An Autaptic Culture System for Standardized Analyses of iPSC-Derived Human Neurons

Graphical Abstract

Highlights
- Quality of human autapses depends on astrocyte type and fetal bovine serum additives
- Standardized assays of key morphological, biophysical, and synaptic properties
- Standardized assays of short-term plasticity, synaptic depression, and synapse recovery
- Some NGN2-induced neurons show multiple/long axons and glutamate-GABA corelease

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In Brief
Rhee et al. establish an autaptic culture system of single iPSC-derived human neurons on astrocyte micro-islands, which allows for standardized assays of neuronal morphology, membrane properties, synapse function, and synaptic short-term plasticity.

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An Autaptic Culture System for Standardized Analyses of iPSC-Derived Human Neurons

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SUMMARY

iPSC-derived human neurons are expected to revolutionize studies on brain diseases, but their functional heterogeneity still poses a problem. Key sources of heterogeneity are the different cell culture systems used. We show that an optimized autaptic culture system, with single neurons on astrocyte feeder islands, is well suited to culture, and we analyze human iPSC-derived neurons in a standardized, systematic, and reproducible manner. Using classically differentiated and transcription factor-induced human glutamatergic and GABAergic neurons, we demonstrate that key features of neuronal morphology and function, including dendrite structure, synapse number, membrane properties, synaptic transmission, and short-term plasticity, can be assessed with substantial throughput and reproducibility. We propose our optimized autaptic culture system as a tool to study functional features of human neurons, particularly in the context of disease phenotypes and experimental therapy.

INTRODUCTION

Modern methods to generate human induced pluripotent stem cells (iPSCs) and to differentiate them by cell type are expected to transform investigations of the molecular and cellular basis of neurological and psychiatric brain diseases (Blanpain et al., 2012; Hanna et al., 2010; Okita and Yamanaka, 2011; Takahashi and Yamanaka, 2016; Yang et al., 2017). Despite this progress, individual iPSC lines can differ substantially, leading to corresponding differences in derived neurons (Osafune et al., 2008; Takahashi and Yamanaka, 2016; Yang et al., 2017). Neurons derived by the same protocol from the same ESC or iPSC line can have different properties (Volpato et al., 2018; Wu et al., 2007), and mass cultured neurons derived from stem cells have massively diverse maturation states (Bardy et al., 2016; Dolmetsch and Geschwind, 2011). The causes of these discrepancies are manifold and include not only the induction protocol and cell line used but also the culturing method, medium composition, starting cell number, and co-cultured astrocytes (Bardy et al., 2016; Johnson et al., 2007; Shi et al., 2012a; Tang et al., 2013; Weick et al., 2010). These sources of heterogeneity in human ESC or iPSC-derived neurons represent a severe limitation to the analysis of—often subtle—disease-related neuronal phenotypes.

To circumvent these problems, we developed an autaptic culture system for iPSC-derived human neurons. This culture system, with single neurons on astrocyte micro-islands, is ideally suited for standardized and systematic analyses of morphological, biophysical, and functional features of neurons and their synapses (Burgalossi et al., 2012), particularly knockout phenotypes (Wojcik and Brose, 2007) and disease-related protein mutations (Lipstein et al., 2017). We optimized medium conditions and astrocyte micro-islands to provide a well-controlled autaptic culture protocol that generates morphologically mature and functionally active human iPSC-derived glutamatergic and γ-aminobutyric acid-ergic (GABAergic) neurons, in which key morphological, biophysical, and synaptic properties can be assessed reproducibly as they develop in vitro.

RESULTS

Generation of Human Neurons

Human iPSC-derived glutamatergic neurons (GlutNs) and GABAergic forebrain neurons (GABANs) were generated by either small-molecule and morphogen treatment (classical differentiation) or transcription factor-based forward programming to generate induced GABAergic neurons (iGABANs) and induced...
glutamatergic neurons (iGlutNs). The forward programming of iGlutNs is described in the accompanying paper in this issue of Cell Reports (Meijer et al., 2019).

To generate GlutNs, a published protocol (Shi et al., 2012b) was optimized to achieve a high degree of purity and reproducibility by defining cell numbers and inhibiting growth factor pathways with the MEK/ERK inhibitor PD0325901 and the γ-secretase/Notch-signaling inhibitor DAPT (Dovey et al., 2001), yielding an almost pure neuronal population after 50 days of differentiation (Figure S1A). GABANs were generated by culturing iPSCs in E8 medium and increasing concentrations of the dual SMAD inhibitors SB431542 and LDN-193189 to obtain a highly standardized, long-term cultures without the risk of overgrowing residual neural precursors, making them suitable for comparative analyses at different sites.

