STEM CELLS
TRANSLATIONAL MEDICINE

Grafted human pluripotent stem cell-derived cortical neurons integrate into adult human cortical neural circuitry

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Abstract

Several neurodegenerative diseases cause loss of cortical neurons, leading to sensory, motor, and cognitive impairments. Studies in different animal models have raised the possibility that transplantation of human cortical neuronal progenitors, generated from pluripotent stem cells, might be developed into a novel therapeutic strategy for disorders affecting cerebral cortex. For example, we have shown that human long-term neuroepithelial-like stem (lt-NES) cell-derived cortical neurons, produced from induced pluripotent stem cells and transplanted into stroke-injured adult rat cortex, improve neurological deficits and establish both afferent and efferent morphological and functional connections with host cortical neurons. So far, all studies with human pluripotent stem cell-derived neurons have been carried out using xenotransplantation in animal models. Whether these neurons can integrate also into adult human brain circuitry is unknown. Here, we show that cortically fated lt-NES cells, which are able to form functional synaptic networks in cell culture, differentiate to mature, layer-specific cortical neurons when transplanted ex vivo onto organotypic cultures of adult human cortex. The grafted neurons are functional and establish both afferent and efferent synapses with adult human cortical neurons in the slices as evidenced by immuno-electron microscopy, rabies virus retrograde monosynaptic tracing, and whole-cell patch-clamp recordings. Our findings provide the first evidence that pluripotent stem cell-derived neurons can integrate into adult host neural networks also in a human-to-human grafting situation, thereby supporting their potential future clinical use to promote recovery by neuronal replacement in the patient's diseased brain.

KEYWORDS

cerebral cortex, human, iPS cells, neural circuitry, regeneration, transplantation

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1 | INTRODUCTION

Brain injury, stroke, epilepsy, and chronic neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis are associated with loss or dysfunction of cortical neurons, leading to motor, sensory, and cognitive deficits.[1-4] Effective treatments to restore function are lacking. Novel strategies to replace the lost neurons and reestablish their afferent and efferent connections in order to reconstruct damaged neural circuitries are highly warranted. Recent studies in animal models provide evidence that this might be possible using cell transplantation. Mouse embryonic cortical neurons implanted into photolytically injured visual cortex of adult mice were shown to receive specific inputs from host neurons.[5] Grafted neurons extended axons, reaching proper targets, and exhibited functional properties indistinguishable from those of the original cells in the visual cortex. Similarly, visual cortical neurons, generated from mouse embryonic stem (ES) cells and transplanted into the ibotenic acid-injured adult mouse visual cortex, reestablished reciprocal synaptic connections with targets of damaged cortex.[6] Some of the grafted neurons responded to visual stimuli. Also human ES cell-derived visual cortical neurons implanted in the same model sent axonal projections resembling the normal ones and could receive functional synaptic input from the host brain.[7]

We have previously demonstrated that cortically fated human-induced pluripotent stem (iPS) cell-derived long-term neuroepithelial-like stem (lt-NES) cells, transplanted into rats in the vicinity of a cortical stroke with massive loss of excitatory projection neurons, differentiate to mature functional cortical neurons.[8-10] These neurons receive afferents from a normal pattern of different brain areas including synaptic inputs from thalamus, which are activated by physiological sensory stimuli.[8,9] Grafted neurons send axonal projections to both hemispheres. These projections become myelinated and form excitatory, glutamatergic synapses on host cortical neurons.[10] Intracortical transplantation of human lt-NES-derived cortical neurons is associated with improvement of stroke-induced sensorimotor deficits,[8-10] at least partly due to nonneuronal mechanisms. Optogenetic inhibition of the grafted, integrated neurons indicates that their activity is involved in the regulation of the stroke-affected animals' motor behavior.[10]

Taken together, available results from several studies in different animal models raise the possibility that transplantation of human cortical neuronal progenitors generated from iPS or ES cells might be developed into a novel therapeutic strategy for disorders affecting cerebral cortex. However, when considering a potential future clinical application, it should be noted that all available studies with human pluripotent stem cell-derived neurons have been carried out using xenotransplantation in animal models. Thus, whether such neurons can survive long-term, differentiate to the appropriate subtype, establish connections, and influence host brain function after transplantation also into the human CNS is unknown. In fact, the only solid in vivo evidence for anatomical and functional integration of human-derived neurons grafted into human brain has been obtained using intrastriatal implantation of human embryonic mesencephalic tissue,

Significance statement

Neuronal replacement by stem cell transplantation could become a novel therapy for neurodegenerative diseases. For example, human pluripotent stem cell-derived cortical neurons, transplanted into stroke-injured adult rat cortex, improve neurological deficits and establish functional connections with host neurons. However, all studies with human pluripotent stem cell-derived neurons have been conducted using xenotransplantation. This study demonstrates that such neurons form functional synaptic networks and differentiate to mature, layer-specific cortical neurons also when implanted onto organotypic cultures of adult human cortex. The findings support the clinical translation of pluripotent stem cell-derived cortical neurons for neuronal replacement in human disorders affecting cerebral cortex.

rich in dopaminergic neuroblasts, in patients with Parkinson's disease.[11]

Here, we have explored, using organotypic slice cultures of adult human cortex, the behavior of human cortically fated lt-NES cells, previously tested in experimental cortical stroke,[8-10] after ex vivo transplantation into the adult human brain tissue environment. We demonstrate, for the first time, that grafted human pluripotent stem cell-derived cortical progenitors survive, develop into different phenotypes of mature cortical neurons, exhibit electrophysiological properties of functional neurons, and establish afferent and efferent synaptic connections with the adult human cortical neurons. Our findings provide supportive evidence for the potential clinical translation of human pluripotent stem cell-derived cortical progenitors as a new tool to reconstruct neural circuitry in human disorders affecting cerebral cortex.

2 | MATERIALS AND METHODS

2.1 | Derivation of iPS cells and lt-NES cell lines

Human iPS cell-derived lt-NES cells were produced as previously described.[12-14] Briefly, human dermal fibroblasts were subjected to sendai virus transduction with the reprogramming factors Oct4, Sox2, KLF4, and c-MYC (CytoTune iPS 2.0 Sendai Reprogramming kit, Invitrogen) and split into plates with mouse embryonic fibroblasts. Colonies were picked and expanded to establish iPS cell lines in feeder-free conditions using mTeSR medium (Invitrogen). On day 0 of neural induction, iPS cells were split using dispase (0.5 mg/mL) in order to collect the whole colonies. Colonies were resuspended in embryoid body (EB) medium (Dulbecco's modified Eagle medium/F12 [DMEM/F12], 10% KSR, 2-Mercaptoethanol [1:1000], nonessential amino acids [NMEAA] [1:100], Glutamine [1:100]) and plated into ultra-low-

attachment culture dishes to generate EBs. The EB medium with freshly dissolved Rock inhibitor, 3 μM Dorsomorphin (Sigma-Aldrich) and 10 μM SB431542 (Sigma-Aldrich) was changed daily. On day 5, EBs were collected and plated on 0.1 mg/mL poly-L-ornithine and 10 mg/mL laminin (both from Sigma) coated six well plates in EB medium with 3 μM Dorsomorphin and 10 μM SB431542. On day 6, medium was changed to N2 medium (DMEM-F12 [without Hepes, +Glutamine], N2 [1:100], glucose [1.6 g/L]) supplemented with 1 μM Dorsomorphin and 10 ng/mL bFGF. Six days later, neural rosettes were carefully picked and grown in suspension in N2 medium with 20 ng/mL bFGF. On day 14, neural rosette spheroids were collected and dissociated with trypsin. The small clumps obtained were grown in adhesion on poly-L-ornithine/laminin-coated dishes in the presence of 10 ng/mL bFGF, 10 ng/mL EGF (both from Peprotech) and B27 (1:1000, Invitrogen). The iPS cell-derived lt-NES cell line was routinely cultured and expanded on poly-L-ornithine/laminin-coated plates into the same media and passaged at a ratio of 1:2 to 1:3 every second to third day. lt-NES cells used for transplantation into rat or ex vivo human tissue were transduced with a lentiviral vector carrying green fluorescent protein (GFP) under constitutive promoter (GFP+ lt-NES cells).

