Correction

Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro

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Human cell reprogramming technologies offer access to live human neurons from patients and provide a new alternative for modeling neurological disorders in vitro. Neural electrical activity is the essence of nervous system function in vivo. Therefore, we examined neuronal activity in media widely used to culture neurons. We found that classic basal media, as well as serum, impair action potential generation and synaptic communication. To overcome this problem, we designed a new neuronal medium (BrainPhys basal + serum-free supplements) in which we adjusted the concentrations of inorganic salts, neuroactive amino acids, and energetic substrates. We then tested that this medium adequately supports neuronal activity and survival of human neurons in culture. Long-term exposure to this physiological medium also improved the proportion of neurons that were synaptically active. The medium was designed to culture human neurons but also proved adequate for rodent neurons. The improvement in BrainPhys basal medium to support neurophysiological activity is an important step toward reducing the gap between brain physiological conditions in vivo and neuronal models in vitro.

BrainPhys | tissue culture milieu | neurobasal DMEM | neuromedium | induced pluripotent stem cells

Induced pluripotent stem cell (iPSC) technology is currently being used to model human diseases in vitro and may contribute to the discovery and validation of new pharmacological treatments (1–3). In particular, neuroscientists have seized the opportunity to culture neurons from patients with neurological and psychiatric disorders and have demonstrated that phenotypes associated with particular disorders can be recapitulated in the dish (4–7). However, the basic culture conditions for growing neurons in vitro have not been updated to reflect fundamental principles of brain physiology. Currently, most human neuronal cultures are grown in media based on DMEM/F12 (4, 5, 7–24), Neurobasal (25–30), or a mixture of DMEM and Neurobasal (DN) (31–34). To promote neuronal differentiation and survival, a variety of supplements, such as serum, growth factors, hormones, proteins, and antioxidants, are typically added to these basal media. Although these media were designed and optimized to promote neuronal survival in vitro, they were not tested for their ability to support fundamental neuronal functions. Using several electrophysiological techniques such as patch clamping, calcium imaging, and multielectrode arrays, we found that widely used tissue culture media (e.g., DMEM basal, Neurobasal, serum) actually impaired neurophysiological functions.

We identified several neuroactive components in these media that acutely interfered with neuronal function. To solve these issues, we designed a chemically defined basal medium: BrainPhys basal. We used human neurons in vitro to demonstrate in a series of experiments that this new medium, combined with the appropriate supplements, better supports important neuronal functions while sustaining cell survival in vitro. Notably, BrainPhys-based medium better mimics the environment present

in healthy living brains, unlike previous media based on DMEM, Neurobasal or serum. Although BrainPhys basal was specifically designed for the culturing of mature human neurons, our studies also showed that BrainPhys provided a functional environment for ex vivo brain slices and for culturing rodent primary neurons.

Results

Electrophysiological recordings and calcium imaging are the prevailing techniques used to assess the functionality of neurons and may be acutely performed in perfusate of artificial cerebrospinal fluid (ACSF) in vitro or ex vivo. ACSF is a basic solution with neurophysiological concentrations of important inorganic salts, energy substrates, and phosphate buffers. We used ACSF as a standard to define the level of optimal electrophysiological activity in vitro. Although ACSF can sustain healthy neurophysiological activity for a few hours, it does not sustain long-term survival or growth in vitro. Indeed, even with the addition of growth factors and other supplements, we found that neurons did not survive in ACSF for more than a few days. Therefore, neurons are usually cultured in an incubator with basal medium such as DMEM/F12 or neurobasal and supplements.

DMEM/F12 Basal Medium Impairs Basic Activity of Human Neurons. We first examined the calcium activity of human neurons in fresh

Significance

Neuronal cultures are very valuable to learn about basic principles of the nervous system. In vivo, neural electrical activity is the essence of nervous system function, controlling emotion, memory, sensory modalities, and behavior. In this study, we discovered that many crucial neurophysiological properties were strongly altered in classic culture media that are widely used by the research community. To overcome this problem, we designed and tested a new tissue culture neuromedium that adequately supports in vitro neuronal activity. The improvements made in this medium reduce the gap between in vivo brain physiological conditions and neuronal models in vitro. Improving physiological conditions in vitro may lead to more successful translation from bench to clinics.

DMEM/F12+sup medium, in which they were cultured for several weeks (Fig. 1A). We found very few active human neurons in
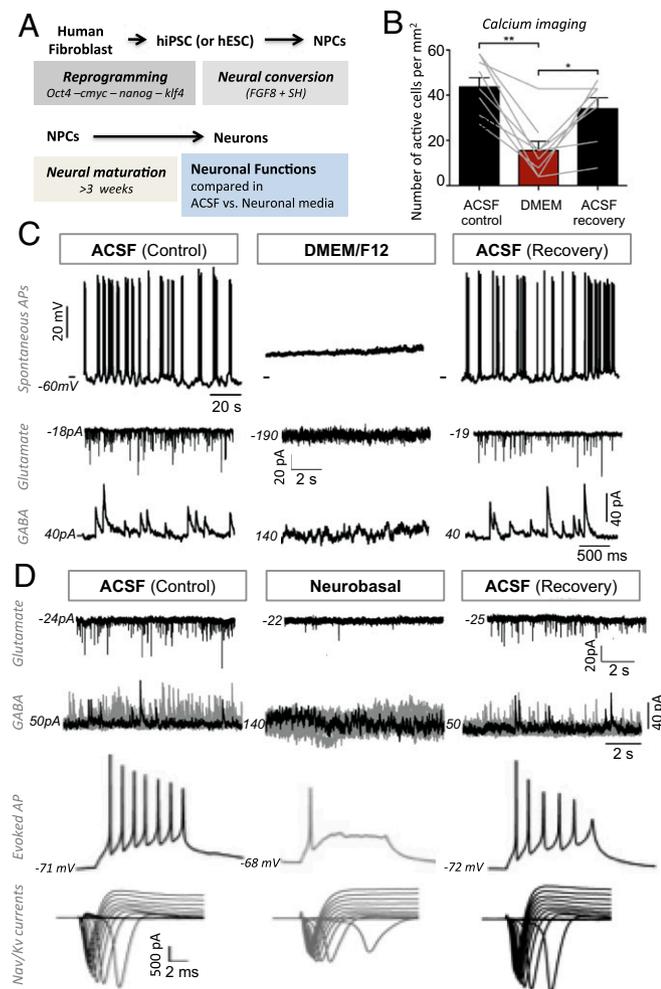

Fig. 1. DMEM/F12 basal and Neurobasal media impair neuronal and synaptic activity. (A) Human neurons derived from iPSCs or ESCs were plated directly on glass coverslips. The basic neuronal functions of single mature neurons were tested and compared in two classic extracellular basal media (DMEM/F12 or Neurobasal-A). The perfusion of extracellular media was switched from ACSF to DMEM/F12 or Neurobasal-A and back to ACSF for recovery. (B) Calcium imaging analysis showing the number of spontaneously active cells in 10-min movies of the same fields of view recorded in ACSF, DMEM/F12 ($n = 9$), and back to ACSF ($n = 8$). Inorganic salt concentration and the osmolarity of ACSF were matched to DMEM. The gray lines represent individual fields of view. Statistics were performed with Wilcoxon test two-tailed, **$P = 0.004$; *$P = 0.016$). See also detailed calcium spikes analysis in *SI Appendix*, Fig. S1A. (C) *Top traces*: Current-clamp recordings reveal that spontaneous APs observed in ACSF quickly disappear in DMEM, together with a large increase in resting membrane potential (*SI Appendix*, Fig. S1). Middle traces: Voltage-clamp recordings (at −70 mV, Cl⁻ reversal potential) reveal that DMEM-based medium induces a large Na⁺ inward current and completely blocks spontaneous AMPA synaptic activity. Bottom traces: Voltage-clamp recordings (at 0 mV, Na⁺ reversal potential) reveal that DMEM-based medium induces large Cl⁻ currents and completely blocks spontaneous GABA synaptic activity (*SI Appendix*, Fig. S2). (D) Upper traces (synaptic activity): Voltage-clamp recordings reveal that Neurobasal-A based medium strongly reduces spontaneous AMPA synaptic activity. In addition, it induces large tonic Cl⁻ currents and completely blocks spontaneous GABA synaptic activity (*SI Appendix*, Fig. S2). *Lower traces* (APs): Neurobasal-A does not affect the resting membrane potential but it strongly impairs evoked APs (500-ms step of current), voltage-dependent Nav/Kv currents (*I-V* traces, clamp −70 mV, voltage steps 5 mV), and spontaneous APs.

