming

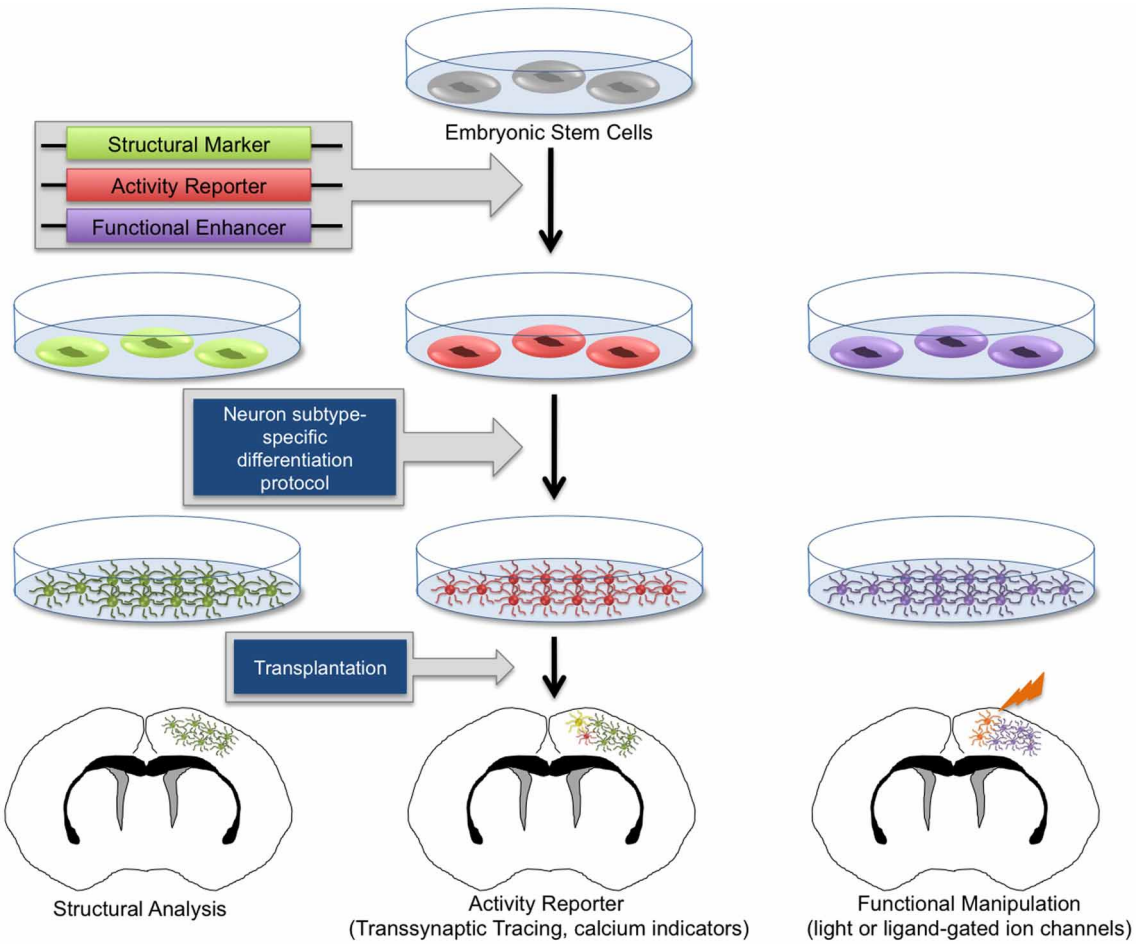

FIGURE 2 | Genetic manipulation of embryonic stem cell-derived neurons. Human and mouse embryonic stem cells (ESCs) can be routinely cultured *in vitro*. Through genetic manipulations, stem cells can be endowed with structural reporters such as fluorescent markers, activity reporters including transsynaptic tracing machinery and calcium indicators, as well as functional enhancers such as light-responsive proteins and exogenous ligand-activated ion channels. Upon neuron subtype-specific differentiation, modified ESCs will give rise to heterogeneous neuronal and glial subtypes. *In vitro*-generated neurons can be transplanted into healthy and diseased brain tissue. With this technology, it is possible to perform morphological analysis, transsynaptic tracing, and activity measurements and manipulation of transplanted neurons, allowing the functional investigation of neuronal circuit integration in the brain.

technology thus provides an elegant means to generate relatively inaccessible or growth-restricted cell lineages from readily obtained tissue samples, with virtually unlimited potential for both basic research and clinical application. One can perhaps envision a near future where biomarkers are used to identify neurological disease patients prior to symptom onset, and where the patient's own somatic cells can be used to validate and study these disorders without having to obtain neural biopsies via invasive measures.

However, similar to difficulties with ESC-derived neurons, efficacy of induced stem cell transplants in animal models can be complicated by tumorigenic transformation (Yamanaka, 2009; Fong et al., 2010) resulting from the genomic integration or sustained expression of reprogramming factors (Okita et al., 2008). Thus, there remains a need for more "factor-free" reprogramming technologies to eliminate oncogenic potential. Moreover, although studies are now beginning to evaluate *in vitro* connectivity of grafted neural cells with host tissue (Tonnesen et al., 2011), the long-term maintenance of these connections and the survival potential for *in vivo* transplants remain unknown. With the development of a more extensive genetic and biological toolset, iNSC technology is poised to overcome such limitations. Genetic manipulation and transplantation of stem cell-derived neurons into live animals will provide a better understanding of different measures of synaptic structure and/or function in the intact brain. Detailed investigations of neuronal organization promise to advance our working knowledge of circuit architecture and operation in both healthy and diseased brain tissue with ever-greater resolution.

VIRAL-BASED APPROACHES TO INVESTIGATE NEURONAL CONNECTIVITY

In order to properly generate desired neuronal subtypes and target areas for transplantation studies, it is necessary to reveal the

precise patterns of cellular connectivity in particular brain regions and in disease models. Classical studies have applied anatomical methods using histology, light microscopy, and ultrastructural analysis to label and identify neurons that make synaptic connections (Stevens et al., 1980; Anderson et al., 1994; Ahmed et al., 1997; Briggman and Denk, 2006; Micheva and Smith, 2007). Structural studies provide great insight into synaptic connectivity, but without additional functional analysis, these connections should perhaps be considered "passive." To further characterize passive connectivity, a number of tracing methods have been implemented, including fluorogold retrograde tracers (Gomez-Nieto et al., 2008; Rossetti et al., 2012), biotinylated dextrans (Shehab et al., 2005; Rossetti et al., 2012), wheat germ agglutinin (Louis et al., 2010), cholera toxin conjugated dyes (Angelucci et al., 1996; Miyashita and Rockland, 2007), fluorescent microspheres (Apps and Ruigrok, 2007; Neely et al., 2009), and lipophilic dyes (Bruce et al., 1997; Makarenko et al., 2000). However, a major limitation to these methods is the gradual signal decline with distance or degree of labeled projections. Additionally, indiscriminate cell-to-cell spread can occur between contacting cells in close proximity, obscuring the interpretation of functional connectivity. As such, these classical technologies label connected neurons anatomically, but do not reveal true functional connections.