2.2 | Generation of cortical neurons

Differentiation of lt-NES cells to neurons with a cortical phenotype was performed as previously described.[8] Briefly, growth factors (bFGF, EGF) and B27 were omitted and lt-NES cells were cultured at low density in differentiation-defined medium (DDM) containing DMEM/F12 with glutamine (Sigma) and supplemented with N2 (1x), NMEAA (0.1 mM), sodium pyruvate (1 mM), bovine serum albumin (500 mg/mL), and 2-mercaptoethanol (0.1 mM) in the presence of bone morphogenetic protein 4 (BMP4) (10 ng/mL, R&D Systems), wingless-type MMTV integration site family, member 3A (Wnt3A) (10 ng/mL, R&D Systems), and cyclopamine (1 mM, Calbiochem) for 8 days. Neural progenitors were then dissociated and plated on glass coverslips in BrainPhys/DDM (1:1) medium supplemented with B27 (1:50 without retinoid acid, Invitrogen).

Derivation of human embryonic neurons was obtained from cerebral cortex of aborted human embryos according to guidelines approved by the Lund-Malmö Ethical Committee, as described in Reference 15.

2.3 | Animals and surgical procedures

Adult (225-250 g) male Sprague-Dawley (SD) rats (Charles River) were used. All procedures were conducted in accordance with the European Union Directive (2010/63/EU) and were approved by the ethical committee for the use of laboratory animals at Lund University and the Swedish Board of Agriculture (Dnr. M68-16). Focal ischemic injury in cerebral cortex was induced by distal middle cerebral artery occlusion,[16-18] and intracortical implantation of cortically fated lt-NES cells

was performed stereotaxically 48 hours later as described previously.[8,9] A total of 300 000 cells were implanted into somatosensory cortex in close proximity to the ischemic area.

2.4 | Organotypic cultures of adult human cortex

Healthy neocortical tissue was obtained with informed consent by resection of a small piece of the middle temporal gyrus from patients undergoing elective surgery for temporal lobe epilepsy (n = 7, both genders, median age 35 years) according to guidelines approved by the Regional Ethical Committee, Lund (Dnr. H15 642/2008). The tissue slices were derived and handled as previously described.[15] Briefly, the surgically resected tissue was immediately kept in ice-cold modified human artificial cerebrospinal fluid and sliced on a Vibratome (Leica VT1200S). Slices of 300-μm thickness were transferred to inserts containing Alvetex scaffold membranes (Reinnervate) in six well plates filled with slice culture medium (BrainPhys medium, Stemcell) supplemented with B27, Glutamax (1:200), Gentamycin (50 μg/mL) (Life Technologies), and incubated in 5% CO2 at 37°C. The organotypic slices were kept in culture for at least 1 week before ex vivo transplantation of GFP+ lt-NES cells.

2.5 | Coculture of lt-NES cells with human organotypic cortical slices

GFP+ lt-NES cells were detached at day 8 of differentiation and resuspended into 50 μL of pure cold Matrigel Matrix (Corning). After partially removing the medium, 10 μL of the suspension mix (1 000 000 cells) were collected into a cold glass capillary and injected as small drops stabbing the semi-dry slices at various sites. Three slices were transplanted with 10 μL of suspension leading to approximately 300 000 cells per slice. Additional medium was added 30 minutes later to fully immerse the organotypic culture. The medium was changed once a week and coculture was maintained for 4 to 8 weeks before electrophysiology recordings or fixation.

2.6 | Electrophysiology

Cortically fated lt-NES cells were grown on coverslips and transferred to the recording chamber for in vitro recordings. Acute slices of adult rat brain or human cortex were prepared following published protocols.[19,20] Whole-cell patch-clamp recordings were performed as described[15] with a double patch-clamp EPC10 amplifier (HEKA) using PatchMaster for data acquisition. Data were analyzed offline with FitMaster, IgorPro, and NeuroMatic. Action potential (AP) threshold was detected as the onset of the AP, AP amplitude was measured from the AP threshold to the AP peak, AP rise time was determined as the time from the AP threshold to the AP peak, the half AP amplitude width was determined as the time between the rising and decaying phase of the AP measured at half the amplitude of the AP,

and the afterhyperpolarization (AHP) was determined as the difference between the AHP peak and the AP threshold.

2.7 | Calcium imaging

Recordings of spontaneous activity in cell culture were analyzed with NETCAL.[21] Active neurons were identified as bright circular objects over the images and their fluorescence brightness along time extracted. These individual fluorescence traces were then normalized as DF/F0 (%) = 100 · (F − F0)/F0, where F0 is the fluorescence value at rest. The timing of neuronal activations (spikes) was inferred with the Schmitt trigger method, which scanned the fluorescence traces for events that first passed a high threshold (set as +2 standard deviations [SDs] above the mean of the trace) and then remained elevated above a second lower threshold (+1 SDs) for at least 2 seconds.

Functional connectivity among neurons was computed on the inferred spike trains using generalized transfer entropy (GTE),[22,23] and by setting a significance threshold of the mean +2 SDs of the joint GTE distribution. The analysis provided the connectivity adjacency matrix, which was visualized as a spatial map with Gephi. Community analysis was carried out using the Brain Connectivity Toolbox[24] in combination with the Louvain's algorithm.[25] The number and size of network communities were portrayed along the diagonal of the adjacency matrix.

2.8 | ΔG-rabies vector production and tracing analysis

The construct for the tracing vector was purchased from AddGene (ID: 30195). High-titre preparations of lentiviral particles were produced according to protocol from Dull and coworkers (Dull et al, 1998) in a biosafety level 2 environment. Pseudo-typed rabies vector was produced following the protocol described previously[26] performing minor adjustments. In the studies of afferent and efferent projections, the lt-NES cells and the adult human cortical tissue, respectively, were first transfected with tracing vector followed by transfection with modified rabies virus. The TVA receptor and rabies GP in the lentiviral tracing vector are expressed under Syn-I promoter and, therefore, after transfection of adult human cortical tissue with this vector, only neurons will express the transgene. Viral infection to study afferent synaptic inputs was carried out by addition of 5 μL of lentiviral tracing vector on lt-NES cell culture. Cells were transplanted afterward and 5 μL of 5% ΔG-rabies vector was added on top of the organotypic slices 8 weeks after grafting. To analyze efferent synaptic outputs, 5 μL of tracing vector were added on the organotypic slices before lt-NES cell ex vivo transplantation. At 8 weeks after grafting, 5 μL of 5% ΔG-rabies vector were added on top of the human coculture. Organotypic cultures were fixed 1 week after ΔG-rabies vector addition, and staining was performed to visualize starter and traced neurons.

