Human embryonic stem cell-derived neurons adopt and regulate the activity of an established neural network

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Whether hESC-derived neurons can fully integrate with and functionally regulate an existing neural network remains unknown. Here, we demonstrate that hESC-derived neurons receive unitary postsynaptic currents both in vitro and in vivo and adopt the rhythmic firing behavior of mouse cortical networks via synaptic integration. Optical stimulation of hESC-derived neurons expressing Channelrhodopsin-2 elicited both inhibitory and excitatory postsynaptic currents and triggered network bursting in mouse neurons. Furthermore, light stimulation of hESC-derived neurons transplanted to the hippocampus of adult mice triggered postsynaptic currents in host pyramidal neurons in acute slice preparations. Thus, hESC-derived neurons can participate in and modulate neural network activity through functional synaptic integration, suggesting they are capable of contributing to neural network information processing both in vitro and in vivo.

Results

hESC-Derived Neurons Adopt the Bursting Behavior of Mouse Cortical Networks via Synaptic Integration. To address whether hESC-derived neurons can fully integrate with an established neural network, we first used long-term cocultures with mouse cortical neurons from embryonic day 16 pups. A unique feature of these cultures is the presence of synchronized network activity referred to as “bursting” (Fig. 1C, i) (23, 24), which may arise from deafferentation because of limited numbers of tonically active neurons (25). Bursting has not been reported in hESC-derived neuron cultures (6, 17, 22), which continuously add tonically active neurons from progenitor cells when differentiated to a “default” dorsal forebrain phenotype (6, 17, 22). These cultures are primarily comprised of both glutamatergic and GABAergic neurons when plated alone or in coculture (6, 22) (Fig. S1A and B).

Current-clamp recordings from GFP-labeled mouse neurons cultured alone (Fig. 1A, Left) revealed the presence of spontaneous bursting as early as 7 d in vitro (DIV) (Fig. S2A, upper trace), which persisted for the duration of the experiment (Fig. S2A, lower trace). We thus considered cortical cultures that displayed bursting to qualify as an established neural network. For cocultures, we plated whole hESC-derived neuroepithelial aggregates (21 DIV) onto DIV mouse cultures. These aggregates contain dividing neural progenitors, as well as postmitotic neurons expressing Channelrhodopsin (ChR2)-mCherry (Fig. 1A, Right). Table S1 illustrates that, compared with measurements for human neurons, mouse neurons demonstrated significantly larger capacitance ($P < 0.001, n = 4$), lower input resistance ($R_{in}; P < 0.001, n = 4$), and more hyperpolarized resting membrane potentials (RMPs) ($P < 0.001, n = 4$) at each time point. Furthermore, mouse neurons had larger inward and outward voltage-gated currents at all times tested (Fig. 1B; 8-wk time point shown).

Similar to previous studies, current-clamp recordings from hESC-derived neurons plated alone (Fig. 1A, Center) showed no bursting activity after 6 wk of culture (Fig. 1C, ii) or at any time point recorded (2 wk; 0 of 24; 4 wk: 0 of 211; 6 wk: 0 of 67; 8 wk: 0 of 53). In contrast, human neurons in coculture with mouse cortical neurons displayed prominent bursting activity in coculture (Fig. 1C, iii and iv, and E). Importantly, bursting measured in current-clamp mode is indicated by AP generation (Figs. 1C and 2 and Figs. S1B and S4). However, we also use bursting generally to refer to any synchronized postsynaptic activity measured in voltage clamp, regardless of whether summation currents are observed. Because hESC-derived neurons demonstrated progressively hyperpolarized RMPs during the study period (Table S1), we quantified the proportion of bursting cells.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1108487108/-/DCSupplemental.
using voltage clamp at a holding potential of −70 mV. Under these conditions, we still observed bursting behavior in mouse (Fig. 1D, i) and human (Fig. 1D, ii) neurons in coculture but not in human neurons alone (Fig. 1D, ii). Fig. 1E illustrates that bursting was observed in few hESC-derived neurons in coculture for 2 wk, a majority of cells at 5 wk, and nearly all neurons after 6 and 8 wk of coculture. Interestingly, human cells displayed significantly smaller burst amplitudes than mouse neurons (Fig. S3A and B), even when corrected for differences in cell size (Fig. S3C). Lastly, we observed the presence of “superbursts” (25) in both mouse (Fig. S2B, upper trace) and cocultured human (Fig. S2B, lower trace) neurons, which exhibited long-duration depolarizations lasting multiple seconds with regenerative spiking. Thus, hESC-derived neurons can develop synchronized bursting activity but only in the presence of an established mouse cortical network.