To elucidate networks between synaptically-coupled partners more precisely, novel technologies using neurotropic viruses have been established (Callaway, 2008; Ugolini, 2010, 2011; Arenkiel, 2011; Arenkiel et al., 2011). Two prominent subtypes of viruses used for transneuronal tracing include rabies virus (RV) and herpes virus (Callaway, 2008; Ugolini, 2010). Both infect cells via retrograde particle transfer, allowing for the identification of neurons presynaptic to an infected source cell. In order to determine the precise identity of synaptic partners, fluorescent proteins have been engineered into the herpes and rabies viral genomes (Kuypers and Ugolini, 1990; Wickersham et al., 2007a; Callaway, 2008), allowing synaptically connected cells to be visualized via detection of viral transfer using fluorescence microscopy. Because these viral particles are self-replicating, their signal is sustained over time and distance, and all synapses are labeled with similar efficiency. The two most common herpes strains used for viral tracing include herpes simplex virus 1 (HSV-1) (Lilley et al., 2001) and pseudorabies virus (PRV) (Enquist, 2002). Both strains are potent neuronal tracers and mark synaptically connected neurons with very high efficiency. However, a major limitation of the use of herpes virus for synaptic tracing studies is its polysynaptic spread (Callaway, 2008; Ugolini, 2010). Herpes virus moves across synapses very quickly, rapidly infecting brain tissue and making it difficult to dissect precise synaptic connections. Additionally, herpes virus integrates into cellular genomes and promptly becomes toxic to the infected animal, leading to death within days (Ugolini, 2010). To overcome these limitations, other transsynaptic viral vectors have been engineered. Like HSV, RV spreads to synaptically connected neurons in a retrograde manner. But because its genome consists of negative strand RNA, RV does not integrate into cellular DNA and is less toxic to infected neurons (Callaway, 2008). Infected cells remain healthy for up to two weeks, allowing for the functional investigation of synaptic partners.

Wickersham et al. have demonstrated that genetically altered RV can be used for precise identification of synaptic partners (Wickersham et al., 2007a). To achieve this, the rabies glycoprotein G gene, which encodes the viral capsid and enables viral assembly and spread is deleted and replaced with the gene for enhanced green fluorescent protein (EGFP). These engineered RV particles infect neurons with high efficiency and use the host cellular machinery to replicate and produce high amounts of EGFP, without generating infectious virions. Because the viral genome is engineered without glycoprotein G, only primarily infected neurons are labeled (Wickersham et al., 2007a). To optimize engineered RV for transsynaptic circuit tracing, Wickersham et al., "pseudotyped" the viral particles with the foreign coat protein EnvA from the avian sarcoma leukosis virus (Wickersham et al., 2007b). EnvA recognizes the TVA receptor that is naturally only found on cell membranes of certain avian species and is not normally present on the mammalian neuronal membrane. By introducing cDNA encoding both the rabies G glycoprotein and TVA receptor, together with a fluorescent marker such as tdTomato for identification purposes, targeted neurons can be infected by the pseudotyped RV, which also encodes EGFP. Additionally, when exogenous glycoprotein G is provided *in trans*, the virus is mobilized to replicate, self-assemble, and carry out a single retrograde jump to its presynaptic inputs. Because presynaptic partners do not express glycoprotein G, and no further glycoprotein G is provided, viral tracing comes to a halt, marking monosynaptically connected input neurons (Wickersham et al., 2007b). Such tracing methods have been extremely powerful to deduce local patterns of neuronal connectivity (Arenkiel et al., 2011; Miyamichi et al., 2011).

Recently, stable mouse ESC lines have been engineered to harbor transsynaptic tracing elements (glycoprotein G, TVA, and a tdTomato reporter) (Garcia et al., 2012). These cells can be differentiated with high efficiency into diverse neuronal lineages as verified via molecular marker expression and electrophysiological analysis. When introduced into slice cultures *in vitro*, or into intact brain tissue *in vivo*, the transplanted cells can be identified by fluorescent reporter expression and subsequently infected with pseudotyped RV to act as source cells for circuit tracing studies (**Figure 3**). In the past, studies with NSC transplants have used the expression of fluorescent reporters (Chang et al., 2012; Steinbeck et al., 2012), magnetic resonance imaging, positron emission tomography (Daadi et al., 2009; Tang et al., 2011; Chang et al., 2012), and phenotypic rescue (Yang et al., 2008; Nagai et al., 2010; Chung et al., 2011; Zhu et al., 2011) to verify the integration of stem cell-derived neurons into brain circuits. However, whether transplanted cells forge functional connections within existing circuitry has remained difficult to ascertain. Through new genetic tracing technologies, it is now feasible to elucidate local networks of synaptic connectivity and to determine the cast of presynaptic contacts that form onto transplanted stem-cell derived neurons. With the knowledge obtained from neuronal tracing studies in healthy and diseased brains, combined with genetic strategies for neuronal activity manipulations, it is possible to genetically target

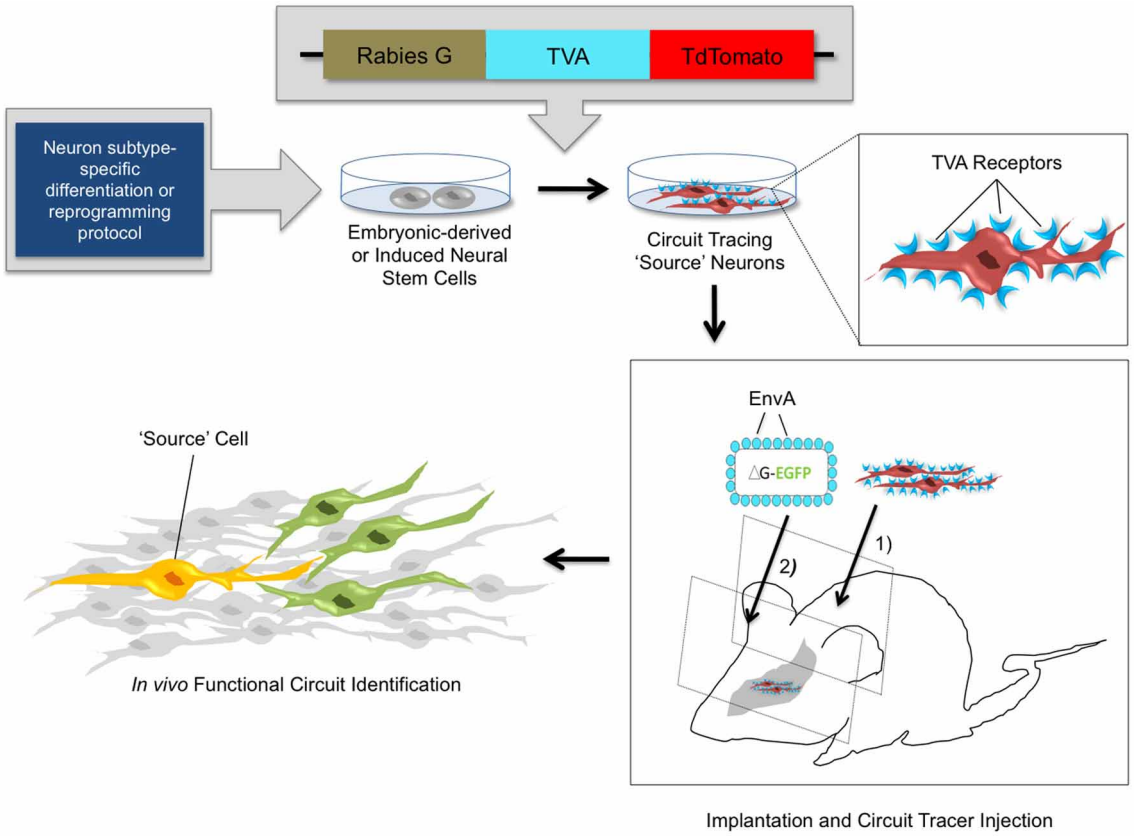

FIGURE 3 | *In vivo* circuit analysis with derived or reprogrammed neurons harboring viral circuit tracing elements. Using neuron subtype-specific differentiation or reprogramming protocols, a specialized population of neural stem cells can be obtained *in vitro*. Electroporation or stable transfection with a transsynaptic tracing vector provides the rabies G coat protein and the TVA receptor, which are components of the monosynaptic circuit tracing process. Incorporation of the viral tracing vector into the stem cell genome transforms these cells into "source" cells for circuit tracing. These cells can be implanted *in vivo* and identified by expression of the tdTomato fluorescent reporter. After a defined time period of maturation of the implanted cells, pseudotyped rabies virus is injected. The virus specifically targets TVA-expressing source cells and marks them with the EGFP fluorescent reporter. The source cells thus fluorescence both red and green, indicated by a yellow color. The circuit tracing system allows a single retrograde jump of virions carrying the EGFP reporter from the source cell to presynaptic neurons. This identifies synaptic connections and functional circuits formed by integrated neural stem cells with high specificity.

healthy and diseased neuronal circuits for precise labeling and/or control of neuronal firing (**Figure 2**).