2.9 | Immunohistochemistry

Cortically fated lt-NES cells plated on glass coverslips were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Rats were sacrificed 2 months after transplantation and brains were fixed overnight at 4°C in 4% PFA before slicing. The organotypic slices were fixed overnight with 4% PFA at 4°C.

To perform staining of in vitro cell culture and rat brain slices, after blocking with 5% of normal donkey serum, primary antibodies were applied overnight in blocking solution at 4°C (Supplementary Table S2) followed by 2 hours incubation of fluorophore-conjugated secondary antibodies (1:200, Molecular Probes or Jackson Laboratories) at room temperature. Nuclei were stained with Hoechst (ThermoFisher Scientific) for 10 minutes and sections were mounted with Dabco mounting medium.

For staining of organotypic cultures, slices were incubated for 12 hours at 4°C in permeabilization solution (0.02% BSA + 1% Triton X-100 in phosphate-buffered saline [PBS]) and overnight at 4°C in blocking solution (5% normal serum +1% BSA + 0.2% Triton X-100 in PBS). Primary antibodies (Supplementary Table S2) were diluted in blocking solution and incubated for 48 hours at 4°C. Secondary antibodies were applied in blocking solution for 48 hours at 4°C. Finally, nuclei were stained with Hoechst for 2 hours before sections were mounted.

Antigen retrieval was performed in all stainings when antibodies recognizing nuclear cortical markers (Tbr1, Satb2, CDP/Cux1, Ctip2, and Brn2) were used. Cells or organotypic cultures were kept in sodium citrate (pH 6.0) with Tween 0.05% for 30 minutes at 65°C.

Images were obtained using bright field (IX51, Olympus, Germany), epifluorescence (BX61, Olympus, Germany), and laser scanning confocal (LSM 780, Zeiss, Germany) microscopes and a Virtual Slide Scanning System (VS-120-S6-W, Olympus).

2.10 | Immuno-electron microscopy (iEM)

Organotypic cultures were fixed with 2% PFA and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Immunostaining and processing of the sample were performed as described.[9] GFP/DAB-positive lt-NES cells were identified based on intense black DAB reaction product within the cytoplasm, whereas nucleus and mitochondria were DAB negative. Sections were mounted on grids, examined, and photographed using a transmission electron microscope JEM-100CX (JEOL, Japan).

2.11 | Statistical analysis

Statistical analysis was performed using Prism 7 software (GraphPad). Unpaired t test was used when data were normally distributed, whereas Mann-Whitney test was used when data did not pass the normality test. Significance was set at $P < .05$. Data are mean ± SEM.

3 | RESULTS

3.1 | Human lt-NES cell-derived cortical neurons form functional synaptic networks in cell culture

Our previous studies demonstrated the capacity of intracortically grafted lt-NES cell-derived progenitors, fated to a cortical phenotype, to differentiate to cortical excitatory neurons and establish afferent and efferent functional projections to cortical and subcortical areas in the stroke-injured rat.[8-10] In this study, we used a new lt-NES cell line which was generated from dermal fibroblasts by optimizing the protocol developed by Koch et al[27] and combining it with dual SMAD inhibition to ensure more efficient neural conversion[12,14] (Supplementary Figure S1A). We first demonstrated that the new cell line

FIGURE 1 Human cortically fated long-term neuroepithelial-like stem (lt-NES) cells generate functional, mature neuronal networks in vitro. A, Confocal immunohistochemical images showing presence of PSD95 and synapsin I in a Map2 positive lt-NES cell (arrow) after 8 weeks of differentiation. Ho, Hoechst. Scale bars = 20 μm for the merged and 10 μm for the isolated images. B-G, Calcium imaging studies performed on days 55 to 60. B, Representative spontaneous activity traces. Blue arrowheads depict activity events (spikes). C, Representative frame of the recording with the regions of interest (ROIs) detected for analysis (yellow squares). Scale bar = 100 μm. D, Raster plot of network activity with 16.8 ± 0.4 firings/neuron per hour. E, Adjacency matrix of neuron-to-neuron effective connections. Functional communities are highlighted in the diagonal and reflect neurons that tend to coactivate together. F, Effective network. Nodes correspond to neurons and links to effective connections. Color of the nodes depicts connectivity degree k (number of incoming and outgoing connections), as stated in the scale below. G, Raster plot of network activity in response to glutamate (arrow), reflecting specific synchronous response. H, Current traces illustrate presence of spontaneous excitatory postsynaptic currents (sEPSCs) in lt-NES cells cocultured with mouse astrocytes and in human embryonic cortical neurons (hCtx). sEPSCs were isolated in the presence of 1 μM TTX and 100 μM PTX, and abolished upon addition of 50 μM D-APV and 5 μM NBQX. I, sEPSC frequency, amplitude, coefficient of variation (CV), and decay time, tau, are illustrated as bar diagrams. *$P < .05$. Recordings and analysis are representative of four replicates with similar results
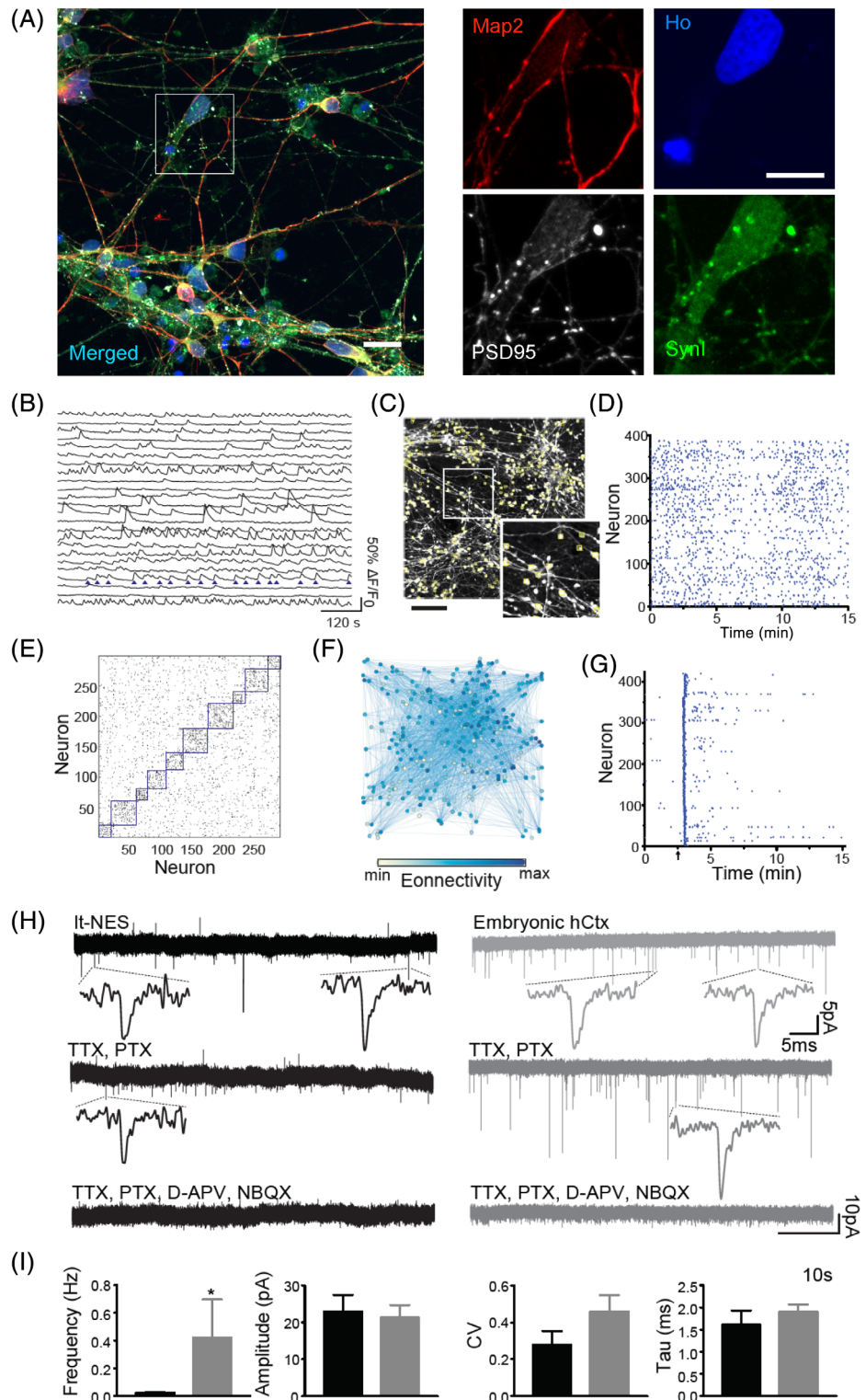
(Supplementary Figure S1B,C) expressed neural markers similar to those described for the lt-NES cell line used in our previous studies.[13] We then committed the lt-NES cells to a cortical neuronal phenotype in vitro following the protocol by Tornero and coworkers.[8] In accordance to previous findings,[8] the generated cells expressed markers of different subtypes of cortical neurons (Supplementary Figure S2A-C)