To determine whether bursting activity in human neurons was attributable to functional integration with the existing mouse network (and not a newly generated human network), we used dual patch-clamp recording of neurons of both species cocultured for 6 and 8 wk. Fig. 2A and B illustrates the presence of nearly simultaneous bursting in a mouse (upper trace) and hESC-derived neuron (lower trace) when bursts were spontaneously generated by the culture. Similar results were obtained from two mouse neurons that also displayed simultaneous bursting (Fig. S4). All dual recordings in which both cells displayed bursting (n = 9) also demonstrated simultaneous bursting. Interestingly, activity in the mouse cell generally preceded that of the human cell by a mean duration of 61.4 ± 9.5 ms (Fig. 2B; range: 1.8–410.1 ms). Furthermore, all bursts recorded in both mouse and human neurons were eliminated by the application of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNOX) (Fig. 2F; 50 μM). Thus, hESC-derived neurons became part of the existing cortical network via excitatory synaptic integration.

hESC-Derived Neurons Regulate the Excitability of an Existing Mouse Cortical Network via Synaptic Output. We next asked whether human neurons could influence mouse network activity via synaptic output. A train of 10 light pulses delivered at 10 Hz to specifically activate hESC-derived neurons was sufficient to induce spiking in the human cell (Fig. 2C and D, lower trace) and bursting behavior in the mouse neuron (Fig. 2C and D, upper trace). Light-induced bursting was highly repeatable (Fig. 2C), and light-induced APs (Li-APs) in hESC-derived neurons preceded bursting activity in mouse cells by a mean duration of 73.7 ± 6.2 ms (range: 38.2–107.9 ms; Fig. 2D, see below). Furthermore, light-induced bursting mimicked the spontaneous bursting activity even when applied within seconds of a spontaneous burst (Fig. 2E). We hypothesized that light-induced bursting was attributable to multiple hESC-derived neurons simultaneously triggering PSCs in mouse cells. Indeed, in mouse cells in which light stimulation did not induce bursting, multiple PSCs were triggered immediately following the light pulse (Fig. S5A). In addition, dual patch-clamp recordings from two human neurons revealed simultaneous excitation upon light stimulation (Fig. S5B). Thus, human neurons are capable of regulating overall...
hESC-derived neurons display a reciprocal synaptic relationship with dissociated mouse cortical cultures. (A) Dual patch-clamp recordings from mouse (upper trace) and human (lower trace) that display repeatable, nearly simultaneous bursting in both cells when bursts are generated spontaneously by the culture. (B) Expanded time scale demonstrates that mouse activity preceded human activity (mean: 61.4 ± 9.5 ms). (C) Repeated 10-Hz light stimulations produced APs in hESC-derived neurons (Lower) and coincident bursting activity in mouse neurons (Upper). (D) Expanded time scale demonstrates that the first light stimulus preceded bursting by a mean duration of 73.7 ± 6.2 ms. (E) Dual patch-clamp recordings from a human (Upper) and mouse (Lower) neuron in which a spontaneous burst was triggered by the culture, followed by a light-induced burst generated by human neurons. (F) All spontaneous bursts and light-induced bursts (but not light-induced APs) were eliminated by application of CNQX (50 μM).

**Fig. 2.** hESC-derived neurons display a reciprocal synaptic relationship with dissociated mouse cortical cultures. (A) Dual patch-clamp recordings from mouse (upper trace) and human (lower trace) that display repeatable, nearly simultaneous bursting in both cells when bursts are generated spontaneously by the culture. (B) Expanded time scale demonstrates that mouse activity preceded human activity (mean: 61.4 ± 9.5 ms). (C) Repeated 10-Hz light stimulations produced APs in hESC-derived neurons (Lower) and coincident bursting activity in mouse neurons (Upper). (D) Expanded time scale demonstrates that the first light stimulus preceded bursting by a mean duration of 73.7 ± 6.2 ms. (E) Dual patch-clamp recordings from a human (Upper) and mouse (Lower) neuron in which a spontaneous burst was triggered by the culture, followed by a light-induced burst generated by human neurons. (F) All spontaneous bursts and light-induced bursts (but not light-induced APs) were eliminated by application of CNQX (50 μM).
cultures are likely glutamatergic (6, 17) (Fig. S1), our findings suggest that inhibitory neurons may functionally integrate more readily after transplantation.