this culture medium, but many more cells became spontaneously active when we imaged the same fields of view in ACSF. The composition of ACSF solutions may vary slightly between laboratories. For example, the calcium concentration is often two or three times higher than physiological levels (∼1.2 mM) (35–37), which artificially increases synaptic release and therefore may also increase calcium neural network activity. To address this issue, we matched the inorganic salt concentration, the pH, and osmolarity of our ACSF to those in DMEM and repeated the calcium-imaging experiments. Even with these precautions, we confirmed that the number of active cells was significantly lower in DMEM basal than in ACSF (Fig. 1B and *SI Appendix*, Fig. S1A; see also logg.salk.edu/files/BrainPhys_movies.pptx).

We then used patch-clamping methods to determine how DMEM basal reduced the overall spontaneous activity of human neurons in vitro (Fig. 1C). We found that DMEM basal consistently depolarized the resting potential of neurons ($n = 22/22$ cells, by 23 ± 3 mV; *SI Appendix*, Fig. S1 B–E). When we examined spontaneously active neurons in ACSF, we found that, on rare occasions, depolarization induced by DMEM increased the firing frequency without saturation ($n = 2/14$; *SI Appendix*, Fig. S1B), but in most cases, it saturated and completely silenced the firing of the cells ($n = 12/14$; Fig. 1C and *SI Appendix*, Fig. S1 C and D).

To investigate the influence of DMEM basal on synaptic function, we performed voltage-clamp experiments at the reversal potential of Cl⁻ (−70 mV) or Na⁺ (0 mV) and found that both spontaneous AMPA and GABA synaptic events, which were recorded in ACSF, completely disappeared in DMEM (Fig. 1C). This effect occurred in every tested neuron and was reversible when switched back to ACSF ($n = 6/6$ mature cells).

At the same time, we also observed that perfusion of DMEM consistently induced large depolarizing Na⁺ and Cl⁻ influxes into the cells (*SI Appendix*, Fig. S1 E and F). Therefore, we suspected that components present in the DMEM but not in ACSF activate Na⁺ and Cl⁻ channels on the neurons. To identify those components, we removed either all of the vitamins, all of the amino acids, or all of the extra components of DMEM. By removing the bulk of the amino acids, we avoided the DMEM-induced depolarization that caused the impairment of action potentials (APs) and synaptic activity. However, this solution failed to sustain the development and survival of neurons for more than a week (*SI Appendix*, Fig. S2; see also logg.salk.edu/files/BrainPhys_movies.pptx). From these experiments, it was clear that neuroactive amino acids in DMEM impaired basic neuronal functions, including AP propagation and synaptic communication.

Neurobasal Medium Reduces Synaptic Communication and AP Firing.
DMEM/F12 basal has been previously modified to optimize the survival of rat primary neurons in culture (38). In this modified version, called Neurobasal (NB or NBA), the developers essentially removed some excitatory amino acids and ferrous sulfate and reduced the osmolarity; they found that NB+B27 improved rat neuron survival in vitro in comparison with DMEM or DMEM/F12. Although cell survival is a critical parameter of cell culture, fundamental electrophysiological properties were not tested in these media. Therefore, we used human iPSC- and embryonic stem cell (ESC)-derived neurons to examine the acute effects of Neurobasal on neuronal functions (Fig. 1A). Unlike DMEM/F12, NBA did not depolarize the resting membrane potential, and at least some excitatory synaptic events could be observed. Nevertheless, NBA strongly reduced or abolished the spontaneous excitatory synaptic activity observed in ACSF (Fig. 1D; $n = 8/8$ mature neurons; Fig. 2E). In Neurobasal, the concentrations of inorganic salts (e.g., NaCl) were almost half of what the neurons are naturally exposed to in the brain (39). As a consequence, we found that Neurobasal significantly reduced the voltage-dependent sodium currents and rapidly inactivating
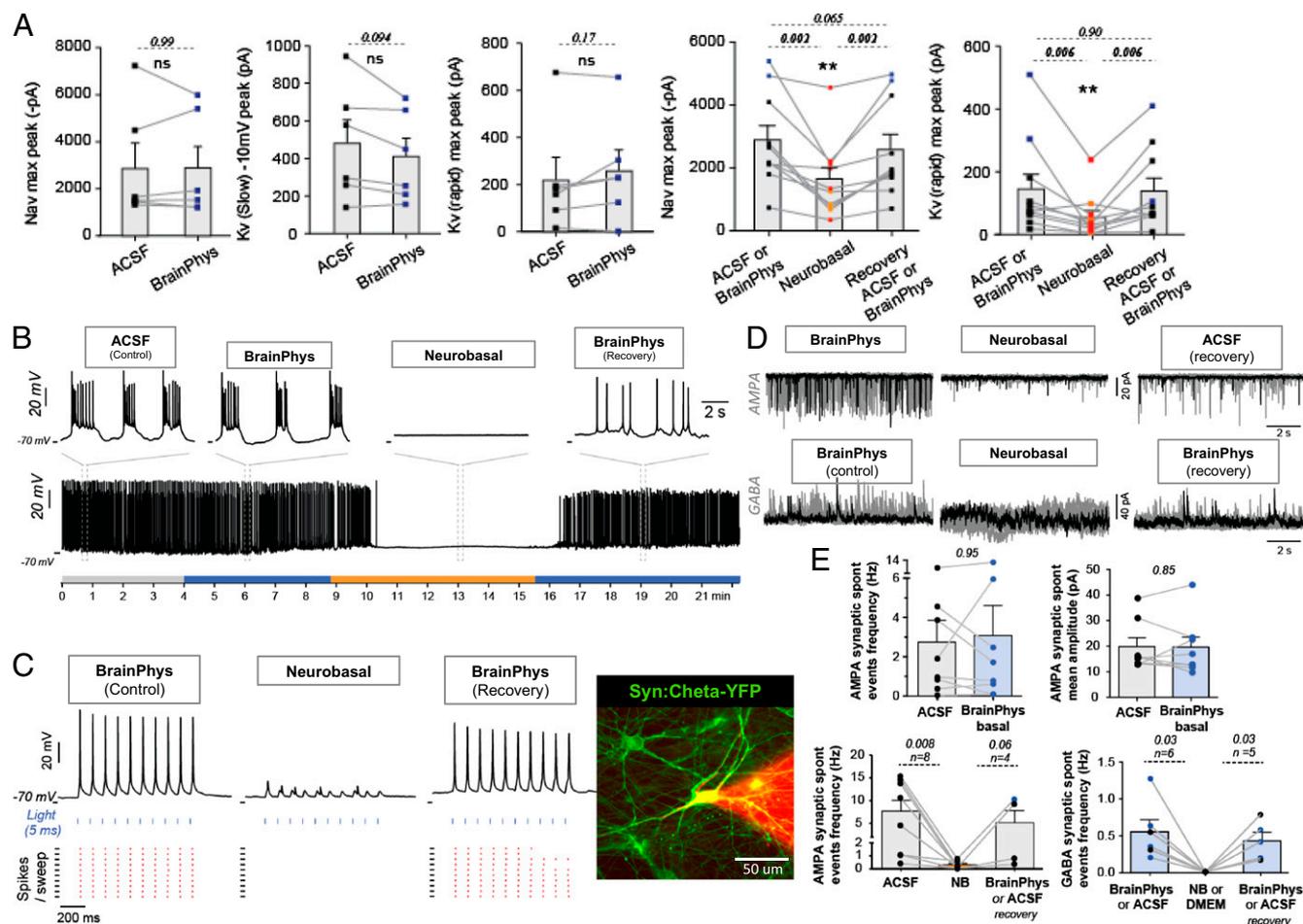
Fig. 2. BrainPhys basal medium supports optimal APs and synaptic activity. (A) Voltage-activated (VA) sodium and potassium currents (measured from *I-V* curves, voltage clamp −70 mV, steps 5 mV) were similar in BrainPhys and ACSF. In the three panels on the left, each neuron (n = 6) was tested both in ACSF and BrainPhys (black vs. blue dots). The maximum amplitudes of voltage-activated sodium and potassium currents of single neurons (n = 10) were significantly reduced in Neurobasal media (red dots, Neurobasal-A; orange dots, Neurobasal) compared with BrainPhys (blue dots) or ACSF (black dots). Nonparametric paired Wilcoxon test *P* values are shown in italics. Histograms represent the mean ± SE. (B) The resting membrane potential, spontaneous and evoked APs were the same in ACSF and BrainPhys. (C) Typical mature patched neuron expressing an optogene (synapsin: ChETA-YFP, green) and filled with rhodamine from the patch pipette (red shadow on the right). Optogenetic control of neuronal activity can be reliably achieved in BrainPhys. Recordings from single mature neurons tested with the same parameters in BrainPhys or Neurobasal-A illustrate that optogenetic control is dramatically improved in BrainPhys. Bottom part of the graphs in C are the corresponding raster plots. See also related results in *SI Appendix*, Fig. S3. (D) Both excitatory (AMPA) and inhibitory (GABA) synaptic activities were clearly apparent in BrainPhys medium. The perfusion of different media while recording the spontaneous synaptic activity of single neurons revealed that BrainPhys better supported synaptic function compared with other media such as Neurobasal-A. Twelve sweeps are superimposed in gray and one of them is highlighted in black for clarity. (E) Patched neurons (n = 22) tested in different basal media are represented by the paired dots. Quantification of AMPA receptor-mediated spontaneous synaptic activity shows similar properties in ACSF and BrainPhys basal (n = 8). Neurobasal significantly reduces AMPA synaptic events (n = 8). Both Neurobasal and DMEM completely block GABA synaptic events (n = 4 + 2). All tests comparing spontaneous activity were performed without any synaptic antagonists or voltage-gated sodium channel blockers. Voltage clamp at respective reversal potential of chloride and sodium was used to distinguish glutamatergic from gabaergic events. Synaptic blockers (NBQX or Gabazine) were used in a subset of cells (n = 15) only to confirm the nature of the receptors mediating the observed synaptic activity. Wilcoxon *P* values are shown in italics. Two-tailed tests were used except for recovery from 0 Hz; then a one-tailed test was used.