and were functional as evidenced by patch-clamp recordings (Supplementary Figure S3A-G). The cortically fated lt-NES cells differentiated to functional neurons also in vivo after transplantation into stroke-injured rat cortex (Supplementary Figure S4A-E).

We explored the ability of the lt-NES cell-derived cortical neurons to form synaptic connections and establish neuronal networks after
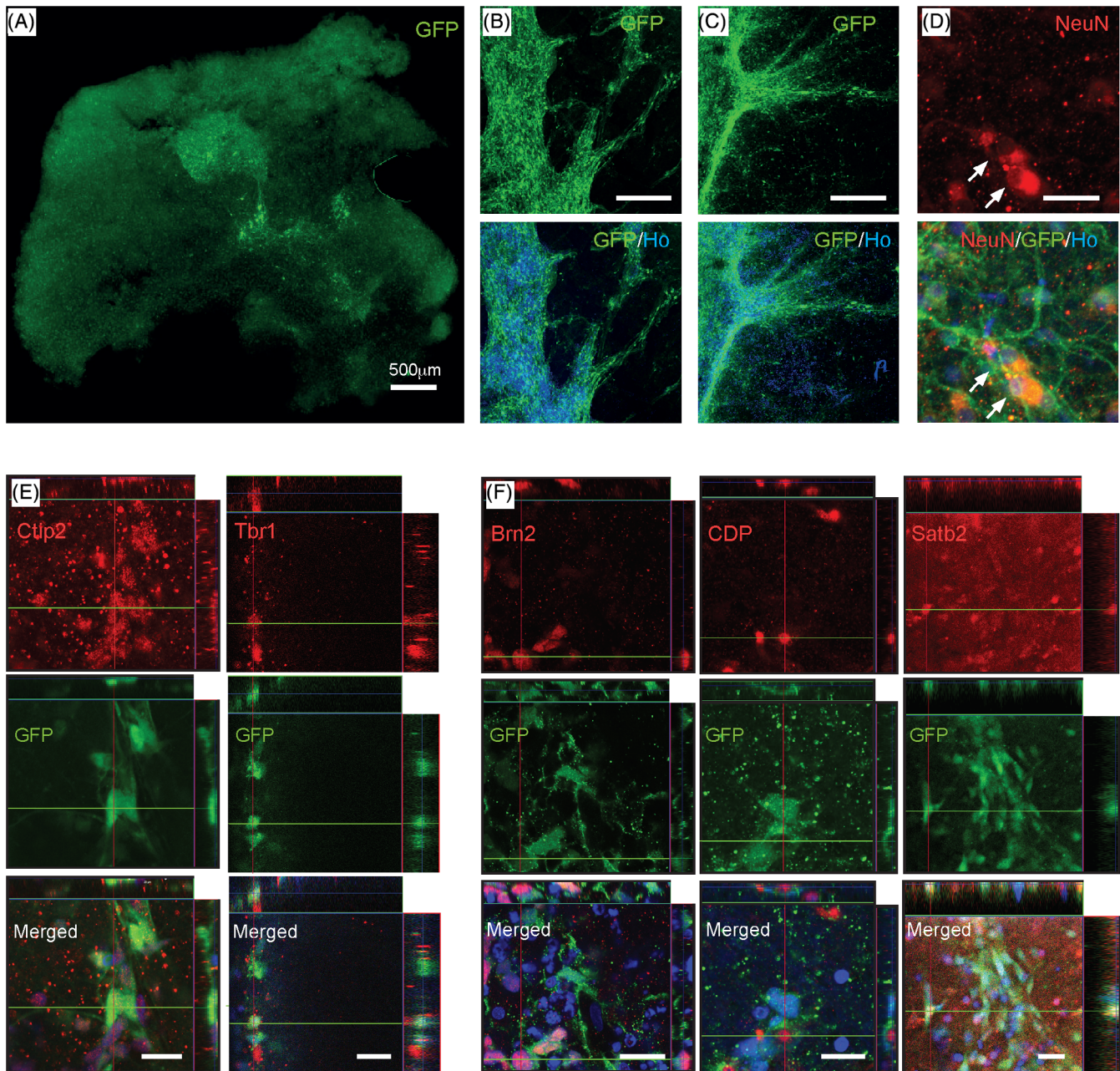

FIGURE 2 Human cortically fated long-term neuroepithelial-like stem (lt-NES) cells survive long-term and express markers of cortical neurons after ex vivo transplantation onto organotypic cultures of adult human cortex. A, Overview of an organotypic culture at 4 weeks after ex vivo transplantation of GFP+ lt-NES cells. B,C, Confocal images of the distribution of GFP+ lt-NES cells at (B) 4 and (C) 8 weeks after ex vivo transplantation. Note: images in A, B, and C are obtained from different slice cultures. Scale bar = 200 μm. D-F, Confocal images of grafted GFP+ lt-NES cells at 4 weeks after ex vivo transplantation showing presence of (D) the mature neuronal marker NeuN, (E) the deep-layer cortical markers Tbr1 and Ctip2, and (F) the upper-layer cortical markers Brn2, CDP, and Satb2. Nuclear staining (Ho: Hoechst, blue) is included in merged panel. Scale bar in D-F = 20 μm