Discussion

Here, we used optogenetic technology to definitively demonstrate that hESC-derived neurons are capable of complete synaptic integration with a preexisting network both in vitro and in vivo and can modulate the excitability of a network via synaptic output. In coculture with bursting mouse cortical neurons, hESC-derived neurons progressively adopted the same bursting behavior, whereby network-derived activation of human neurons led to depolarization and spiking behavior. We verified that the human neurons were part of the existing network via dual patch-clamp recording and that bursting in human cells was driven by synaptic activity via antagonism of glutamatergic neurotransmission. Furthermore, optical stimulation of ChR2+ human neurons caused excitatory and inhibitory postsynaptic responses in mouse neurons and could trigger bursting behavior. Lastly, activation of ChR2+ human neurons in slices taken from transplanted mouse brains revealed light-induced PSCs in pyramidal neurons.

The integration of hESC-derived neurons within an established network displays interesting parallels with the incorporation of nascent dentate granule cells (DGCs) in mature hippocampus. Newly born DGCs in the subgranular zone display physiological properties distinct from mature cells, such as elevated RMPs, high R \text{in}_o, reduced thresholds for long-term potentiation and depression (26, 27), and potentially increased excitability (28). hESC-derived neurons exhibit similarly depolarized RMPs, higher R \text{in}_o (Table S1) (6), and potentially increased excitability even after extended time periods (22). It will be interesting to know whether these properties are critical for integration and whether hESC-derived neurons display comparable synaptic plasticity characteristics to those of immature DGCs. Secondly, postsynaptic maturation, indicated by robust dendritic spine growth of newly born DGCs, occurs 3–4 wk after terminal differentiation (29). Our results demonstrate a significantly greater proportion of bursting cells (i.e., postsynaptically mature) after 4 wk compared with the 2-wk time point (Fig. 1E). Furthermore, it is thought that DGCs require 1–2 mo of maturation to fully integrate with the established circuitry in the hippocampus (28, 30, 31). Here, integration of hESC-derived neurons occurred over a similar time course, where significantly greater presynaptic integration (Fig. 3E) was observed after 6–8 wk than at earlier time points.

A similar time course is observed for the improvements in behavioral symptoms of neurodegeneration after stem cell transplantation, supporting the idea that synaptic integration is crucial for long-term outcomes of cell replacement in disease models (32). Significant effects of transplanted dopamine (DA) neurons on rotational behavior in Parkinson models are not typically observed at 4 wk after transplantation but are observed after 6–8 wk (7, 14), consistent with our findings for presynaptic innervation (Fig. 3E). In addition to the temporal correlation, hESC-derived neurons are capable of integrating synaptic
currents to produce spiking (Fig. 1C), can make excitatory and inhibitory connections with mouse neurons (Fig. 3C and D), and pre- and postsynaptically integrate in vivo (Fig. 4). Although these data suggest that hESC-derived neurons may participate in network information processing, future research is necessary to demonstrate a causal link between synaptic integration and the behavioral changes observed after transplantation.

The combination of directed neural differentiation of ESCs and iPSCs with optogenetics may have broad utility for evaluating the physiological mechanisms underlying outcomes of stem cell transplants. Similar to previous studies that examined synaptic connectivity between brain regions (33, 34), ChR2 expression could be used to map local and distant neuronal connections between transplanted and endogenous neurons in disease models. In vivo, the use of implantable light-stimulation devices (35) will give researchers unprecedented real-time access to examine the physiological underpinnings of successful cell replacement for neurodegenerative disorders. For instance, whereas the forebrain glutamatergic or GABAergic neurons used in this study may be useful for treatment of frontotemporal dementia, ischemia, or epilepsy, optogenetics can be used to target a number of potentially therapeutic populations such as midbrain DA neurons (7, 8) and spinal motor neurons (13). These methods may be particularly necessary to interrogate more subtle, modulatory effects of metabotropic transmitters such as DA (36), which has been the focus of many neuronal cell-replacement studies (37). The ability to stimulate multiple neurons simultaneously (Fig. S4) could allow for detection of the downstream consequences of DA release, such as its effect on sodium currents (38). This would allow for a direct demonstration of presynaptic integration of transplanted DA neurons, which has been historically difficult using traditional techniques (39).