potassium currents, and it impaired the amplitude and frequency of evoked and spontaneous APs (n = 8/8 cells; Fig. 1D). We tried to increase the concentration of NaCl in Neurobasal (40); however, despite some slight improvements in the amplitude of sodium currents through voltage-gated channels, this modification was not sufficient to optimize the electrical and synaptic activity of the human neurons. We suspected that, as in DMEM, several neuroactive components in Neurobasal were preventing optimal neurophysiological activity. Because neither DMEM nor Neurobasal was capable of optimally sustaining essential electrophysiological neuronal properties, we decided to design a new basal neuro-medium (BrainPhys basal) that would be better adapted to neuronal function in culture.

BrainPhys Basal Medium Supports Functional AP Firing. Neuronal function is fundamentally based on the generation and propagation of APs. Voltage-gated sodium (Nav) and potassium (Kv) channels are crucial to achieving high firing rates of APs. APs reflect the sequential activation of Nav and Kv channels that trigger a large influx of sodium and an efflux of potassium, largely depending on ionic gradients. Therefore, the concentrations of major inorganic salts (Na^+, Cl^-, K^+) in the media are critical for APs generation and propagation. The concentrations of NaCl are higher in BrainPhys basal, ACSF, and DMEM compared with Neurobasal (~120 vs. ~70 mM) and closer to neurophysiological levels. As a result, the observed amplitudes of voltage-activated currents through important ionotropic channels
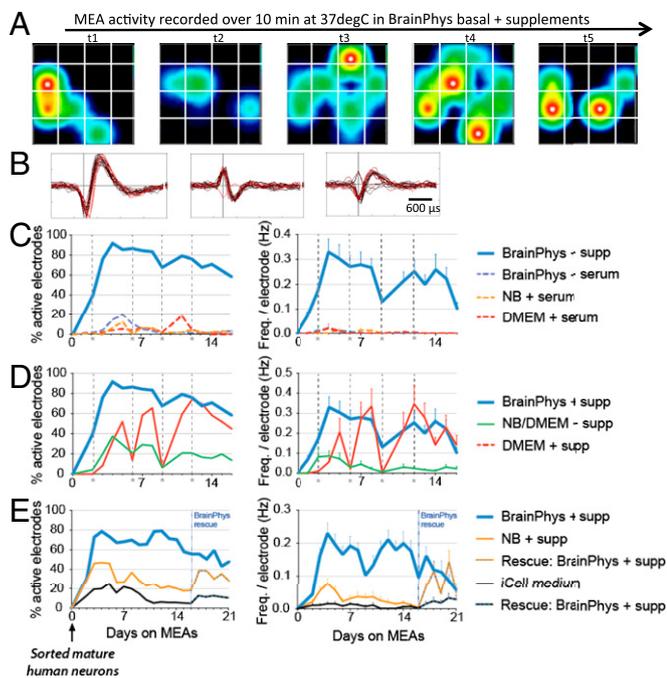
Fig. 3. Long-term exposure of mature human neurons to BrainPhys basal (+supplements without serum) supports optimal and stable neuronal activity on MEAs. (*A–E*) Electrical activity of purified mature human iPSC-derived neurons (iCell neurons from Cellular Dynamics) was recorded in 48-well multielectrode array (MEAs) plates (Axion) in different neuronal maturation media at 37 °C every day for 2–3 wk. (*A*) Example illustrating the heatmap activity of seven snapshots of the same array in a well. Recordings (10 min) from neurons in BrainPhys with supplements (for 5 d) (see logg.salk.edu/files/BrainPhys_movies.pptx). (*B*) Examples of spikes detected by individual electrodes in BrainPhys+sup medium. (*C–E*) Percentage of active electrodes (>0.005 Hz) and frequency of detected spikes per electrode averaged from daily 10-min recordings. Each value represents the average of at least 96–192 electrodes spread in four to eight wells per conditions. The same batches of cells were compared in the 48-well plates with different media. Dashed lines in *C* and *D* mark the feeding dates. Recordings were performed before feeding. Each experiment was repeated at least twice independently with different set of cells and combined in the figures. For clarity, the experiment in *C* and *D* was split into two graphs, but the same control (BrainPhys+sup) is represented twice. See analysis of spikes properties in *SI Appendix*, Table S1.

(Nav, Kv) did not differ with perfusion of ACSF or BrainPhys medium (Fig. 2*A*). In contrast, the amplitudes of Nav and rapidly inactivating Kv currents were systematically larger when recorded in BrainPhys or ACSF compared with Neurobasal (Fig. 2*A* and *SI Appendix*, Fig. S3*A*).

Consequently, the spontaneous and evoked APs in single neurons tested in BrainPhys reached levels similar to those measured in ACSF (Fig. 2*B* and *SI Appendix*, Fig. S3*B*). APs were also tested with optogenes such as channelrhodopsin (ChR2 or ChETA) that could be efficiently expressed in human neurons, allowing us to control the firing activity of the neurons in BrainPhys with brief flashes of light (Fig. 2*C*). In sharp contrast, light stimulations of neurons that successfully evoked responses in BrainPhys failed to evoke clear APs in either NBA- or DMEM/F12-based medium (Fig. 2*C* and *SI Appendix*, Fig. S3*D*). Consistent with the overall improvement in firing activity, the Ca²⁺ activity measured with imaging in ACSF and BrainPhys was comparable (see logg.salk.edu/files/BrainPhys_movies.pptx).