8 weeks of differentiation. The pre- and postsynaptic markers Synapsin I and PSD95, respectively, were localized in close proximity in our lt-NES cell cultures (Figure 1A), providing evidence for the occurrence of synapses. We used fluorescence calcium imaging to monitor spontaneous activity in the network formed by the lt-NES cell-derived neurons and to quantify its functional traits. Neurons exhibited variations in calcium levels characteristic of mature neurons (Figure 1B). About 76% of all identified regions of interest (example in Figure 1C) were active. Analysis of fluorescence data provided onset times of neuronal activations (blue arrowheads) and basic statistics of network activity. Neuronal activity was rich and sustained along the recording (Figure 1D). As shown in the adjacency matrix of functional connections (Figure 1E), neurons tended to interact functionally in small communities of typically 25 neurons each (blue boxes along the diagonal of the matrix) that were not isolated from the rest of the network as evidenced by functional connections outside the diagonal. Functional analysis revealed that a neuron typically connected to 35 other neurons, both within the community and between communities. Network map indicated that functional connections could cross the entire network, illustrating the high integration of the system (Figure 1F). We note that communities are not shown in the map for clarity, since neurons belonging to the same community are physically distant, a trait that illustrates the complexity of network formation. Neurons with a high number of incoming or outgoing connections (darker colors) were uniformly spread, revealing a similar formation of connections across the culture (Figure 1F). Evoked responses to the addition of glutamate indicated the presence of receptors for this neurotransmitter in most neurons (Figure 1G).

Whole-cell patch-clamp recordings showed the presence of fast-decaying glutamatergic (Figure 1H, top trace) spontaneous excitatory postsynaptic currents (sEPSCs), which were inhibited by the presence of the NMDA and AMPA receptor antagonists, D-APV and NBQX (Figure 1H, bottom traces), in cortically fated lt-NES cells cocultured with mouse astrocytes. This finding demonstrates the functionality of glutamatergic synapses on the lt-NES cell-derived cortical neurons. The frequency of sEPSCs in the lt-NES cell-derived cortical neurons (0.022 ± 0.005 Hz, n = 7) was significantly lower as compared with that in cultured embryonic human cortical neurons (0.422 ± 0.276 Hz, n = 13) in the absence of inhibitors (Figure 1H top traces and Figure 1I). In contrast, the sEPSC amplitude, coefficient of variance, and decay time were similar (Figure 1I). Thus, the sEPSCs recorded from lt-NES cell-derived cortical neurons exhibit similar size and shape as sEPSCs observed in cultured embryonic human cortical neurons. Taken together, our findings indicate that the lt-NES cell-derived cortical neurons probably form fewer glutamatergic synapses, as indicated by reduced sEPSC frequency, but exhibit characteristics similar to those of embryonic human cortical neurons.

3.2 | Grafted human lt-NES cell-derived cortical neurons establish afferent and efferent synaptic connections with adult human cortical neurons

We wanted to determine if grafted lt-NES cell-derived cortical neurons could integrate in adult human cortical circuitry. Cortically fated
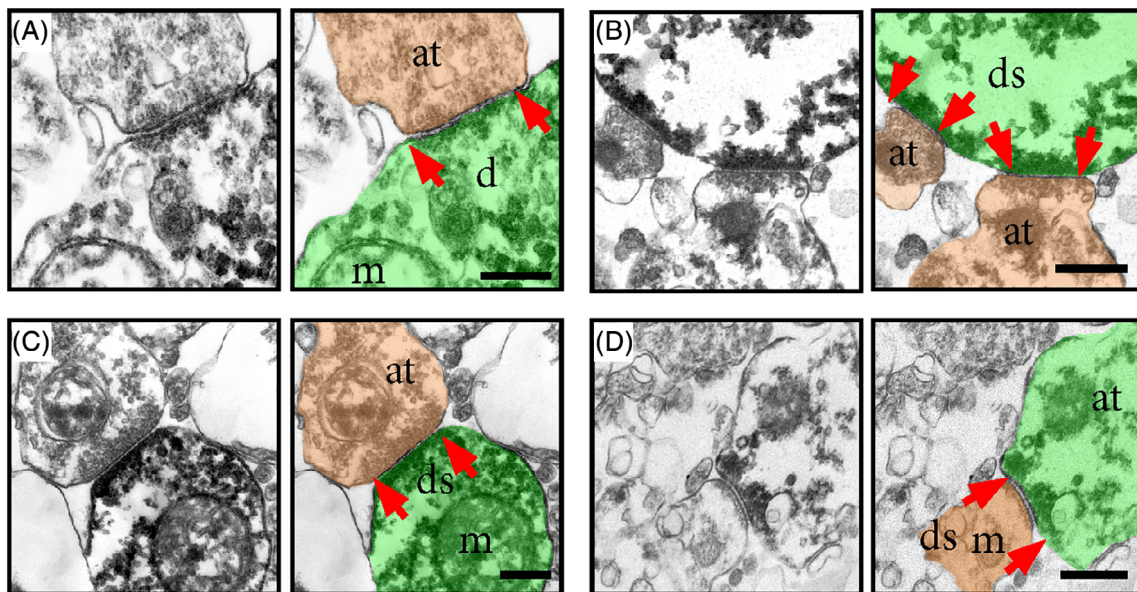

FIGURE 3 Grafted human long-term neuroepithelial-like stem (lt-NES) cell-derived cortical neurons establish afferent and efferent synapses with adult human cortical neurons in organotypic cultures. A-C, Representative immuno-electron microscopy (iEM) images showing asymmetric synapses with continuous postsynaptic densities (red arrowheads) in grafted GFP+ (green) lt-NES cell dendrites (d) or dendritic spines (ds), connected with host presynaptic axon terminals (at, brown) at 4 weeks (A,B) and 8 weeks (C) after ex vivo transplantation. D, GFP+ axon terminal (green) forming efferent synaptic contacts with host GFP− dendritic spines (brown). m, mitochondria. Note: For better visualization in the electron microscopical images, GFP/DAB-positive grafted cells and axon terminals were colored in green and adult cortical neurons and axon terminals in brown. Scale bar = 0.2 μm