Materials and Methods

Cell Culture and Transplantations. Animal experiments were carried out according to the protocols approved by the University of Wisconsin–Madison Animal Care and Use Committee. Mouse cortical neurons (embryonic days 14.5–16.5) were provided by Dr. E.W. Dent (University of Wisconsin–Madison), cultured according to previously published methods (40), and plated at a density of 5 × 10^4 cells/10-mm coverslip. hESCs (WA09; passages 24) were cultured and differentiated to neurons essentially as described previously (41), with the addition of B27 (1:200; Invitrogen), 37.5 mM NaCl, and 0.3% glucose to differentiation media (DM). For cocultures, four to five hESC-derived neuroepithelial aggregates (21 div) were plated onto 7div mouse cultures.

For transplantation experiments, SCID mice (8–10 wk of age) were anesthetized with 1% isoflurane mixed with oxygen and received 2 μL of 35div to 40 div cell suspension (5–7 × 10^6 cells/μL) unilaterally to the CA3 region of the hippocampus using the following stereotaxic coordinates: anterior–posterior = Q:12 ± 2.46 mm; left–right lateral = ±2.25 mm; and dorsoventral = −2.25 mm. Three to 4 mo following transplantation, mice were either killed for sectioning and staining according to previously published methods (15) or were prepared for ex vivo recordings.

Lentiviral Vectors and Transduction. Channelrhodopsin-2 constructs used were either the Syn-ChR2-mCherry described previously (22) or Syn-ChR2(H134R)-mCherry transfer vector created using methods described previously (41), by replacing the CamKIIa promoter with the synapsin-1 promoter. Lentiviral production and transduction, as well as the pGK-GFP lentivirus, have been described previously (42). Viral particles were concentrated by ultracentrifugation (SW28 rotor; Beckman Coulter) at 20,000 × g for 3 h, resuspended in DM and triturated using the Lenti-X qRT-PCR kit (Clontech). hESC-derived aggregates or 12-mm coverslips containing mouse neurons were incubated
with respective viruses (10^6pfu/mL) for 24 h and then washed with DM.

**Immunohistochemical Staining.** Immunolabeling of hESC-derived neurons was performed according to previously established methods (6, 15) using the following primary antibodies: polyclonal DiScRed (1:1,000; Millipore), monoclonal β-tubulin (1:1,000; Sigma), polyclonal GABA (1:1,000; Sigma), and a human-specific nuclear antibody (1:400; Millipore). To detect primary antibodies, we used Alexa-Fluor secondary antibodies (1:1,000; Jackson Immunoresearch) conjugated to fluorophores FITC, Cy3, and Cy5, which were visualized using a Nikon confocal workstation (D-Eclipse C1) running EZ-C1 software (version 3.5). 3D reconstruction and surface rendering were performed using Imaris software (version 7.3; Bitplane).

**Electrophysiological Recordings and Light Stimulation.** Whole cell patch-clamp recordings were performed as previously described (22), with the following modifications. The extracellular solution was a modified HBS that contained (in mM) 120 NaCl, 3 KCl, 1 MgCl_2, 15 Hepes, and 23 glucose (pH 7.4, 300 mOsm). The intracellular recording solution contained the following (in mM): 121 K-gluconate, 22 KCl, 10 NaHepes, 10 EGTA, and 4 Mg-ATP (pH 7.2, 290 mOsm). Pharmacological antagonists picrotoxin (50 μM) and CNQX (50 μM) and AP5 (25μM; Sigma) were applied using a gravity-fed drug barrel system or bath applied via extracellular solution. The number of neurons recorded at each time point ranged from 12 to 30 for each group (human and mouse). Acute slices from transplanted SCID mice were prepared according to previously published methods (22), using the intracellular solution described above.