GENETIC APPROACHES TO MANIPULATE NEURAL CIRCUIT ACTIVITY

The desire to use cell-based therapeutic approaches toward the treatment of damaged or diseased nervous tissue is rapidly gaining popularity. However, prospects for these approaches depend both on the ability to guide selective programs of neuronal differentiation, and to engineer cells whose output can be precisely controlled. In the adult brain, it has been established that diverse forms of activity govern the integration and survival of new neurons (Nilsson et al., 1999; van Praag et al., 1999; Rochefort et al., 2002; Leuner et al., 2004; Rochefort and Lledo, 2005; Yamaguchi and Mori, 2005). Through application of novel genetic tools, it is now possible to artificially "induce" or "silence" activity in brain circuits using light (Nagel et al., 2005; Arenkiel et al., 2007; Zhang et al., 2007a,b; Chow et al., 2010; Deisseroth, 2010; Zhang et al., 2010; Tonnesen et al., 2011; Madisen et al., 2012), or exogenous chemical ligands (Tan et al., 2006; Lerchner et al., 2007; Arenkiel et al., 2008; Conklin et al., 2008; Drenan et al., 2008; Wehr et al., 2009). Furthermore, genetically encoded calcium indicators provide a method for functional readout in neurons (Pologruto et al., 2004; Mao et al., 2008; Tian et al., 2009; Akerboom et al., 2012; Chen et al., 2012). This capability is especially valuable for cell-based circuit and tissue repair, where integration and functional control may be desired. Early efforts toward manipulating neuronal activity in select cell types have used exogenous ligands not normally present in the brain (Arenkiel et al., 2008; Drenan et al., 2008; Wulff and Arenkiel, 2012). Chemical genetic technologies have demonstrated both the activation (Arenkiel et al., 2007; Conklin et al., 2008; Drenan et al., 2008) and silencing (Tan et al., 2006; Lerchner et al., 2007; Wehr et al., 2009) of select neuronal cell types in intact brain tissue both *in vitro* and *in vivo*. For example, the transgenic overexpression of ligand-gated ion channels (LGIC) in neuronal subtypes allows cellular activation upon

exogenous ligand administration (Drenan et al., 2008; Magnus et al., 2011). Examples of chemical genetic tools for neuronal activation include expression of modified opiate receptors (Coward et al., 1998), custom G_q-protein coupled receptors with high sensitivity for synthetic ligands (DREADD hM3Dq) (Armbruster et al., 2007), hyperdopaminergic activity via ectopic acetylcholine receptor activation (Drenan et al., 2008), as well as expression of the transient receptor potential cation channel subfamily V member 1 (TRPV1), which is normally expressed in nociceptive peripheral neurons and can be potently activated by administration of capsaicin (Arenkiel et al., 2008). Alongside this, several chemical genetic tools have also been developed to artificially silence neuronal activation, and include ectopic expression of a glutamate-gated chloride channel (GluCl) from *Caenorhabditis elegans*, which is activated by the antihelminthic drug ivermectin (Slimko et al., 2002; Lerchner et al., 2007), G_i-coupled receptors with high sentitivity for synthetic ligands (DREADD hM4Di) (Arenkiel et al., 2007), as well as expression of the *Drosophila* allatostatin receptor (AlstR) which induces G_i-coupled silencing in the presence of the insect peptide allatostatin (Birgul et al., 1999). Using chemical genetics, it becomes possible to precisely control neuronal output with high specificity. However, the use of exogenous ligands in living organisms poses several challenges. For example, it is necessary to consider the intrinsic properties of the ligand itself, as well as potential homeostatic mechanisms present in living tissues. Notably, engineered ligands must be inert and able to cross the blood-brain barrier. Further, ligands should be non-toxic to the animal and rapidly degraded in order to allow for precise time-dependent control of neuronal manipulation. Because of the challenges posed by chemical genetic technologies, researchers have also implemented optical genetic methods for controlling neuronal activity with light (Nagel et al., 2005; Arenkiel et al., 2007; Chow et al., 2010; Deisseroth, 2010).

Optogenetic approaches allow for fast and precise control of neuronal activity using light-activated ion channels via a spectrum of different wavelengths (Zhang et al., 2007a,b, 2010). The most popular light-activated ion channels used for neuronal depolarization are the channelrhodopsins, originally identified in the green algae *Chlamydomonas reinhardtii* (Nagel et al., 2005; Deisseroth, 2010). In response to blue light (480 nm), channelrhodopsin-2 (ChR2)-expressing neurons rapidly influx cations, which depolarize the cell membrane, leading to firing of action potentials. ChR2-assisted circuit mapping (CRACM) has allowed precise dissection of neuronal circuits by stimulating and activating ChR2-expressing neurons while recording electrophysiological responses in postsynaptic neurons (Petreanu et al., 2007). Alternatively, action potential inhibition can be achieved via light-activated hyperpolarizing channels. This may be accomplished by either light-directed anion influx, or proton efflux. Halorhodopsin (NpHR) is a light-driven chloride pump that is activated with yellow-green light (570 nm) to drive chloride ions into cells, whereas archaerhodopsin actively drives protons out of the cell upon illumination with yellow-green light (Chow et al., 2010; Madisen et al., 2012). *In vitro* differentiated neurons harboring ChR2 and NpHR have recently revealed important

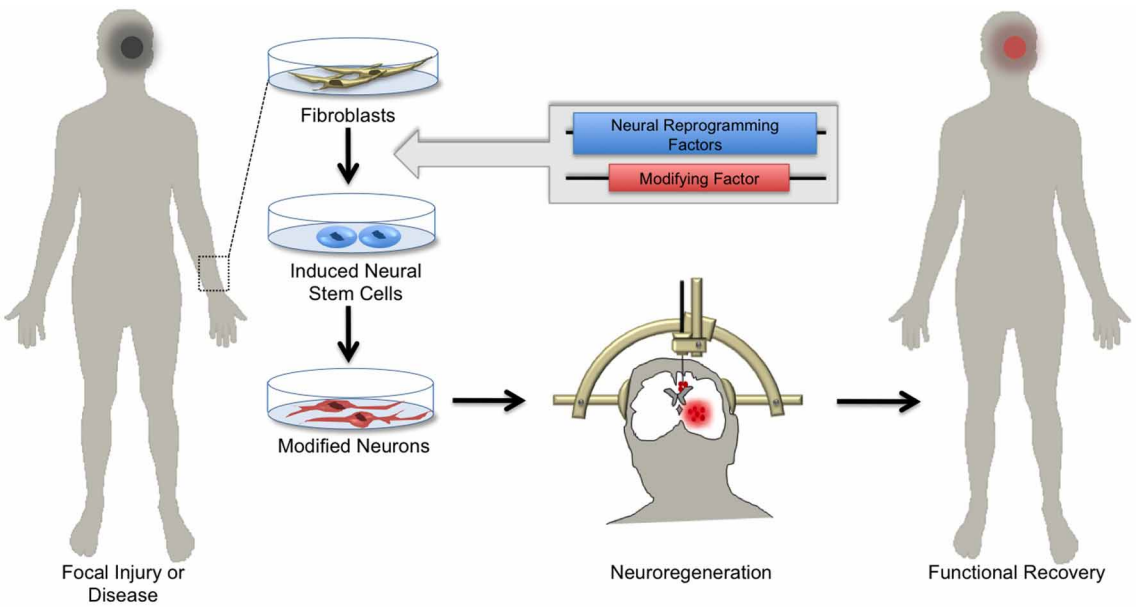

FIGURE 4 | Hopes for using induced neuronal stem cells for regenerative medicine. Skin biopsies from patients with focal neurological injury or neurodegenerative disease are a ready source of fibroblasts. Using the approach of cellular reprogramming and subsequent genetic manipulations, patient-derived fibroblasts can be differentiated into induced neural stem cells (iNSCs) harboring functional elements. Depending on the patient's needs, iNSCs can be differentiated *in vitro* into specialized neuronal subtypes. Patient-derived neurons can then be transplanted by local injection into areas of diseased or damaged brain tissue. Upon integration, these cells regenerate parenchymal tissue, replacing dysfunctional cells and ultimately supporting functional recovery.

information regarding the functional integration of intrastriatal grafts in a Parkinson's disease model (Tonnesen et al., 2011).