BrainPhys Basal Medium Supports Functional Excitatory and Inhibitory Synaptic Activity. Integrated neural network activities—and ultimately, brain functions—are achieved by synaptic commu-

nication. Therefore, we optimized BrainPhys basal to support both excitatory and inhibitory synaptic function. The summation of excitatory and inhibitory synaptic neurotransmitters determines the probability of neurons to fire APs. Thus, simply optimizing AP firing may act indirectly to promote synaptic activity. In addition, specific ions such as calcium play critical roles in the synapse in triggering fast vesicular release. Aiming to reduce the differences between culture media and in vivo brain conditions, we adjusted calcium levels in BrainPhys basal to be close to those in human cerebro-spinal fluid in vivo (Ca²⁺ ~ 1.1 mM) (37).

In the brain, glutamate mediates most of the synaptic excitation, whereas GABA mediates most of the synaptic inhibition. To examine whether the different media could support basic synaptic functions, we tested the levels of spontaneous synaptic activity mediated by AMPA or GABAa receptors (identified in voltage clamp by reversible blockades with their respective antagonists, NBQX or SR95531). Voltage-clamp experiments showing strong Na⁺ and/or Cl⁻ currents on perfusion of DMEM/F12 or NBA in comparison with ACSF strongly suggested that synaptic function was impaired by the presence of receptors agonists present in these media but not in ACSF. Therefore, in BrainPhys basal, we excluded or reduced the neuroactive amino acids in classic media that could directly influence glutamatergic synaptic activity (glutamate, aspartate) and/or GABAergic synaptic activity (glycine, alanine, serine). Although spontaneous activity might be variable between cells, paired analysis of single mature neurons clearly showed that, unlike in DMEM or Neurobasal, the levels of spontaneous AMPA synaptic activity in BrainPhys were not significantly different from those in ACSF (Fig. 2*E*) and were therefore largely improved compared with DMEM or Neurobasal (Figs. 1 and 2 *D* and *E*). We also observed that both Neurobasal and DMEM completely blocked synaptic inhibitory phasic activity (*n* = 6/6 cells, 4 in NBA, 2 in DMEM/F12; Fig. 2 *D* and *E*). The blockade of GABA synaptic activity was systematically accompanied by a large influx of chloride currents (*SI Appendix*, Fig. S1 *E* and *F*) and therefore suggested that the blockade was due to the excess of neuroactive components activating chloride channels (e.g., glycine, alanine, serine). Following up on this hypothesis, we indeed found that reducing the concentration of these elements improved GABAergic synaptic activity. The spontaneous GABA synaptic activity was proven to be functional in BrainPhys (*n* = 7 cells; Fig. 2*D* and *SI Appendix*, Fig. S1*G*) and comparable to levels of activity in ACSF (*n* = 3 cells tested in both solutions; Fig. 2 *D* and *E*).

BrainPhys Basal Medium Better Mimics the Human Brain's Energy Levels and Osmolarity. Our primary objective was to design a medium that better supported neuronal functions and activity in vitro. In addition, we wanted to design a medium that would better mimic the basic conditions in the human brain. These improvements are not only important for studies directly focusing on neuronal activity but are also potentially critical for disease-modeling studies, as more realistic experimental models will increase translational success for patients.

To reach that goal, it seems essential to provide energy levels close to those observed in healthy human brains, especially because several neurological disorders have been shown to be related to mitochondria dysfunctions (41–43). The mammalian brain uses glucose as one of its main sources of energy (44). Disruption of glucose homeostasis may affect brain physiology and lead to brain disorders (44, 45). Surprisingly, the glucose levels in DMEM and Neurobasal are at least two to five times higher than those in the brain of hyperglycemic patients (46). We therefore balanced the energetic components in BrainPhys to provide glycemic levels similar to those reported for the brains of healthy patients (~2.5 mM).

Furthermore, we set the osmolarity to approximate that of typical human cerebrospinal fluid (~300 mOsmol/L). In contrast,
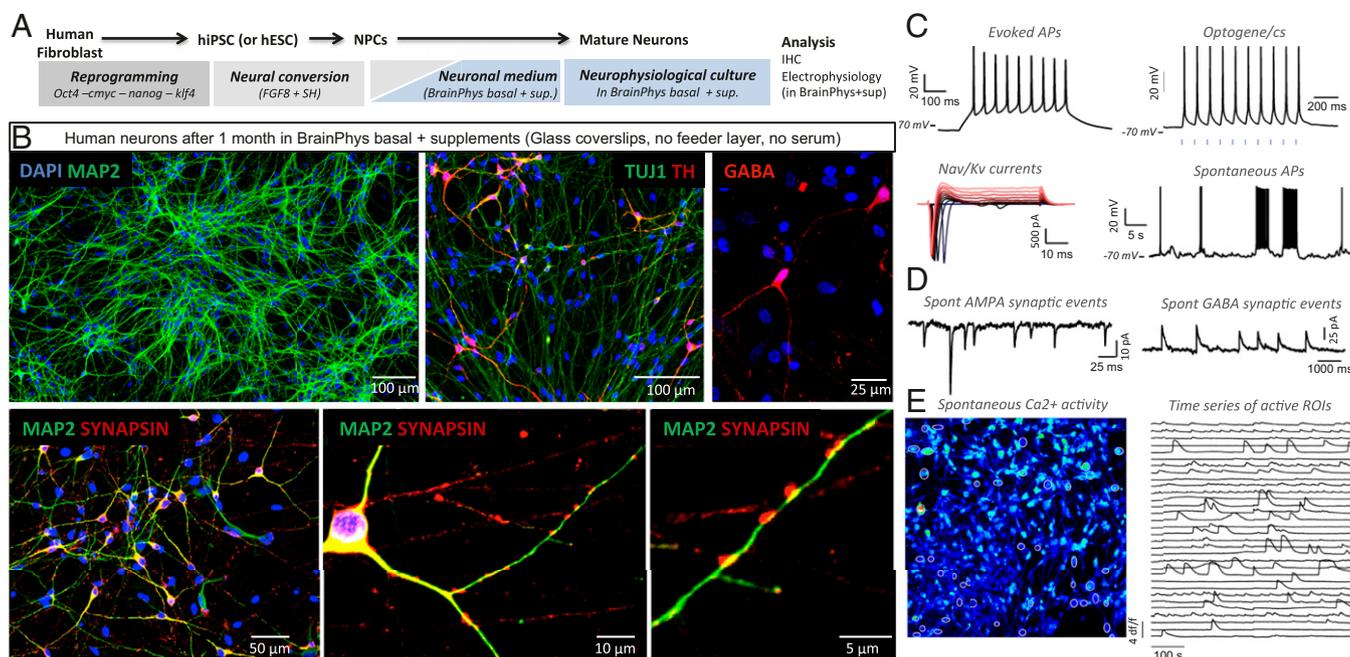
Fig. 4. Characterization of human neurons cultured for several weeks in BrainPhys basal + supplements. (*A*) Human NPCs derived from iPSCs or ESCs were plated directly on glass coverslips. Human neurons were matured in neuronal medium (BrainPhys + sup) for 3–6 wk. Analysis of electrophysiological properties was performed in the same neuronal medium: BrainPhys +sup. (*B*) Immunostaining of typical human IPSC-derived neuronal cultures grown in BrainPhys with supplements for 4 wk. (*C* and *D*) Electrophysiological activity of typical functional neurons after 3–6 wk in BrainPhys-based neuronal medium. Patch-clamp recordings were also performed in BrainPhys medium with supplements. (*C*) From left to right: Train of APs evoked by a small 500-ms depolarizing step of current or brief flashes of light (syn: ChETA-YFP). *I-V* traces (clamp −70 mV, steps 5 mV) showing typical voltage-activated Na^+ and potassium currents. Spontaneous APs recorded in current clamp. (*D*) Spontaneous synaptic events mediated by AMPA receptors (sensitive to NBQX, voltage clamp at −70 mV) and GABA receptors (sensitive to Gabazine, voltage clamp at 0 mV). (*E*) Calcium imaging showing typical activity of neuronal cultures grown and recorded in BrainPhys + supplements. Time series analysis is plotted for the active regions of interest (white circles) (see logg.salk.edu/files/BrainPhys_movies.pptx); 91% of the active ROIs showed clear calcium spikes; the remaining showed slow calcium waves. Statistics for the calcium spikes of 41 active ROIs (mean ± SE): 8 ± 1 calcium spikes per 10 min, df/f = 1.1 ± 0.1; rise 10–90% = 3 ± 0.3 s; decay 37% = 11 ± 1s; half-width = 12 ± 1s.