GFP+ lt-NES cells were transplanted ex vivo onto organotypic slice cultures of adult human cortex, obtained from epileptic patients undergoing resective surgery, and kept in culture up to 8 weeks. We found that neurons and astrocytes in the organotypic slices were preserved after both 4 and 8 weeks of culture, as shown by the presence of the neuronal marker NeuN and the astrocyte markers GFAP and Vimentin, respectively (Supplementary Figure S5A,B). The grafted lt-NES cells exhibited extensive arborizations and extended neurites throughout the whole organotypic culture, also toward areas devoid of lt-NES cells at 4 and 8 weeks after ex vivo transplantation (Figure 2A-C). The lt-NES cell-derived neurons expressed the mature neuronal marker NeuN (Figure 2D) as well as markers of different cortical layers: the deep cortical layer markers Tbr1 and Ctip2 (layers V-VI) (Figure 2E), and the upper cortical layer markers Brn2, CDP/Cux1 (layers II-III), and Satb2 (layers II-V) (Figure 2F). Astrocytes derived from the lt-NES cells were also observed (data not shown). Our findings show that cortically fated lt-NES cells are able to generate mature neurons with characteristics of both upper and deep cortical layers not only in vitro or after grafting into stroke-damaged rat brain but also after ex vivo transplantation into the adult human cortical environment. Teratoma or secondary tumor formation was not observed.
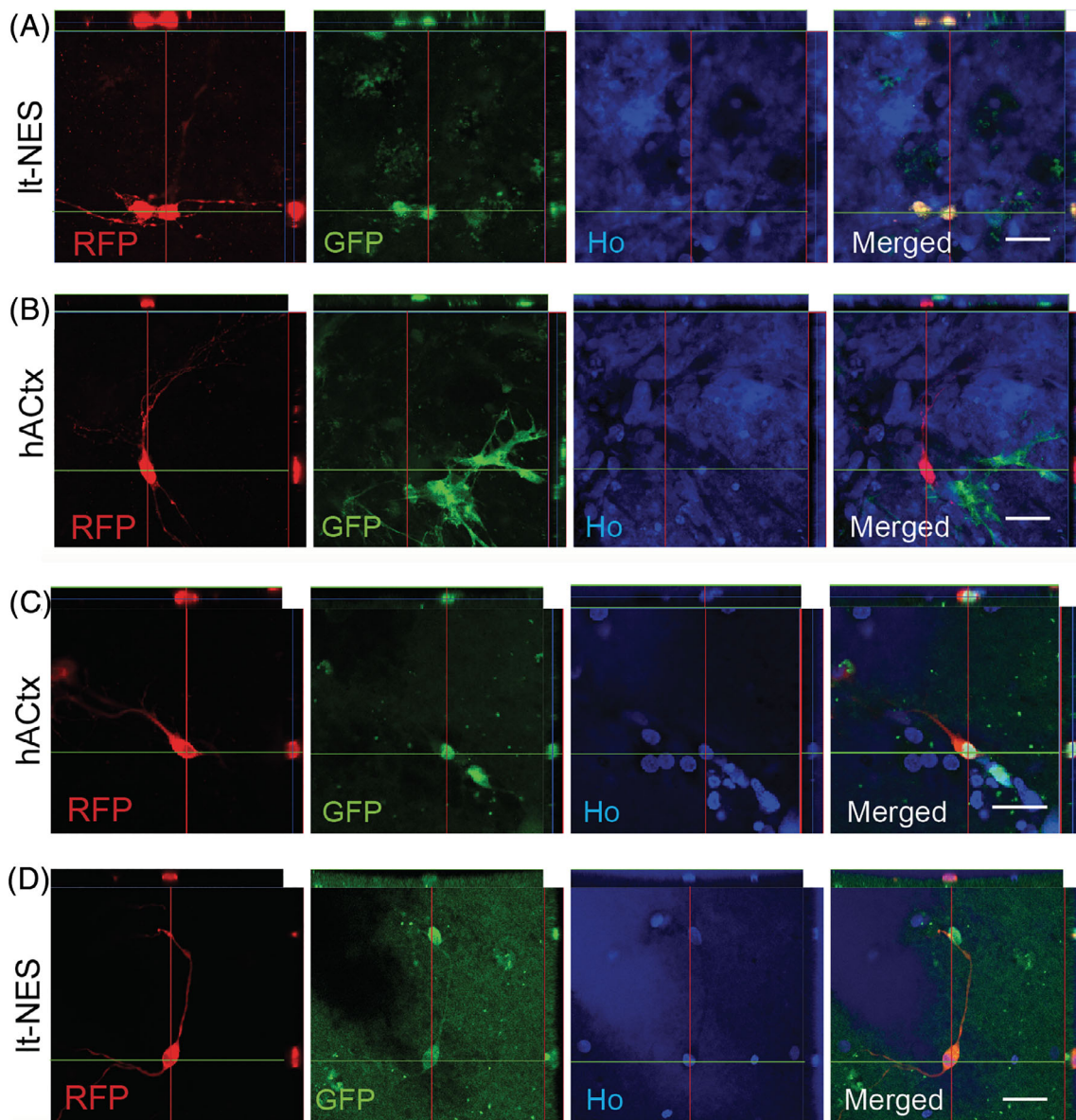

FIGURE 4 Grafted human long-term neuroepithelial-like stem (lt-NES) cell-derived cortical neurons form afferent and efferent connections with adult human cortical neurons in organotypic cultures. A,B, Grafted lt-NES cell-derived neuron infected with TVA and the retrograde tracing AG-Rabies vector (A, RFP [red] cytoplasm/GFP [green] cytoplasm/green nuclei) connects monosynaptically with adult human cortical neuron (hACtx) in the organotypic culture (B, red cytoplasm). C,D, Adult human cortical neuron infected with TVA/AG-Rabies vector (C, red cytoplasm/ green nuclei) establishes monosynaptic contact with grafted lt-NES cell-derived neuron (D, red cytoplasm/green cytoplasm). Nuclear staining (Ho, Hoechst, blue) is included in merged panel. Arrows indicate colocalization. Scale bar = 20 μm

iEM showed that the majority of the GFP+ lt-NES cell-derived cells in the organotypic cultures exhibited the ultrastructural features of mature neurons[28] at 4 weeks after ex vivo transplantation. The grafted neurons received axodendritic (Figure 3A) or axospinous (Figure 3B) inputs from GFP− axon terminals originating in the adult human cortical neurons. These contacts displayed the typical features of synapses, including clustering of synaptic vesicles close to the presynaptic membrane (more than four presynaptic vesicles within 100 nm of the presynaptic membrane), a clearly defined synaptic cleft, and postsynaptic membrane with evident postsynaptic densities (PSDs) (Figure 3A,B).

At 4 weeks after ex vivo transplantation, the vast majority (85.1%) of the spines of GFP+, lt-NES cell-derived neurons made contacts with several GFP− axon terminals (Figure 3B). Four weeks later,