Additionally, genetically encoded calcium indicators such as GCaMP allow for functional readout of manipulated neurons (Pologruto et al., 2004; Mao et al., 2008; Tian et al., 2009; Akerboom et al., 2012; Chen et al., 2012). GCaMP is a modified GFP fused to calmodulin. Upon calcium influx, such as in the case of depolarization, GCaMP undergoes a conformational change, leading to increased fluorescence intensity. In the absence of calcium influx, the GCaMP-expressing cells fluoresce dimly.

The emerging array of genetic tools for precise manipulation of neuronal activity gives hope to transitioning stem cell therapy from the conceptual to the clinical realm. For example, it may soon be possible to generate NSCs expressing detectable markers and/or activity-induced ion channels from patients with damaged or diseased nervous tissue and transplant these cells back into the affected patient (**Figure 4**). Such genetic strategies might enable monitoring of the integration and survival of transplant-derived neurons, and perhaps through controlled activity manipulations result in symptomatic relief in select neurological disorders.

CONCLUDING REMARKS

Advances in stem cell research combined with powerful genetic technologies now allow unprecedented levels of investigation and manipulation of complex neuronal circuits. Previous efforts to implement cell-based therapies have been hindered by tumorigenesis, graft rejection, cell death, lack of circuit integration, and the inability to follow grafted cells *in vivo*. Scientific advances are beginning to tackle these challenges. Further, transsynaptic tracing has enabled high resolution dissection of neuronal circuits, which has begun to reveal insights into the molecular mechanisms that guide synapse formation and circuit integration in the living brain. Genetic approaches to manipulate neural circuit activity have allowed for the selective "activation" and "silencing" of discrete neuronal subtypes. This knowledge, combined with the ability to generate high numbers of neurons *in vitro*, promises to yield significant advances in cell therapy. In the future, it might be possible to manipulate injured and/or diseased brain circuits with artificially grafted cells, allowing for sustained symptomatic relief in a range of neurological disorder and neuronal injury models.

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Post-stroke inflammation and the potential efficacy of novel stem cell therapies: focus on amnion epithelial cells

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Ischemic stroke is a debilitating disease for which there are currently no effective treatments besides the clot-buster, tissue plasminogen activator (t-PA), which is administered to less than 10% of patients due to a limited (4.5 h) time window of efficacy. Thus, there is an urgent need for novel therapies that can prevent or reverse the effects of stroke-induced brain injury. Recent encouraging reports have revealed that stem cells derived from human tissue, including embryonic, induced pluripotent, neural, and mesenchymal cells, can rescue injured brain tissue and improve functional recovery in experimental models of stroke. However, there are potentially major limitations to each of these types of stem cells that may ultimately prevent or restrict their use as viable mainstream treatment options for stroke patients. Conversely, stem cells derived from the placenta, such as human amnion epithelial cells (hAECs), appear to have several important advantages over other stem cell lineages, in particular their non-tumorigenic and non-immunogenic characteristics. Surprisingly, so far hAECs have received little attention as a potential stroke therapy. This brief review will firstly describe the inflammatory response and immune cell involvement following stroke, and then consider the potential for hAECs to improve stroke outcome given their unique characteristics. These actions of hAECs may involve a reduction of local inflammation and modulation of the immune response, promotion of neural recovery, differentiation into neural tissue, re-innervation of lost connections, and secretion of necessary cytokines, growth factors, hormones and/or neurotransmitters to restore cellular function.

Keywords: stem cells, human amnion epithelial cells, stroke, immune cells, inflammation, cerebral ischemia, therapy

INTRODUCTION

Stroke occurs following a sudden disruption of blood flow to the brain, thereby starving the tissue of oxygen and nutrients and initiating neuronal death within minutes (Broughton et al., 2009). This crippling disease is the world's second leading cause of death, with approximately 15 million strokes occurring each year, and accounting for 9.5% (i.e., 6 million) of all deaths per annum (WHO, 2002). Alarmingly, the incidence of stroke more than doubles each successive decade for people over the age of 55. Thus, with the annual number of strokes increasing due to the ageing population, an increasingly greater financial and social burden will be caused to survivors and to the community. Thus, major advances to prevent and treat stroke are of paramount importance.

TREATMENT OF ISCHEMIC STROKE

Ischemic stroke, which occurs when the blood supply to the brain is obstructed by an embolus or a thrombus, accounts for approximately 87% of all stroke cases (WHO, 2002). It is most disappointing that still the only available "pharmacological" intervention to reduce brain damage after stroke is the "clot-buster," tissue plasminogen activator (t-PA). t-PA is an enzyme that works by catalyzing the conversion of plasminogen to plasmin which can then break down either the embolus or thrombus causing cerebral ischemia (Sloan, 1987). However, t-PA can only be administered within 4.5 h of the onset of ischemia and only after a CT scan has verified that the stroke is due to a thrombus rather than a hemorrhage (Del Zoppo et al., 2009). After 4.5 h, there is no evidence for a net beneficial effect of t-PA due to the increased risk of hemorrhagic transformation. Consequently, only 2–8% of stroke patients currently receive this treatment (based on United States statistics) (Reeves et al., 2005; Kleindorfer et al., 2008). At best, t-PA can only restore blood flow, and it cannot target mechanisms of cellular injury or others that promote healing. Other treatment options for ischemic stroke include anti-coagulants, such as heparin that inhibits clot formation, and anti-platelet agents, such as aspirin, that reduce the risk of platelet aggregation. However, these treatments have no effect on stroke outcome and are mainly used in the prevention of a secondary stroke. Although a plethora of neuroprotective compounds have shown promise in animal models of stroke, no other treatment has achieved efficacy in clinical trials (Dirnagl, 2006).

Therefore, new therapies that may protect neural tissue from post-ischemic damage and/or promote functional recovery are desperately needed to reduce mortality and long-term neurological deficits in stroke victims. An important step in this process will be to understand the key mechanisms that contribute to injury following stroke.