the osmolarity of NBA is ∼30% lower than neurophysiological levels (∼220 for NB or 250 mOsmol/L for NBA). It is important to note that evaporation of the medium often occurs in tissue culture and can drastically affect the osmolarity. To reduce cytotoxic osmotic stress, the cells were kept in an appropriately humidified incubator and half of the medium was replaced at least two or three times a week.

Supplements to Basal Medium That Do Not Acutely Impair Neuronal Activity. To sustain cell survival and/or neural differentiation in vitro, supplements such as antioxidants, growth factors, hormones, and proteins should be added to basal media. Many of these molecules can be found naturally in serum; therefore, serum is often used. However, in addition to introducing variability from batch to batch, we found that serum (both chemically defined and FBS) dramatically impaired neuronal activity (*SI Appendix*, Fig. S4). As an alternative to serum, we tested the effect of a combination of several chemically defined supplements on acute and long-term neural activity of human neurons in vitro. We patch-clamped single mature human neurons in various perfusates and found that adding a defined set of supplements [N2, B27, retinoic acid, brain-derived neurotrophic factor (BDNF), glia cell-derived neurotrophic factor (GDNF), ascorbic acid, cAMP, laminin, and cholesterol; *SI Appendix*, Table S6] to BrainPhys basal medium did not acutely affect the firing rate or excitatory/inhibitory synaptic activity (*n* = 6 cells; *SI Appendix*, Fig. S4).

BrainPhys Basal+sup Supports Stable Long-Term Electrical Activity of Mature Human Neurons in Vitro. Our patch-clamping experiments comparing the effect of different media on neuronal activity

demonstrated that BrainPhys basal medium was more efficient to support physiological action potential firing and synaptic activity. To study brain disorders in vitro, mature neurons should be cultured in a medium that supports their basic function for some time before analysis.

To find out whether BrainPhys basal + supplements was adequate to sustain long-term neuronal activity, we recorded the electrical activity of mature human neuronal cultures over several weeks. In those experiments, we used a multielectrode array (MEA) system integrated on a 48-well tissue culture plate (Axion; 16 electrodes per well; Fig. 3 *A* and *B*). We obtained frozen stocks of characterized and purified mature human iPSC neurons (commercially available iCell neurons from Cellular Dynamics), thawed the purified neurons directly onto the MEA plates, and started recording their activity 2 d later, every day, for about 2 wk. With this system, we compared the electrical activity of neurons over time in BrainPhys+sup and other media. First, regardless of the basal media used, we confirmed our patch-clamping results that adding serum dramatically impaired neuronal activity. The impairment caused by serum lasted more than 2 wk (Fig. 3*C*). In NBA+sup or NBA/DMEM mixture+sup, we found that the overall neuronal activity was relatively low in the first few days and deteriorated rapidly within about a week (Fig. 3*E*). The poor neuronal performance in the NBA+sup could be rescued, at least partially, within a few days when replaced with BrainPhys+sup (Fig. 3*E*). In DMEM+sup, the neuronal activity was somewhat higher than in NBA+sup or NBA/DMEM+sup but also highly fluctuated over time, presumably in synchrony with the feeding cycles (twice a week in those experiments; Fig. 3*D*). In contrast, BrainPhys+sup (with the same serum-free
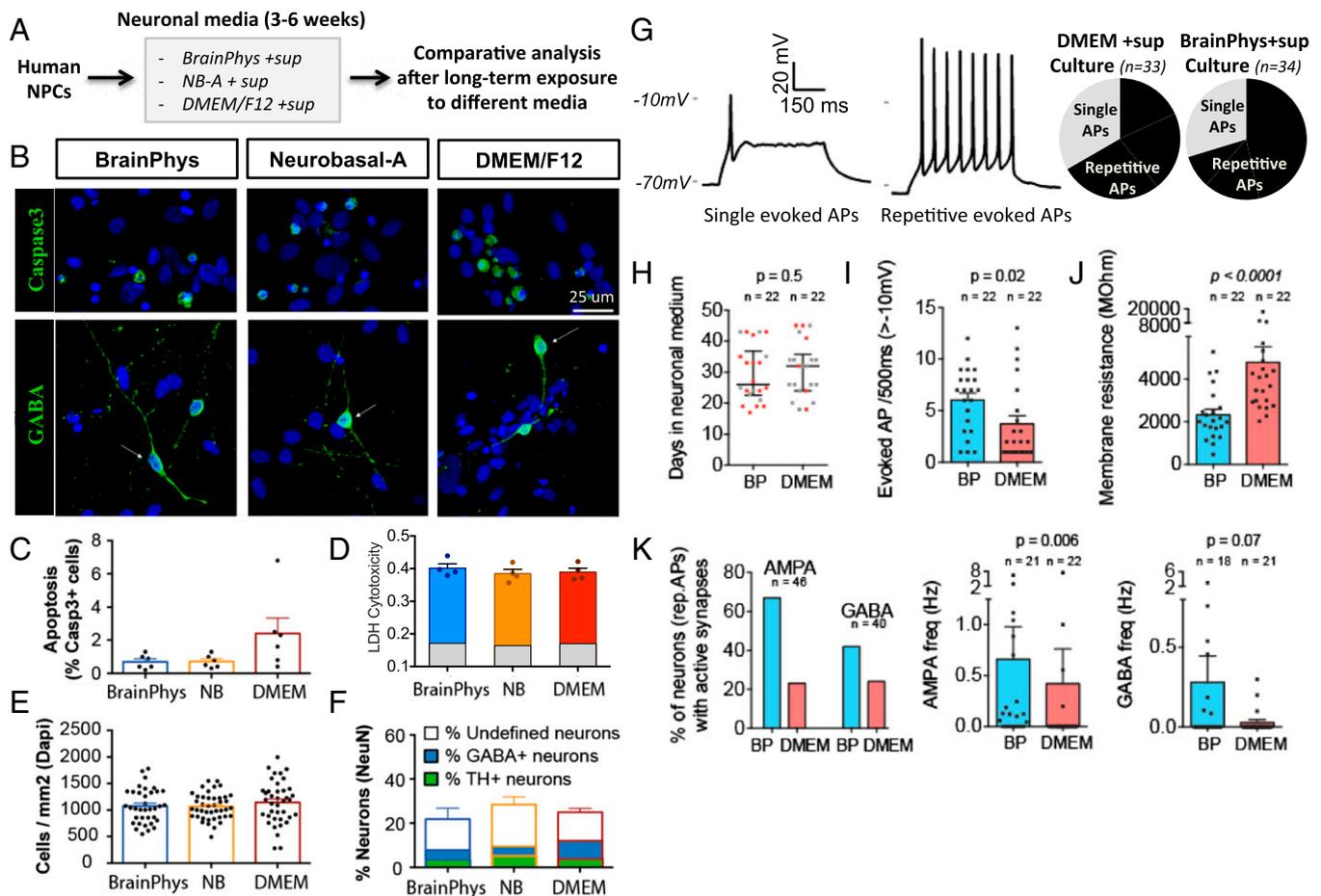
Fig. 5. Comparison of human neurons cultured in BrainPhys vs. classic media based on DMEM or Neurobasal. (*A*) In these experiments, we seeded human NPCs on coated glass coverlips (no feeder layer) in neural progenitor medium (*SI Appendix, Methods*). We then gradually switched to three different media: BrainPhys basal, DMEM/F12, or Neurobasal-A with the same serum-free supplements (*SI Appendix, Methods*). Half-media changes were performed every 3 d for 3–6 wk before analysis. (*B–F*) Representative immunostaining and analysis of cells cultured in the same plate with three different conditions. Nuclei were stained with DAPI (blue). Despite some variability, no clear differences in cell survival or proportion of neuronal subtypes (e.g., dopaminergic, GABAergic) were found between the three conditions. Quantification on all FOV with 20× objective (tiles 2 × 2) (for TH, n = 23 FOV; for NeuN, n = 20 FOV; for GABA, n = 23 FOV). (*G*) To characterize the influence of BrainPhys basal on functional properties, we compared the neurons matured in BrainPhys+sup with those in DMEM/F12+sup for 2–6 wk. Alternative blind patch clamping of the two groups was performed in ACSF for unbiased comparison. Electrophysiological types of neurons were defined based on their optimal firing patterns in response to 500-ms depolarizing steps of currents in ACSF. Similar proportion of cells with single or repetitive APs was found in the two media cultures. (*H–K*) For better sample homogeneity, the analysis was focused on neurons with repetitive APs. (*H*) The cells with repetitive APs spanned throughout the time windows of recording in both groups. Red dots represent neurons with evoked firing >10H (threshold −10 mV). Median and interquartile ranges are shown. (*I*) The evoked firing frequency (threshold −10 mV) of neurons with repetitive APs was significantly higher in the BrainPhys+sup cultures. (*J*) The membrane resistance of neurons with repetitive APs was significantly lower in the BrainPhys+sup cultures. (*K*) BrainPhys+sup culture also showed higher proportions of neurons receiving active AMPA synaptic inputs (NBQX sensitive) and GABA synaptic inputs (Gabazine sensitive). The presence of active synapses was determined by detecting at least four spontaneous synaptic events with distinctive kinetics over a 4-min recording in voltage clamp (−70 or 0 mV, respectively).