FIGURE 5 Human cortically fated long-term neuroepithelial-like stem (lt-NES) cells become functional after ex vivo transplantation into organotypic cultures of adult human cortex. A, Post hoc identification of patched lt-NES cells at 4 (n = 13, left, black) and 8 (n = 13, right, blue) weeks after grafting (indicated by arrowhead). Individual and merged channels are shown. Scale bar = 50 μm. B, Representative voltage trace illustrating the ability of the lt-NES cell-derived neurons to generate action potentials (APs) during a current step of 150 pA at 4 and 8 weeks after ex vivo transplantation. C, Bar diagram illustrating maximum number of APs generated during current steps (10-200 pA in 10 pA steps) (*P < .05). Plot illustrating the number of APs against the current steps. D, APs induced by a current ramp (0-300 pA) from a holding potential of −70 mV at 4 and 8 weeks after ex vivo transplantation. First AP generated (*, expanded in the box) was used for determining AP characteristics. E, AP characteristics of lt-NES cell-derived neurons at 4 and 8 weeks after ex vivo transplantation. F, Expanded current traces illustrating the inward sodium current (denoted by *) activated during voltage steps ranging from −70 to +40 mV in 10 mV steps. The sodium current was blocked by the presence of 1 μM TTX. The plot illustrates the sodium current peak, unaltered between 4 and 8 weeks after ex vivo transplantation. G, Current traces illustrating the outward potassium current (*) activated during voltage steps ranging from −70 to +40 mV in 10 mV steps. The potassium current was blocked by the presence of 10 mM TEA showing no alteration between 4 and 8 weeks after ex vivo transplantation. H, Current traces illustrate the presence of spontaneous postsynaptic currents in lt-NES cell-derived neurons at 4 and 8 weeks after ex vivo transplantation. Nuclear staining (Ho, Hoechst, blue) is included in merged panel
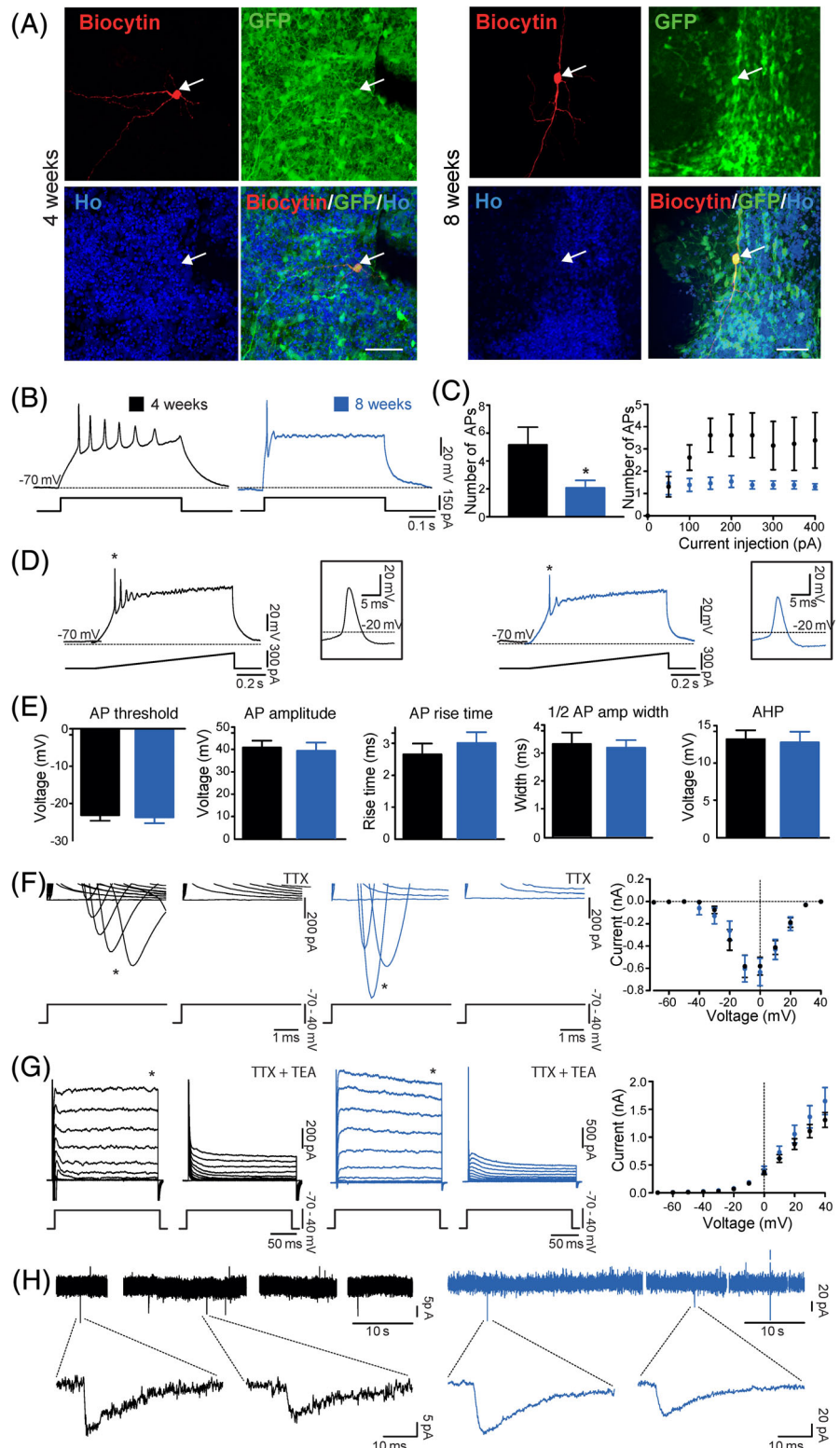
GFP+ spines formed synapses only with single GFP− axon terminals devoid of other synaptic partners (Figure 3C). At this time point, we observed a variety of mature synaptic inputs including axodendritic (96.2%) and axosomatic (3.8%) synapses. The iEM analysis showed that among axodendritic synaptic contacts, 87.2% of the axon terminals were in contact with dendritic spines (identified on basis of shape, size, and presence of a spiny apparatus) (Figure 3C). The other GFP− axons terminated on GFP+ dendritic shafts or on structures that could not be identified as small shafts or dendritic spines (12.8%). The axodendritic contacts exhibited the ultrastructural characteristics of asymmetric excitatory/glutamatergic synapses, such as a prominent PSD, wide synaptic cleft, and spherical synaptic vesicles (Figure 3C).

At both time points (4 and 8 weeks after ex vivo transplantation), we observed GFP+ terminals forming efferent synaptic contacts on GFP− adult human cortical neurons, but these contacts were much fewer (18.3%) as compared with the afferent inputs (81.7%) (Figure 3D). The efferent contacts, similar to the afferent ones, exhibited ultrastructural characteristics of asymmetric excitatory/glutamatergic synapses. The GFP+ axon terminals displayed spherical synaptic vesicles, and particularly docked vesicles at the presynaptic membrane, indicating the presence of a readily releasable pool of synaptic vesicles and functional activity of synapses (Figure 3D).[28]

To confirm that the grafted lt-NES cell-derived cortical neurons were able to establish afferent and efferent monosynaptic connections with the adult human cortical neurons, we used rabies virus retrograde tracing. The cortically fated lt-NES cells (to identify the afferent synaptic inputs on these cells) or the organotypic slice cultures (to disclose the efferent synaptic outputs) were transduced to stably express the TVA receptor under control of the human synapsin I promoter (tracing vector, Figure 4), making them susceptible to rabies virus infection. We found that the grafted lt-NES cell-derived neurons infected with TVA/△G-rabies vector (Figure 4A) established monosynaptic afferent inputs from adult human cortical neurons in the organotypic cultures (Figure 4B). Conversely, the adult human cortical neurons infected with TVA/AG-rabies vector (Figure 4C) received monosynaptic inputs from the grafted lt-NES cell-derived cortical neurons (Figure 4D). These results show that lt-NES cell-derived neurons are able to establish both afferent and efferent connections with adult human cortical neurons in organotypic cultures.

3.3 | Grafted human lt-NES cell-derived cortical neurons become functionally integrated with adult human cortical neurons

We then determined if the grafted lt-NES cell-derived cortical neurons were functional and responded to afferent synaptic inputs. Using whole-cell patch-clamp technique, we found that all 13 recorded lt-NES cell-derived cells exhibited similar basic electrophysiological characteristics (Figure 5A) at 4 and 8 weeks after ex vivo transplantation (Table 1). Already at 4 weeks, the cells were able to fire multiple APs (Figure 5B,C; Table 1). The AP

characteristics, that is, threshold, amplitude, rise time, half amplitude width and AHP, as well as the size of Na and K currents were similar at both time-points (Figure 5D-G; Table 1). Fast-decaying, most likely glutamatergic, postsynaptic currents with a frequency of 0.08 ± 0.04 Hz (n = 3) and 0.12 ± 0.09 Hz (n = 2) were detected at 4 and 8 weeks after grafting, respectively (Figure 5H). Taken together, our findings show that the human cortically fated lt-NES cells are able to differentiate to mature functional neurons and provide further evidence that they receive functional synaptic inputs after ex vivo transplantation onto adult human neocortical organotypic cultures.