ACTIVATION OF THE IMMUNE SYSTEM AND BRAIN INFLAMMATION AFTER STROKE

It is now understood that the immune system plays an integral role in the pathogenesis of ischemic stroke and it contributes to infarct formation (Iadecola and Anrather, 2011). In the post-stroke acute phase (minutes to hours), microglia and cerebral endothelial cells within the affected zone are activated by hypoxia, shear stress, and the production of reactive oxygen species (ROS) (Jin et al., 2010). This causes the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecules (VCAMs), selectins (in particular, P-selectin and E-selectin), and integrins (in particular, Mac-1 and LFA-1), on endothelial cells, leukocytes, and platelets (Yilmaz and Granger, 2010). Adhesion molecule expression is also induced on circulating leukocytes. Simultaneously, oxidative stress and locally-derived pro-inflammatory mediators (cytokines and chemokines) produced by the injured tissue alter the permeability of the blood brain barrier (BBB). As the ischemic cascade progresses, cell death leads to a new phase of the inflammatory response. Dying and dead cells release "danger signals" that activate the immune system (Iadecola and Anrather, 2011; Magnus et al., 2012). Some of these signals, such as the nucleotides adenosine triphosphate (ATP) and uridine triphosphate (UTP) and high-mobility group protein B1 (HMGB1), are released by cells under stress when the cell membrane is still intact, and thereby set the stage for the subsequent immune response. A result of these processes is a time-dependent infiltration of immune cells (**Figure 1**). These immune cells include neutrophils, macrophages, dendritic cells, and T and B lymphocytes (Stevens et al., 2002; Gelderblom et al., 2009, 2012; Brait et al., 2010; Jin et al., 2010; Kleinschnitz et al., 2010, 2012). Neutrophils, which are thought to be the first immune cell to enter the brain post-stroke, undergo granule exocytosis to release a variety of pro-inflammatory molecules such as large quantities of nitric oxide (NO) derived from inducible NO synthase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived ROS, and matrix metalloproteinases (MMPs) (Yilmaz and Granger, 2010). Both $CD4^+$ and $CD8^+$ T lymphocytes contribute to brain injury by producing pro-inflammatory mediators, such as the potent cytokines interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-17, and tumor necrosis factor (TNF) (Kleinschnitz et al., 2010, 2012; Brait et al., 2012; Gelderblom et al., 2012). T lymphocytes contribute further to the state of oxidative stress by also producing NADPH oxidase-derived superoxide (Brait et al., 2010). In addition, $CD8^+$

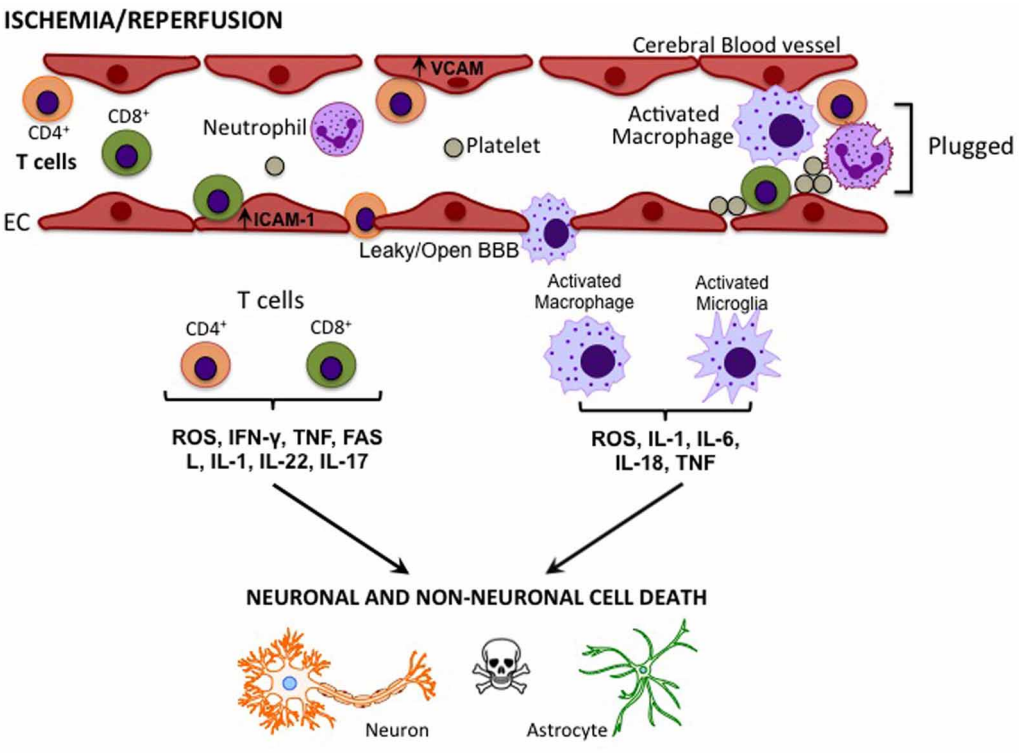

FIGURE 1 | Cytokines/effector molecules involved in post-stroke inflammation in the brain. Following cerebral ischemia-reperfusion, elevated levels of adhesion molecules, such as ICAM-1 and VCAM in vascular endothelial cells (EC), attract circulating T cells and macrophages. These immune cells may cross the compromised blood-brain barrier (BBB) to infiltrate the injured tissue and cause cell death via the release of various cytokines and effector molecules.

T lymphocytes induce apoptosis in already compromised neuronal cells following stroke (Barry and Bleackley, 2002; Brait et al., 2012), and both macrophages and activated microglia produce a number of pro-inflammatory cytokines and ROS to exert neurotoxic effects (Jin et al., 2010).

Interestingly, the spleen appears to play an important role in the acute influx of immune cells into the brain after stroke. For example, splenectomy prior to experimental stroke reduces infarct size (Ajmo et al., 2008). Moreover, these authors showed that splenectomy-induced neuroprotection following stroke correlated with a decrease in activated microglia, macrophages, and neutrophils in the ischemic hemisphere, thus suggesting that the spleen is a possible source of detrimental immune cells following stroke (Ajmo et al., 2008). In addition, Offner et al. found that T lymphocytes derived from blood and lymph nodes secreted increased levels of pro-inflammatory mediators, and expressed elevated levels of chemokine receptors post-stroke (Offner et al., 2006). More recently, it was shown that the spleen contributes to neurodegeneration following stroke through IFN-γ signaling (Seifert et al., 2012).

THE SYSTEMIC IMMUNE RESPONSE FOLLOWING STROKE

The immune response following stroke is not restricted to the brain, as effects on immune function are also seen in the periphery. While both innate and adaptive immune cells contribute to early post-stroke neuronal injury, the circulating levels of these cells are then rapidly reduced. For example a profound systemic immunodepression—or "stroke-induced immunodeficiency syndrome"—occurs as early as 12 h after ischemic stroke (Gendron et al., 2002; Prass et al., 2003). This phenomenon is triggered by hyperactivity of the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis due to post-stroke brain damage, which leads to reduced numbers of T and B lymphocytes and also of NK cells within the spleen, thymus, bone marrow, and lymph nodes (Gendron et al., 2002; Prass et al., 2003; Offner et al., 2006; Liesz et al., 2009). This leads to increased apoptosis and increased release of immune cells from these primary and secondary lymphoid organs, resulting in tissue atrophy and this consequently predisposes patients to infection (e.g., commonly resulting in pneumonia or sepsis), a major determinant of stroke morbidity and mortality.

Interestingly, although the liver is not a secondary lymphoid organ, a recent publication investigated the effect stroke had on invariant natural killer T (iNKT) cells within that organ (Wong et al., 2011). Wong and colleagues showed that cerebral ischemia-reperfusion slowed the migration of resident hepatic iNKT cells and increased the expression of the immunosuppressive cytokine, IL-10, in association with an increased susceptibility to bacterial infection. In support of these findings, the onset of bacterial infection occurred much earlier in iNKT cell-deficient mice subjected to stroke, whereas brain infarct size was unchanged. This indicated that iNKT cells play a key role in systemic protection against infection after stroke. Furthermore, those researchers found that the increased release of noradrenergic neurotransmitters from sympathetic nerves innervating the liver following stroke can undermine systemic immunity by a direct inhibitory effect on hepatic iNKT cells. Thus, the authors suggested that blockade of stress pathways could improve outcomes in stroke patients by helping to protect systemic immune function and thereby preventing infections. Collectively, evidence suggests that various components of the immune system in the brain as well as in secondary lymphoid and visceral organs may play critical roles in the development of post-stroke damage and mortality.