Light stimulation was achieved by a custom-built LED device that used a ~950-mW blue light-emitting diode (LED) (470 nm; Thor Labs) coupled to a fiber optic cable that was placed 2–5 mm from ChR2-expressing neurons. Power to the LED was delivered through a current-controlled LED driver (Thor Labs). Light intensity could be modulated by a potentiometer and ranged from 0.1 to 1 mW/mm^2, with most stimulations using ~0.4 mW/mm^2. Triggered light pulses were controlled via the open source Arduino microcontroller platform (SmartProjects) with timing (time high and frequency) regulated by custom Arduino programs.

**Statistical Analyses.** One-way ANOVA followed by Newman–Keuls post hoc tests were used to determine whether mean differences between groups were different and were considered significant when P < 0.05. Data are reported as means ± SEM.

**ACKNOWLEDGMENTS.** We thank Erik W. Dent for providing mouse cortical cultures; Justin C. Williams and Tom Richner for support with our LED stimulation device; the Laboratory for Optical and Computational Instrumentation (LOCI), Kevin Eliceiri, Johannes Schindelin, and Jimmy Fong for assistance with 3D reconstructions; and Eva D. Zarnowska for helpful discussions. This study was supported by National Institutes of Neurological Disorders and Stroke Grants NS045926 and NS05778 and, in part, by National Institutes of Health Core Grant P30 HD03352 to the Waisman Center from the National Institute of Child Health and Human Development.

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Supporting Information

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Fig. S1. hESC-derived forebrain cultures contain significant numbers of GABAergic neurons. (A) Confocal images of 6-wk-old human cultures alone (Left) and mouse–human cultures (Right) that display GABA⁺ neurons (green). Note that in cocultures, GABA⁺ cells (arrows) and GABA⁻ cells (arrowheads) express the human-specific nuclear antigen (blue). (B) Pooled data demonstrate that hESC-derived cultures are comprised of ∼40% GABAergic neurons, which is not affected by coculture with mouse cortical cells.

Fig. S2. Spontaneous bursting is maintained in mouse cortical networks for at least 2 mo. (A) Representative whole cell recordings (−70-mV holding potential) from mouse cortical neurons at 1 wk (upper trace) and 8 wk (lower trace) in vitro. (B) Representative traces from mouse (Upper) and human (Lower) that illustrate “superbursts” in each cell type.
Fig. S3. Bursting amplitudes in hESC-derived neurons are significantly smaller than those in mouse neurons. (A) Representative voltage-clamp traces of individual bursts in hESC-derived neurons at 2 wk (i), 4 wk (ii), and 8 wk (iii) in coculture or bursts in a mouse neurons (iv). (B) Pooled raw data demonstrate a significantly smaller mean bursting amplitude in hESC-derived neurons compared with mouse cortical neurons (P < 0.001 for all time points; n = 4). (C) Pooled data normalized to cell size demonstrate a significantly smaller bursting amplitude in hESC-derived neurons at 2, 4, and 8 wk (*P < 0.05; n = 4). Data are means ± SEM.

Fig. S4. Mouse cortical neurons display simultaneous bursting. Representative current-clamp recordings from two mouse cortical neurons at 6 wk in vitro.
Fig. S5. Multiple postsynaptic currents are triggered in individual mouse neurons during light stimulation. (A) Whole-cell recording from a mouse cortical neuron during brief (10-ms) light stimulations. Lower trace reveals multicomponent, large-amplitude postsynaptic responses between ~10 and 100 ms following an individual light stimulation. (B) Whole-cell patch-clamp recordings from two hESC-derived neurons in coculture with mouse neurons for 8 wk illustrates simultaneous AP generation in both cells upon light stimulation.

Fig. S6. Spontaneous postsynaptic currents make light-induced currents difficult to detect in some neurons. Representative traces from two different mouse cortical neurons during light stimulation after 6 wk in coculture with ChR2-expressing human neurons.

Fig. S7. Morphology of transplanted hESC-derived neurons. (A) Compressed confocal z-stack of a transplanted hESC-derived neuron expressing both ChR2-mCherry and the human nuclear antigen. (B) 3D reconstruction of the neuron in A demonstrating multipolar morphology. Scale bars, 50 μm.
Table S1. Basic physiological properties of hESC-derived neurons in coculture with mouse cortical cultures

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H, human; M, mouse.
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