supplements) significantly improved the spontaneous activity of human neurons within a few days and, importantly, kept it stable for more than 2 wk (Fig. 3 *A–E*, see statistics in *SI Appendix, Table S1*; for illustration of live MEA activity in different media; see also logg.salk.edu/files/BrainPhys_movies.pptx).

Taken together, these results demonstrate the effectiveness of BrainPhys basal with serum-free supplements to keep mature human neurons physiologically active and stable.

Characterization of Human Neurons Maturing Several Weeks in BrainPhys+sup Medium. The methodological variations to obtain different types of human neurons in vitro (e.g., cortical, dopaminergic) are probably countless. Generally, somatic cells (e.g., fibroblasts) or pluripotent stem cells (e.g., iPSCs, ESCs) are converted to neural progenitor cells (NPCs) in neural conversion media with various growth factors. Then, those NPCs are cul-

tured in neuronal media and usually kept in the same media until analysis. We found that BrainPhys+sup was efficient for use with mature neurons obtained with different protocols, and it provided an active and neurophysiological environment for several weeks or more. Then we tested whether neural progenitors could be switched gradually from their neural progenitor medium to neuronal medium based on BrainPhys with supplements.

After expansion, human NPCs (derived from ESCs or iPSCs) were seeded on transferable, treated glass coverslips (no feeder layer). Within 24 h after plating the cells, the NPC medium was gradually replaced with subsequent feeds of BrainPhys+sup (Fig. 4*A*). We found that human NPCs plated in BrainPhys+sup differentiated effectively into mature and synaptically active neurons within 3–5 wk. After about 4 wk in BrainPhys neuronal medium, the cultures formed dense neuronal networks and stained abundantly for dendritic/axonal markers (MAP2 and

TUJ1), synaptic markers (Synapsin), and neuronal markers (TH, GABA, VGlut; Fig. 4*B*). Consistent with our previous observations, human NPCs differentiated not only into neurons but also into a population of astrocytes staining positively for GFAP (*SI Appendix*, Fig. S5).

The neurons survived on glass coverslips in BrainPhys+sup for several weeks or even several months for the best cell lines (tested up to 5 mo). We tested the neurophysiology of these BrainPhys cultures with path-clamping directly in BrainPhys+ sup, in which they were cultured, and we found many mature neurons firing healthy trains of APs (Fig. 4*C*) and receiving active AMPA and GABA synapses (Fig. 4*D*, see summary of electrophysiological properties of large sample, *n* = 106, in *SI Appendix*, Table S2). For the best cell lines, those types of neurons were found around 3 wk after plating in maturation medium and remained active for several months (tested up to 5 mo). Similarly, calcium imaging experiments supported the finding that BrainPhys-based neural cultures generated and sustained functionally active neural networks (Fig. 4*E*; see also logg.salk.edu/files/BrainPhys_movies.pptx).

BrainPhys+sup Medium Supports Brain Cell Survival in Vitro. DMEM and Neurobasal media were specifically optimized to promote the survival of cells in vitro. To test the viability of the cells in different basal media (DMEM/F12, NBA, and BrainPhys basal, all with the same set of serum-free supplements), we performed matched experiments using the same cell lines, plated at similar densities and at the same time, but fed for several weeks with different media (Fig. 5*A*). We found that, after 1 mo in either medium, the proportion of apoptotic cells (active caspase 3), the overall cell density (DAPI), and the concentration of lactate dehydrogenase released in the supernatant by dying cells did not change significantly between the three groups (Fig. 5 *B–E*).

BrainPhys+sup Supports the Basic Function of Many Classes of Neurons. Human cell reprogramming is used to differentiate a multitude of neuronal classes. We collected electrophysiological evidence that BrainPhys+sup is suitable to culture many classes of human neurons derived from iPSCs (*SI Appendix*, Table S4). Importantly, after ≥2 wk in BrainPhys+sup, each of these classes held mature functional neurons (evoked APs > 10 Hz and spontaneously active synapses).

When we compared the differentiation of NPCs in BrainPhys+ sup and other media (DMEM+sup and NBA+sup), we did not find significant differences in the proportion of NeuN-positive neurons (Fig. 5*F*). Our iPSC differentiation protocol generated a variety of neuronal classes in the same culture (glutamatergic, GABAergic, dopaminergic). The proportion of NeuN+ neurons that positively stained for GABA or TH and the remaining NeuN+ neurons (presumably mostly glutamatergic) did not vary significantly in different neuronal media (Fig. 5*F*). Therefore, when used for approximately 2–4 wk at the latest stage of the culture process (Fig. 4*A*), these three basal media did not appear to greatly influence the neuronal identity. Instead, it is more likely that early steps of the neuronal conversion from iPSC to NPC, and/or specific supplements, played a more critical role in the neuronal identity fate.

In addition, in BrainPhys+sup, NPCs differentiated not only into neurons but also into astrocytes that expressed GFAP protein (immunostaining). We also used GFAP promoter linked with fluorescent reporters to reveal astrocyte-like electrophysiological properties (low resting potentials, absence of sodium currents).