THE POTENTIAL FOR STEM CELL THERAPY FOLLOWING STROKE

Given the complex nature of post-ischemic brain injury and the failure of effective stroke treatments targeting single molecular pathways, ultimately successful approaches may include cell-based therapies that have the potential to target multiple injury mechanisms and cell types when administered at an appropriate time(s) after the stroke event. Hence, there is now considerable interest in stem cell therapy as a possible treatment for stroke. Stem cells are undifferentiated cells capable of self-renewal and are broadly classified as being of embryonic, fetal, or adult origin (Yu et al., 2009).

Embryonic stem cells (ESCs) are pluripotent, meaning that they can give rise to all cell types of the organism, whereas fetal and adult stem cells are multipotent, such that they can give rise to cells of multiple, but limited number of lineages. A variety of stem cells, including embryonic, bone marrow, neural, and induced pluripotent stem cells (iPSCs) have been shown to improve stroke outcome (Daadi et al., 2008; Schwarting et al., 2008; Hicks et al., 2009; Kawai et al., 2010). As a result of such promising studies, the first fully regulated clinical trial (PISCES study—Pilot Investigation of Stem Cells in Stroke) using ESCs to treat stroke patients has recently commenced in Glasgow. Unfortunately however, there tends to be major limitations with the use of most stem cell types, which may offset their use as a clinical treatment for stroke patients.

LIMITATIONS AND BENEFITS OF STEM CELL LINEAGES FOR TRANSPLANTATION INTO THE CNS

ESCs were expected to have broad potential due to their pluripotent capabilities, and transplantation of human ESC neural derivatives into a rodent model of stroke has been reported to improve functional outcome (Daadi et al., 2008; Hicks et al., 2009). Nevertheless, several problems exist regarding human ESCs, including ethical/political issues (i.e., due to the destruction of human embryos), immune rejection, and their fetal "age" (i.e., they lack key functional characteristics of adult cells) (Xi et al., 2010). Moreover, ESCs may form teratomas (developmental tumors) following transplantation (Knoepfler, 2009).

iPSCs were first derived in 2006 (Takahashi and Yamanaka, 2006) by re-programming mouse and human fibroblasts into pluripotent ESC-like cells. Since then, many types of iPSCs have been created using diverse cell types (Kiskinis and Eggan, 2010). iPSCs possess most of the key properties of ESCs but avoid the ethically controversial issues surrounding embryo destruction. These cells have been used to treat central nervous system (CNS) injuries such as spinal cord injury and stroke in rodents (Kawai et al., 2010; Tsuji et al., 2010), but in both cases tumor formation from iPSCs was observed.

Fetal neural stem cells (NSCs) are derived from human fetal brains (isolated from aborted material) and are capable of differentiating into neurons, astrocytes, or oligodendrocytes (Lindvall and Kokaia, 2011). Because of the invasive nature of obtaining autologous adult human neural cells, fetal NSCs have been evaluated as an alternative expandable source of neural cells. Although NSCs also involve ethical issues, these cells were considered to be safer than human ESCs regarding tumor formation after transplantation, however, brain and spinal cord tumors have been reported to develop following fetal NSC treatment (Dirks, 2001; Schmidt et al., 2005; Amariglio et al., 2009).

Mesenchymal stem cells (MSCs), derived from bone marrow or umbilical cord blood, can differentiate into neuronal-like cells, astrocytes, or endothelial cells, and their administration can reduce infarct volume and improve functional outcome in experimental stroke (Wu et al., 2008; Liu et al., 2009). Transplantation of MSCs can also reduce apoptosis and promote endogenous cellular proliferation after stroke, and long-term follow-up data have revealed improved survival in patients that received bone marrow MSCs compared with controls (Lee et al., 2010). Similar to ESCs, however, concerns and limitations associated with MSC use in stroke include poor cell survival and engraftment after transplantation, no direct evidence of functional neuronal differentiation, limited sources, and the fact that their extraction from bone marrow requiring invasive procedures, although they do not appear to form tumors after transplantation (Zimmermann et al., 2003).

HUMAN AMNION EPITHELIAL CELLS

hAEC characteristics

While the above stem cell types may certainly have therapeutic potential if their respective issues can be addressed, currently those limitations seriously offset their likely routine use in clinical stroke. An alternative stem cell lineage that is gaining interest as a potential stem cell therapeutic is the human amnion epithelial cell (hAEC). hAECs are derived from the amniotic sac, a thin avascular tissue that encloses the fetus and is attached to the placenta. The amnion consists of an inner layer of epithelial cells that is in direct contact with the amniotic fluid, referred to as the amniotic epithelium. Directly beneath the epithelial layer is the amniotic mesoderm, which includes a compact stromal layer and also a fibroblast layer. These two cell types have a different embryological origin. hAECs are derived from the embryonic ectoderm and amniotic mesodermal cells originate from the embryonic mesoderm (Parolini et al., 2008). Whilst amniotic mesodermal cells are also a potential cell-based therapy for stroke, this review will limit its focus to the potential of hAEC therapy.

A considerable advantage of hAECs over other stem cell lineages is that they possess very few of the limitations of other stem cell types outlined above (see **Table 1**). hAECs are easily obtained from separating the amnion sac from the term placenta, which are usually discarded after birth (Miki et al., 2005). As such, hAECs are readily available, they require no invasive procedure for harvesting, and they largely lack ethical barriers to their use (Yu et al., 2009). Furthermore, native hAECs do not express the polymorphic antigens HLA-A, HLA-B and HLA-C (class IA), and HLA-DR (class II), on their surfaces (Akle et al., 1981; Terada et al., 2000) but express the non-polymorphic, non-classical human leukocyte antigen G (HLA-G) (Houlihan et al., 1995), which does not elicit an immune response but rather suppresses it. Thus, hAECs are considered to be immunologically inert and would thus be expected to have a very low risk of rejection upon transplantation. These properties are, of course, consistent with the functions of the amnion to protect the fetus from the mother's immune system and to secrete various nutritive factors (Liu et al., 2008). Moreover, hAECs have low tumorigenicity (Miki et al., 2005) because they lack telomerase, an enzyme that preserves chromosomal sequences commonly lost during successive cell division (Hiyama and Hiyama, 2007). It would therefore be expected that hAECs are unlikely to promote tumor formation in the recipient.

Table 1 | Beneficial characteristics of five major stem cell lineages.

Benefits	ESCs	BMSCs	iPSCs	NSCs	hAECs
Readily available	X	✓	✓	✓	✓
Do not require invasive extraction	✓	X	✓	X	✓
Pluripotent properties	✓	X	✓	X	✓
Differentiate into functional neural tissue	✓	X	✓	✓	✓
Non-immunogenic	X	[a]✓	X	X	✓
Immunomodulatory properties	✓	✓	✓	✓	✓
Non-tumorigenic	X	✓	X	X	✓

[a] *Autologous transplantation only.*

ESCs, embryonic stem cells; BMSCs, bone marrow-derived stem cells; iPSCs, induced-pluripotent stem cells; NSCs, neural stem cells; hAECs, human amnion epithelial cells.