Finally, we compared the electrophysiological characteristics of the lt-NES cell-derived cortical neurons with those of cortical neurons in acute slices of adult human brain tissue (Supplementary Figure S6A). We found that the adult human cortical neurons fired multiple APs (8.6 ± 0.9 APs during a 500 ms current step) (Supplementary Figure S6B). The basic AP characteristics were significantly different from those of the lt-NES cell-derived cortical neurons in the organotypic slices, except the AHP (Supplementary Figure S6C; Supplementary Table S1). Moreover, the adult human cortical neurons expressed 10 times larger Na current peak and 3 times larger K current peak as compared with the human lt-NES cell-derived neurons (Figure 5F,G; Supplementary Figure S6D,E). All adult human cortical neurons exhibited spontaneous fast-decaying, most likely glutamatergic, PSCs with a frequency of 0.50 ± 0.2 Hz (n = 7). In contrast, only a subset (3/13) of the lt-NES cell-derived cortical neurons exhibited sPSCs with a frequency approximately 1/5 of that of adult human cortical neurons (Figure 5H; Supplementary Figure S6F). Thus, after 4 weeks in organotypic cultures, the electrophysiological properties of the lt-NES cell-derived neurons differed significantly from those of cortical neurons in acute slices of adult human brain.

TABLE 1 Basic electrophysiological characteristics of cortically fated lt-NES cell-derived neurons at 4 and 8 weeks after transplantation onto organotypic cultures of adult human cortex

	ctx-NSPCs in situ	
	4 weeks (n = 13)	8 weeks (n = 12-13)
V_{rest}	−42.3 ± 8.3 mV	−44.1 ± 3.9 mV
R_{input}	1502 ± 232 MΩ	1003 ± 147 MΩ
C	2.0 ± 0.4 pF	6.7 ± 3.0 pF
Max # APs	5 ± 1	2 ± 1*
AP threshold (mV)	−23.2 ± 1.5	−23.7 ± 1.6
AP amplitude (mV)	40.9 ± 3.0	39.3 ± 3.7
AP rise time (ms)	2.7 ± 0.3	3.0 ± 0.3
½ AP amp. width (ms)	3.3 ± 0.4	3.2 ± 0.3
AHP (mV)	13.2 ± 1.2	12.8 ± 1.4

Note: Unpaired *t* test or Mann-Whitney test. * indicates significant difference between 4 and 8 weeks (Mann-Whitney *P* = .0194).
Abbreviations: AHP, afterhyperpolarization; AP, action potential; lt-NES, long-term neuroepithelial-like stem.

4 | DISCUSSION

Here, we present experimental evidence demonstrating, for the first time, that grafted human pluripotent stem cell-derived cortical neurons can integrate into adult human cortical neural circuitry. We have previously shown that cortically fated human lt-NES cell-derived neurons integrate morphologically and functionally into the stroke-injured rat brain after intracortical transplantation.[8-10] In this study, we found that these cells, which formed functional synaptic neuronal networks in cell culture, differentiated to mature, layer-specific cortical neurons when transplanted ex vivo onto organotypic cultures of adult human cortex. The grafted neurons were functional and established both afferent and efferent synapses with the adult human cortical neurons in the slices, as evidenced by iEM, rabies virus retrograde tracing, and by the occurrence of sEPSCs during whole-cell patch-clamp recordings.

The development of new therapies for human neurodegenerative disease, such as stem cell-based approaches, is to a large extent dependent on the use of animal models. In many cases it is unclear, though, whether the results obtained in such models can be translated to the adult human brain. Organotypic slice cultures of human brain tissue have become useful tools to explore the functional and morphological integration of transplanted neurons into adult human neuronal networks. This model system preserves cellular elements, morphological and electrophysiological parameters of pyramidal neurons, complexity of the neuronal three-dimensional architecture as well as synaptic connectivity and microenvironment.[29-31] However, mechanisms of integration can only partly be explored in organotypic slice cultures due to absence of components of the vascular and immune systems and somewhat decreased viability and survival of the neurons with long-term culturing. Moreover, the resected human tissue is subjected to severe injury response, involving proliferation of reactive cells and progressive neurodegeneration.[32,33] Nonetheless, recent studies show that it is possible to ameliorate such a response by optimizing the slice culture medium, which makes it possible to partially preserve long-term neuronal viability and robust electrophysiological single cell and network function.[34,35]

The long-term functionality and the afferent and efferent connectivity of the grafted human pluripotent stem cell-derived cortical neurons shown here in organotypic slices provide important evidence demonstrating their ability to survive, differentiate, and integrate into local neural networks in the adult human cortical tissue environment. In this regard, they resemble human embryonic dopaminergic neurons, transplanted into striatum of patients with Parkinson's disease. These grafted neurons can survive for many years and reinnervate the putamen with high specificity,[36] form synaptic contacts with host neurons,[37] and become integrated into neural circuitries in the patient's brain.[38] However, in Parkinson's disease, the human embryonic dopaminergic neuroblasts are implanted in the target area due to their inability to significantly reconstruct the nigrostriatal pathway after implantation in the substantia nigra.[39] In contrast, repairing the stroke-injured brain will necessitate the reformation also of long-distance pathways. For example, the monosynaptic, transcallosal projections of the grafted cortically fated lt-NES cells to the contralateral cortex are probably involved in their effects on motor function in the stroke-injured rat brain.[10] Such axonal projections are manyfold longer in the human as compared with the rodent brain. Currently, it is unknown whether the grafted human cortical neurons will have the capacity to form long-distance connections with other areas in the diseased human brain and if lack of guidance cues and presence of inhibitory molecules will produce an environment that is restrictive to axonal growth.

5 | CONCLUSION

The present findings are particularly interesting when considering moving stem cells toward the clinic in stroke and other disorders affecting the cerebral cortex. We provide the first evidence that human pluripotent stem cell-derived neurons integrate in adult host neural networks not only after xenotransplantation in rodents but also in a human-to-human grafting situation. Our study represents an early but important step in clinical translation of neuronal replacement strategies to promote functional recovery in the injured brain.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Marita Grønning Hansen: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Cecilia Laterza: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Sara Palma-Tortosa: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Giedre Kvist: collection and/or assembly of data, manuscript writing, final approval of manuscript. Emanuela Monni: collection and/or assembly of data, final approval of manuscript. Oleg Tsupykov: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Daniel Tornero: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Naomi Uoshima: collection and/or assembly of data, final approval of manuscript. Jordi Soriano: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Johan Bengzon: provision of study material or patients, final approval of manuscript. Gianvito Martino: provision of study material or patients,

final approval of manuscript. Galyna Skibo: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Olle Lindvall: conception and design, manuscript writing, final approval of manuscript. Zaal Kokaia: conception and design, manuscript writing, final approval of manuscript, financial support.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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