Culture in BrainPhys+sup Can Increase Action Potential Firing Frequencies and the Proportion of Synaptically Active Human Neurons. Despite the failure of classical basal media to support optimal neurophysiological activity, when combined with the right supplements, these media manage to support survival and neuronal differentiation in vitro.
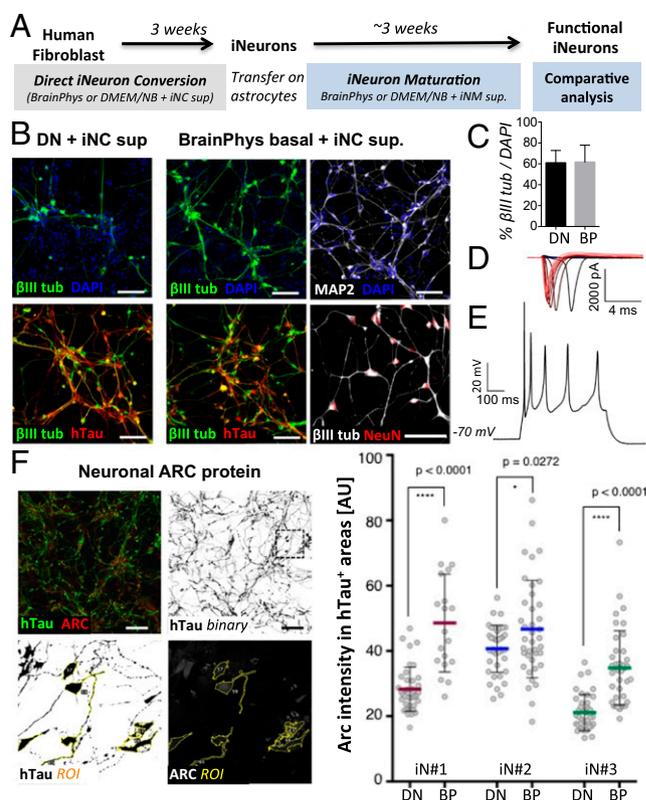

Fig. 6. BrainPhys basal with the appropriate supplements permits efficient direct neuronal conversion, as well as development of human iNs, and increases ARC protein expression. (*A*) Experimental design: iN-competent human dermal fibroblasts were converted in neuronal conversion (NC) medium for 3 wk. For maturation, iN cells were relocated onto astrocytes and further cultured in neuronal maturation (NM) medium (see *Methods* for more details). (*B*) Immunofluorescence staining for β-III-tubulin, human Tau (hTau), MAP2ab, and NeuN following 3-wk conversion in media based on DMEM/F12:Neurobasal (*Left*) or BrainPhys (*Right*) with the same supplements. (Scale bar, 50 μm.) (*C*) The percentage of TUJ1+/DAPI was counted after 3 wk of conversion in different basal media with the same supplements. No significant difference was observed in the efficiency of neuronal conversion when either basal medium was used. (*D* and *E*) Following 3 wk of conversion, the iNs were replated on mouse astrocytes for 2 wk in BrainPhys basal+sup before patching and showed strong sodium/potassium currents and repetitive evoked APS. (*F*) Representative image of immunofluorescence staining for hTau and ARC in iN cells after maturation on astrocytes (3-wk conversion + 3-wk maturation). Quantification using ROI selection and measurement in ImageJ. (Scale bar, 100 μm.) Plot illustrating the quantification of neuronal ARC protein levels in iN cultures derived from three donors. Gray points represent individual cells (average *n* = 38 per group, minimum *n* = 19 per group). Bar show means ± SD of each group. Unpaired *t* test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Electrical activity is known to play an important role in neuronal development and synaptic function (47–49). Typically in the brain, most newly generated glutamatergic synapses lack functional AMPA receptor-mediated transmission. Over time, these synapses are eliminated if kept silent, whereas those exposed to correlated electrical activity will mature and prevail (47). We asked whether BrainPhys+sup could improve the synaptic function of mature neurons. To address that question, we randomly patched a homogeneous sample of 65 synapsin-GFP–positive neurons in cultures growing side by side in DMEM/F12 or BrainPhys basal (with the same supplements). To avoid possible bias due to tissue culture variability, we compared the effects of the media on neurons from the same NPCs, plated at the same density ($\sim$2 × 10^5 cells per well; 190 mm^2), in the same plate, at the same time, and we discarded the few coverslips in
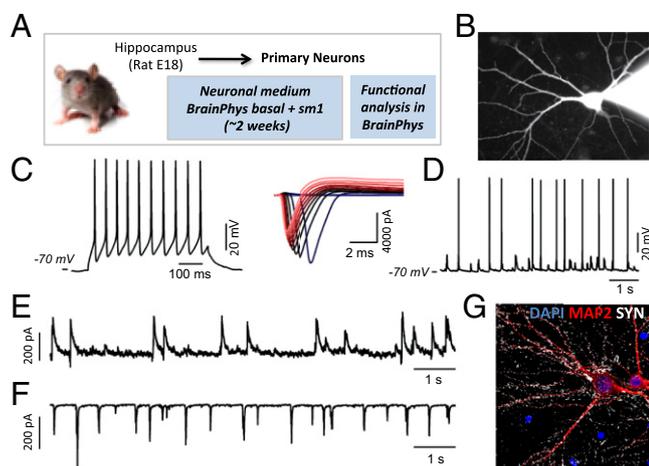
Fig. 7. Differentiation of healthy and active rat primary hippocampal neurons in BrainPhys basal+sm1. (A) Rat hippocampal neurons were plated on coated glass coverslips (no feeder layer, no serum) in BrainPhys basal medium (with sm1) for ~2 wk before characterization. (B) Images of a typical patch-clamped neuron filled with Rhodamine. On the right, the shadow is the Rhodamine-filled patch pipette. Electrophysiological characterization was performed in BrainPhys. (C) Train of APs evoked by a small 500-ms depolarizing step of current. I-V traces (clamp −70 mV, steps 5 mV) showing typical voltage-dependent Na⁺ and K⁺ currents. Colored traces correspond to increasing voltage steps. (D) Spontaneous APs recorded in current clamp (0 pA). (E) Spontaneous GABA synaptic events recorded in voltage clamp at (0 mV). (F) Spontaneous AMPA synaptic events recorded in voltage clamp (−70 mV). (G) Coverslips were fixed after patching and processed for immunohistochemistry. Dendrites were stained with MAP2, presynaptic terminals with Synapsin, and nuclei with DAPI.

each groups where some cells detached from the coverslips and/or formed large clumps. We blindly alternated the patching of neurons that were cultured in BrainPhys or DMEM/F12 (with supplements); therefore, the time that the neurons spent in either neuronal medium was virtually identical in both groups (average 28 and 29 d, respectively, ranging from 17 to 45 d). The neurons that were patch-clamped had on average the same number of primary neurites (BrainPhys: 3.5 ± 0.2, DMEM: 3.5 ± 1.2; *SI Appendix*, Table S3). The patched neurons were cultured in either DMEM/F12+sup or BrainPhys+sup media, but because patching the cells in DMEM impairs activity, we acutely assessed their functional properties in ACSF. In these conditions, we were reasonably confident that the only difference between the coverslips (n = 22) selected for analysis was the basal medium used for feeding. Despite our efforts to homogenize the sampling of recorded neurons, we obtained more neurons with high APs firing rates in the BrainPhys+sup cultures. To quantify this result, we defined different electrophysiological types of neurons based on their firing patterns in response to 500-ms depolarizing steps of currents (Fig. 5G). We divided the neurons into two groups: those with single evoked APs and those with repetitive evoked APs. After 2–6 wk in maturation media, electrophysiological testing in ACSF revealed a similar proportion of cells with single or repetitive APs. Overall neurons with particularly high evoked APs frequencies were found throughout the time window of our analysis in both culture groups (Fig. 5H). However, the cells with repetitive firing showed significantly higher firing frequency when cultured in BrainPhys+sup instead of DMEM+sup (Fig. 5I). Consistently, in neurons from BrainPhys-based cultures, the Nav and rapidly inactivating Kv currents were significantly higher, but the voltage activation threshold was similar to those of neurons in DMEM-based cultures (*SI Appendix*, Table S3). In addition, the membrane resistance of BrainPhys-cultured neurons was significantly lower (Fig. 5J)

and the capacitance was significantly higher than in DMEM-cultured neurons, characteristics that also typically correlate with more mature-like properties (*SI Appendix*, Table S3).

The numbers of neurons with repetitive evoked APs receiving active excitatory AMPA and inhibitory GABA synaptic inputs were about two to three times higher in the BrainPhys cultures (Fig. 5K). Interestingly, these synaptic enhancements occurred without obvious differences in the number of proximal synaptic puncta measured by immunohistochemistry (IHC; *SI Appendix*, Fig. S6). This finding suggests that spontaneous electrical activity in BrainPhys+sup activates/strengthens silent synaptic contacts rather than increasing the formation of additional synapses.