Due to the fact that amnion epithelial cells originate from the epiblast, from which they separate early in embryonic development (day 8), hAECs possess a high level of pluripotency. For example, hAECs can differentiate into all three germ layers: endoderm, ectoderm, and mesoderm (Toda et al., 2007). Notably, they can generate clinically relevant cell types, such as myocytes (including cardiomyocytes), osteocytes, adipocytes, pancreatic cells, hepatocytes, as well as neural, and astrocytic cells (Toda et al., 2007). These latter cell types are, of course, of particular importance for treating stroke. More specifically, hAECs may express markers of glial and neuronal progenitor cells and display multiple neuronal functions, such as synthesis and release of acetylcholine, catecholamines, and neurotrophic factors (Elwan and Sakuragawa, 1997; Bailo et al., 2004). Recent studies have shown that hAECs can facilitate neuroregeneration in CNS disorders such as Parkinson's disease (Kakishita et al., 2000). Thus, there is good reason to predict that hAECs may exert a neuroprotective effect if administered after stroke, but they have so far received little attention as a potential stroke therapy. Before further considering this potential, we will briefly summarise the known effects of hAECs in CNS diseases.

hAECs in the treatment of CNS diseases

In animal models of CNS disorders, accumulating evidence suggests that hAECs can exert neuroprotection and facilitate

neuroregeneration. For example, hAECs transplanted into the striatum of a rat model of Parkinson's disease were found to not only survive but were functional (i.e., producing dopamine) and prevented neuronal degeneration (Kakishita et al., 2000, 2003). In addition, after injection of hAECs into the transection cavities of a primate model of spinal cord injury, those cells survived for up to 60 days during which there was no evidence of inflammation, suggesting that the cells can avoid immunological rejection within the CNS (Sankar and Muthusamy, 2003). Furthermore, improved performance in locomotor tests was observed in hAEC-treated animals compared to lesion control animals, suggesting neuroprotection and improved function of the motor neuron tracts controlling locomotion. It has also been reported that administration of hAECs assists in the penetration of host axons, and completely abolishes glial scar formation in rats with spinal cord injury (Wu and Hui, 2006). More recently, McDonald et al. reported preliminary findings that intraperitoneal injection of hAECs can suppress clinical symptoms, as well as decrease CNS inflammation, demyelination and axonal degeneration in the spinal cord and brain of a experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (McDonald et al., 2011). These authors also found that hAECs can reduce proliferation of myelin oligodendrocyte glycoprotein-specific T cells, and also decrease their secretion of pro-inflammatory cytokines, IFNγ, and TNF. Interestingly, these data point to a possible immunomodulatory mechanism by which amnion epithelial cells can suppress the development of EAE. Consistent with these findings, Liu et al. recently reported that intravenously administered hAECs reduced infiltration of T lymphocytes and monocyte/macrophages, and consequently attenuated demyelination, within the CNS of an EAE mouse (Liu et al., 2012). These authors demonstrated that this effect was due to the secretion of transforming growth factor-β and prostaglandin E2 from hAECs to suppress splenocyte proliferation. As a further example of the beneficial neuroprotective effects of hAECs, conditioned media collected from these cells has been found to be neurotrophic for rat cortical cells (Uchida et al., 2000), and to support the survival of chicken neural retinal cells (Tcheng et al., 1994). Overall, there is mounting evidence that hAECs may have substantial protective and regenerative properties that could be amenable to the treatment of neurological diseases.

hAECs in the treatment of stroke

Thus far, only one published study has tested the effect of hAECs on ischemic stroke outcome (Liu et al., 2008). It found that direct intra-cerebral (i.c.) injection of hAECs, 24 h after middle cerebral artery occlusion (MCAO) in rats, resulted in a reduced infarct volume and improved behavioral and neurological outcomes at 16 days post-stroke. Furthermore, apoptosis—as detected via cleaved caspase-3 levels—was reduced in the vicinity of the transplanted cells. It is worth noting that the same research group has also reported that i.c. injection of human amnion mesenchymal cells can similarly improve stroke outcome in rats (Tao et al., 2012). In analogous studies to those in ischemic stroke, intraventricular injection of hAECs was reported to reduce brain edema and to improve motor deficit in a rat model of i.c. hemorrhage (Dong et al., 2010). Moreover, intra-cerebroventricular transplantation of amniotic fluid-derived stem cells at 3 days post-MCAO resulted in the attenuation of stroke-associated cognitive deficits (Rehni et al., 2007).

Despite these very promising early experimental findings, i.c. injection of stem cells still seems unlikely to become a feasible routine method of delivery for stroke patients. Reasons for this include the fact that i.c. injections would require routine access to suitable imaging facilities and surgical expertise, and in any case they may involve significant adverse effects, such as the breakdown of the BBB and a heightened inflammatory response within the brain (McCluskey et al., 2008). Furthermore, any protective effects from i.c. injection of stem cells will presumably be localized to the immediate region of brain and would not also target the detrimental systemic/immunological effects of stroke. Therefore, future studies would ideally employ a less invasive and more clinically amenable delivery route of stem cells, such as intravenous (i.v.) injection.

As i.v. administration is a minimally invasive procedure, it poses a substantially lower risk of adverse clinical events when compared to i.c. transplantation. In fact, Tarjiri and colleagues have reported that i.v. administration of amniotic fluid-derived stem cells at 35 days post-stroke significantly reduces infarct damage and behavioral deficits as assessed at 60–63 days after MCAO (Tajiri et al., 2012). There is therefore great scope to further explore the ability of hAECs to limit injury and/or promote tissue repair and functional recovery when administered systemically following stroke.

Potential mechanisms of action by hAECs in the treatment of stroke

There are various possible mechanisms by which hAECs might exert therapeutic effects following stroke. Firstly, hAECs could secrete neurotrophic factors that promote neuronal recovery of damaged cells in the penumbra (Lindvall and Kokaia, 2004; Liu et al., 2008). Such factors could also promote synaptogenesis to re-innervate lost connections. Secondly, as hAECs have pluripotent properties, they could differentiate into a neuronal phenotype and replace damaged or dead cells (Lindvall and Kokaia, 2004; Liu et al., 2008). Thirdly, hAECs could act as "biological minipumps" within the CNS, secreting necessary cytokines, growth factors, hormones, and/or neurotransmitters to restore cellular function. Lastly, hAECs could potentially improve stroke outcome by modulating the inflammatory response that contributes to brain injury (Lindvall and Kokaia, 2004; Meisel and Meisel, 2011). Included in this mechanism is the protection of neurons from immune cell-mediated apoptosis (see **Figure 2**).

Immunomodulatory properties of hAECs

hAECs can exert immunomodulatory actions by actively suppressing T lymphocyte proliferation, reducing the expression of the potent pro-inflammatory cytokines IL-1α and IL-1β (Solomon et al., 2001), and via producing inhibitors of MMPs and proteolytic enzymes associated with inflammatory reactions. In addition, although expression of HLA-G on hAECs enables their evasion of the immune system, this protein has also been shown to be anti-inflammatory by inducing apoptosis of activated $CD8^+$ T lymphocytes and inhibiting $CD4^+$ T lymphocyte proliferation (Banas et al., 2008). Furthermore, hAECs transplanted to the ocular surface can create a local environment that reduces

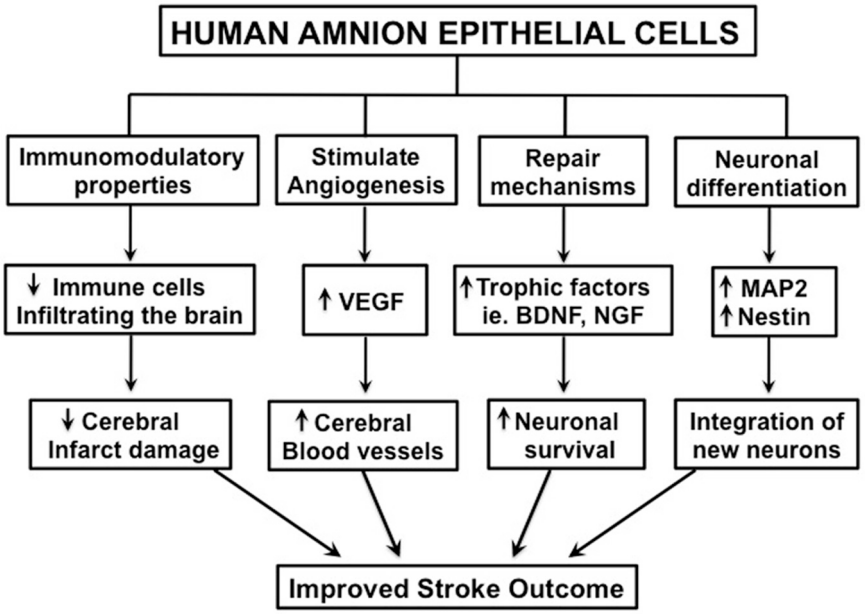

FIGURE 2 | Schematic diagram illustrating the potential mechanisms by which human amnion epithelial cells may improve stroke outcome. "↑" = increased; "↓" = decreased.