BrainPhys+sup Medium Can Be Used in Direct Neuronal Conversion Protocols (Induced Neurons). Most of our tests were performed on human iPSC- or ESC-derived neurons. However, direct conversion of human fibroblasts to induced neurons (iNs), without passing through the pluripotent stage, represents an attractive alternative (Fig. 6A). To that end, we tested whether BrainPhys basal medium could support neural conversion and/or maturation of human iNs (Fig. 6B). Human iNs in BrainPhys+sup displayed healthy neuronal morphologies and positively stained for standard neuronal markers (Fig. 6B). The efficiency of neural conversion using BrainPhys basal instead of classic basal media (DN: 50:50 mixture DMEM/F12 and NBA) was not significantly different (~60% of neurons with both media; Fig. 6C). iNs cultured in BrainPhys +supplements for 2 wk after conversion displayed healthy functional AP properties (Fig. 6 D and E). These results demonstrate the proof of concept that BrainPhys basal can be used with direct conversion methods.

BrainPhys+sup Medium Increases ARC Protein Expression in Human Neurons Directly Induced from Fibroblasts (iNs). To further examine the influence of long-term exposure to BrainPhys+sup on human neurons using an alternative measure, we examined the cellular level of Arc protein. Transcription of the immediate early gene Arc is known to be increased by neuronal activity, or natural activating stimuli in vivo (50, 51). An increase in neuronal Arc protein levels triggers critical molecular mechanisms that strengthen synaptic communication and play a role in memory consolidation (51, 52). We compared iN cultures exposed to different basal media (BrainPhys basal vs. DN basal) with the same supplements (N2, B27, ascorbic acid, retinoic acid, laminin, BDNF, and GDNF). Strikingly, within 3 wk, BrainPhys basal significantly increased the levels of ARC protein in the human iNs (Fig. 6F). These results demonstrate that the use of BrainPhys basal in direct neuronal reprogramming techniques does not directly influence the reconversion efficiency but increases the neuronal levels of functional activity.

BrainPhys Basal Is also Applicable to Rodent Neurons. Although we developed BrainPhys basal to culture human neurons, we demonstrated that it could also be used for electrophysiological recordings in acute mouse brain slices or to culture rat primary neurons. Mouse hippocampal granule cells patched in BrainPhys basal were fully functional (*SI Appendix*, Fig. S7), and their properties recorded in BrainPhys basal were virtually identical to those in ACSF (n = 4 cells). We also checked that rat primary neurons could grow successfully in BrainPhys basal medium (supplemented with sm1) and found that they formed active neural networks within 2 wk in vitro [Fig. 7; n = 7/7 patched rat primary neurons had strong evoked AP firing (>10 Hz) and were synaptically active].

Discussion

Improving Electrical and Synaptic Activity of Mature Human Neurons in BrainPhys Basal with Supplements. In vivo, electrical activity is the essence of nervous system function. In this study, classic cell culture media were fully tested for their influence on fundamental neuronal activity. We discovered that many crucial neurophysiological properties were altered in virtually all classic

Table 1. Properties of various basal neuromedia

		DMEM	Neurobasal	ACSF	BrainPhys
Cell culture (basal Neuromedium+ supplements)	Neuronal survival in vitro	✓	✓	Less than few days	✓
	Neuronal differentiation in vitro	✓	✓	X	✓
Neuronal activity and fundamental neuronal functions	Spontaneous and evoked action potentials	Impaired	Impaired	✓	✓
	Network spontaneous calcium activity	Impaired	—	✓	✓
	Excitatory synaptic activity	Blocked	Low	✓	✓
	Inhibitory synaptic activity	Blocked	Blocked	✓	✓
	Neuronal function in vitro	Not physiological	Not physiological	✓ Short-term only	✓ Physiological
Neurophysiological properties of the media	Inorganic salt	✓	Not physiological	✓	✓
	"Neuroactive" components	Saturating neural activity	Saturating neural activity	✓	✓
	Glucose level	Hyperglycemic	Hyperglycemic	Hyperglycemic	✓Physiological
	Osmolarity (mOsmol)	✓ (315)	Low (220-250)	✓ (~300)	✓ (305)
	pH	✓ (7.4)	✓ (7.4)	✓ (7.4)	✓ (7.4)

We identified unphysiological properties in widely used basal media and resolved them in a new neuronal medium (BrainPhys).

media based on DMEM, Neurobasal, or serum (Table 1). Less physiological conditions certainly reduce the clinical relevance of experimental models. To mimic electrical activity in vivo (53, 54), we designed a new neuronal culture medium (*SI Appendix*, Table S5). Over time, the enhancement of neuronal activity in BrainPhys medium was not detrimental to the survival of human neurons. To the contrary, we found that long-term exposure to BrainPhys medium enhanced neuronal synaptic function. Unlike other classical media, BrainPhys basal with a mixture of serum-free supplements optimally supported fundamental electrical and synaptic activity.

Maturation of Human Neurons in More Physiological Conditions in Vitro. We did not design BrainPhys basal to specifically accelerate maturation. However, the superiority of BrainPhys basal in supporting spontaneous electrical and synaptic activity may indirectly enhance neuronal development (40, 47–49).

In classic medium, without a feeder layer, the probability of patching mature and synaptically functional human neurons can be relatively low (4, 55). Like other basal media, BrainPhys basal needs to be used with appropriate supplements. Further investigation might reveal new sets of growth factors that will better foster neuronal development. In our hands, BrainPhys basal with a specific set of supplements (*Methods* and *SI Appendix*, Table S6) was able to support the culturing of mature human neurons from several cell lines (greater than eight) obtained from a variety of patients. It is important to note, however, that we also encountered some cell lines, clones, or batches that failed to generate mature and active neuronal cultures. Whenever these cell lines failed to meet our neurogenic standards, they also failed in other media. In addition, it is possible that enhancement of neuronal activity affects, to different degrees, various neuronal populations based on their maturity and synaptic profiles. Nevertheless, conditions supporting basic neuronal function in vitro will have overall important cellular implications. For example, in vivo, both ARC and synaptic activity play vital roles in the molecular/cellular mechanisms underlying memory formation and consolidation. As a result ARC down-regulation has been implicated in multiple neurological disorders (51). We found that ARC protein expression was significantly increased in human neurons exposed to BrainPhys+sup medium. ARC expression is known to correlate with neuronal activity and regulate synaptic strength (51); therefore, an increase in ARC in healthy neurons exposed to BrainPhys+sup might play a role in the unsilencing of synapses of mature neurons. Although further investigation will

be needed to clearly identify the neurodevelopment mechanisms involved, our results highlight the need to use more physiological media to differentiate brain cells in vitro.

Modeling Neurological Disorders in a Dish with More Realistic Conditions. We report that classic media, which we and others have used to differentiate and culture human neurons, are suboptimal to support electrical activity and, therefore, provide very different conditions than those observed in the living brain (53, 56). Most neurological disorders are chronic and progressive and are very closely related to neuronal activity and synaptic function; thus, when modeling human neurological and psychiatric disorders in vitro, the lack of accurate physiological conditions may mask the real mechanisms of the pathologies. Although relevant phenotypes have been found between patient and control iPSC-derived neuronal cultures differentiated in currently used media (3), we predict that new phenotypes might be revealed from studying neurons in conditions that promote their electrophysiological activity. Neural models closely mimicking the living brain will be more likely to recapitulate the dysfunctions occurring in patients' brains and, in turn, lead to the discovery of more effective treatments for neurological and psychiatric disorders. The development of a new neuronal medium, such as BrainPhys basal, that sustains physiological neural activity in vitro takes us one step closer to this goal.

Methods

Human iPSC-derived neurons were obtained with previously described protocols, using the four Yamanaka factors. The BrainPhys basal medium tested in this study was custom-made. Detailed methods can be found in the *SI Appendix*.

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