the surrounding inflammatory response (Hori et al., 2006). This effect is thought to be due to hAECs reducing infiltration of major histocompatibility complex class II antigen-presenting cells into the inflamed cornea. Moreover, we have demonstrated that hAECs transplanted into a bleomycin-induced lung injury model reduces the immune response, preventing lung fibrosis and loss of function (Moodley et al., 2010; Murphy et al., 2011). These results were associated with an *in vivo* reduction in the pro-inflammatory cytokines, TNF, IFNγ and IL-6, and an increase in the anti-inflammatory cytokine, IL-10 (Murphy et al., 2011). As a consequence of these actions of hAECs on the immune system, there is a reduction in the infiltration of immune cells to the area of damage.

hAECs are believed to secrete a number of immunomodulatory factors. In fact, supernatant from hAEC culture can inhibit both innate and adaptive immune cells (Li et al., 2005). For example, hAECs produce alpha-fetoprotein, a protein that reduces immune cell reactivity and suppresses neuroinflammation in a mouse model of multiple sclerosis (Irony-Tur-Sinai et al., 2009). Furthermore, hAECs secrete macrophage inhibitory factor, which inhibits neutrophil and macrophage migration and natural killer cell-mediated cytolysis (Li et al., 2005). Fas ligand and TNF-related apoptosis-inducing ligand are both members of the TNF family that are produced by hAECs, can regulate the immune response through apoptosis of lymphocytes (Li et al., 2005). Moreover, hAECs express transforming growth factor-β, which suppresses immune cell numbers through apoptosis as well (Li et al., 2005). Overall, the immunomodulatory properties of hAECs lead us to speculate that these stem cells may be able to limit the inflammatory response that contributes to infarct formation following stroke.

Migration of intravenously injected hAECs after stroke

Due to the acute nature of stroke onset, an i.v. injection is ideal so that therapeutics can be administered quickly after the event. However, i.v. administration of stem cells has two initial obstacles that must be overcome: (1) the ability of the cell to pass through the extensive capillary network of the lungs; and (2) whether the cells can effectively home to stroke-affected regions of tissue in sufficient numbers to provide efficacy. Whether this may occur remains to be tested, but the relatively small diameter of hAECs (8–15 μm) probably increases the likelihood of these cells passing through the lungs, compared with larger stem cell lineages, such MSCs, which do not easily passage across the lungs (Fischer et al., 2009). Indeed, we have reported that only a minor percentage of i.v.-injected hAECs persist in the lungs of control mice, and even in mice in which lung injury has been induced using bleomycin (Moodley et al., 2010). Thus, it is conceivable that i.v.-administered hAECs may have minimal impact on lung function and that a substantial proportion of these cells can pass into the systemic circulation.

Stem cells communicate with each other and their environment via paracrine signaling (Burns et al., 2009). In order to understand why and how cells migrate to their target organs, the relevant chemotactic signal(s) must be identified. While very little is known about the chemotaxis response involved in hAEC migration from the circulation following i.v. transplantation, several studies have defined the mechanisms that attract other types of stem cells to injured sites following stroke. For example, it has been shown that there is an increase in levels of stromal cell-derived factor-1α (SDF-1α) in brains of experimental animal models of stroke (Hill et al., 2004; Robin et al., 2006) and a subsequent decrease in stem cell migration after the addition of

an antagonist of the chemokine receptor type 4 (CXCR4) (Robin et al., 2006; Wang et al., 2008). SDF-1α is a growth factor produced by multiple types of mouse and human neural cells, and which functions as a chemokine that is thought to be important for neural progenitor migration during development. It is well-documented that the chemokine interaction between SDF-1α and CXCR4, its cognate receptor commonly expressed on the surface of stem cells, plays a major role in stem cell migration (Robin et al., 2006; Wang et al., 2008). More research is required to clarify whether CXCR4 and/or other factors play a role in hAEC homing and signaling pathways.

Ability of hAECs to engraft and differentiate and/or promote neuronal repair

Stem cell therapy was initially considered to be an opportunity for treating stroke patients by ultimately replacing dead neurons with new neurons in the post-stroke infarcted brain. As indicated, hAECs can indeed differentiate toward a neural lineage, which may ultimately add to their potential for post-stroke therapy (Elwan and Sakuragawa, 1997; Bailo et al., 2004; Yu et al., 2009). In fact, i.c. injections of hAECs in rats at 24 h after MCAO were found to migrate to ischemic areas and to then express astrocyte (glial fibrillary acidic protein) and neuronal markers (microtubule-associated protein 2 and nestin) (Liu et al., 2008). Correlating with these observations, the hAEC-treated rats showed improved behavioral and neurological outcomes, as well as reduced infarct damage. Thus, the authors postulated that the functional improvement following hAEC treatment may have been partially due to the newly differentiated neuron-like cells re-establishing connections with surviving host neurons. Similarly, in a hemorrhagic stroke model, hAECs transplanted into the brain were found to express neuron-specific antigens and to improve motor deficits after 4 weeks (Dong et al., 2010). As a further example, i.v. administration of amniotic fluid-derived stem cells resulted in an increased number of cells expressing microtubule-associated protein 2, and the cell proliferation marker, Ki67, in the dentate gyrus and in the subventricular zone of stroked animals, indicating increased neurogenesis (Tajiri et al., 2012). Collectively, the existing evidence supports the concept that hAECs can undergo neural differentiation *in vivo*. Future studies must identify if these newly formed neurons are functional and might be able to integrate within the existing network of cells to substantially replace dead tissue.

As indicated above, secreted paracrine factors may play a key role in hAEC-mediated recovery after stroke. If administered as a delayed post-stroke treatment, it is postulated that hAECs could improve long-term stroke outcome via the release of factors that promote re-innervation, thus restoring synaptic transmitter release to stimulate plastic responses, orchestrating rescue and repair processes, and improving or preserving survival and function of existing neurons. Indeed, it has been shown that hAECs can secrete trophic factors such brain-derived neurotrophic factor, neurotrophin-3, nerve growth factor (Sakuragawa et al., 1996; Meng et al., 2007), and novel epidermal growth-like factors (Venkatachalam et al., 2009). Whilst repair of stroke-induced damaged neurons has yet to be described, hAECs have shown an ability to promote recovery of injured tissue and facilitate functional plasticity in other diseases of the CNS. For example, transplanted hAECs produce neurotrophic substances and stimulate repair and regeneration of host neurons in a primate model of spinal cord injury (Sankar and Muthusamy, 2003). In addition, hAECs transplanted into the cerebral lateral ventricle of a transgenic mouse model of Alzheimer's disease were found to rescue damaged cholinergic neurons (Xue et al., 2012). The authors reported that hAEC treatment increased the number of cholinergic neurons, as well as the level of acetylcholine produced by these cells, which was suggested to be largely responsible for the reversal of cognitive decline in this animal model. Thus, in such a manner, hAECs may possess an ability to both repair and replace lost neuronal tissue and, together with their other anti-inflammatory characteristics, they may represent a promising cell-based clinical therapy for neurodegenerative diseases, including stroke.

CONCLUSIONS

In summary, hAECs appear to have several advantages over other stem cell lineages as a cell-based therapy, particularly their non-immunogenic and non-tumorigenic properties. There is now evidence that hAECs can cross the BBB where they can engraft, survive for up to 60 days, differentiate into neurons, reduce inflammation and promote regeneration of damaged CNS tissue in animal models of neurological diseases. We suggest that a future concerted experimental focus to characterise the efficacy of post-stroke hAEC therapy may yield valuable information that could be routinely applied in the clinical setting.

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