All experiments were based on GlutNs, GABANs, iGlutNs, iGABANs, and mouse cortical neurons for comparison (Table S1).

Human Cortical Glut Neurons Thrive in Autaptic Culture

Because human iPSC-derived neurons mature more slowly in vitro than rodent neurons, we tested the ability of rat and mouse astrocytes and of a human astrocyte cell line to support long-term autaptic cultures of GlutNs (Figure S1). We did not use purchasable human primary astrocytes, because (1) our intention was to develop an inexpensive routine method and (2) at least with mouse and rat primary astrocytes, freshly prepared, unpassaged cells are needed for micro-island cultures (Burgalossi et al., 2012). We tested the optimal fetal bovine serum (FBS) concentration (range 0.25%–10%) in N3 medium to maintain astrocytes in long-term culture and found that concentrations above or below 0.5% led to cell loss. We then assessed the optimal seeding number of human neurons (range 0.5 × 10^3 to 100 × 10^3 per 100 mm² micro-island astrocyte coverslip) (Burgalossi et al., 2012) and found 3,500–8,000 cells per 100 mm² micro-island astrocyte coverslip to be optimal.

We next tested rat, mouse, and human astrocytes more systematically using N3 medium (Shi et al., 2012a, 2012b) or N3 medium with 0.5% FBS (the osmosality of the different culturing conditions was screened) (Figures S1K and S1L). Cells were fixed after 2, 4, 6, and 8 weeks in vitro and labeled for the dendrite and soma marker MAP2, the presynaptic marker VGlut1, and the postsynaptic marker Shank2. To assess neuronal growth, we assayed dendrite complexity by Sholl analysis (Deshpande et al., 2017; Sholl, 1953). Differences between astrocyte types and culture conditions were apparent at 8 weeks (Figure 1A). GlutNs co-cultured with mouse astrocytes without/with FBS (GlutM-FBSNs/GlutM-FBSNs) and rat astrocytes without/with FBS (GlutR-FBSNs/GlutR-FBSNs) developed better in culture (average dendritic intersections of 143.1 ± 20.7, 95.9 ± 11.4, 171.6 ± 13.6, and 132.8 ± 17.4, respectively) than neurons on human astrocytes without/with 20 μM flocuridine (H/H+FUDR, added to prevent further astrocyte division) (54.3 ± 9.1 and 70.3 ± 27.3 intersections, respectively) (Figure 1B). At 2 weeks, cultured neurons in the presence of FBS showed less complex dendrites, but this changed around 4 weeks, when neurons in FBS improved (e.g., GlutR-FBSNs, 350.6 ± 24.0 intersections, and GlutR-FBSNs, 265.8 ± 21.9 intersections) (Figure 1B). All conditions allowed neurons to thrive, except when human astrocytes were used; these did not support neuron survival beyond 4 weeks (Figures 1A–1D).

To assess synaptogenesis, we counted colocalizing puncta of immunolabeled VGlut1 and Shank2 (Figures 1E and 1F). GlutNs cultured with human astrocytes hardly formed any synapses, while GlutR-FBSNs and GlutM-FBSNs developed the highest numbers of synapses (160.0 ± 13.7 and 131.2 ± 12.9, respectively) (Figure 1G). In comparison, GlutM-FBSNs and GlutR-FBSNs developed significantly fewer synapses (73.2 ± 8.1 and 117.0 ± 18.2, respectively) (Figure 1G). In parallel, we quantified neuronal survival, expressed as the ratio of neurons per micro-island (Figure 1H). At 2 weeks, ratios of 1.3 ± 0.1, 0.9 ± 0.04, 1.1 ± 0.1,
and 0.8 ± 0.1 were observed for the M–FBS, M+FBS, R–FBS, and R+FBS conditions, respectively. Ratios decreased over time under all conditions, most prominently with R+FBS cells, whose ratio was only 0.26 ± 0.02 at 8 weeks, while the M–FBS condition had the highest survival rate after 8 weeks (0.8 ± 0.1). These data show that cultured human GlutNs are viable for at least 8 weeks with mouse and rat astrocytes, with mouse astrocytes being superior in terms of neuronal survival (Figure 1H).

We assessed the maturation of GlutM+FBSNs by labeling MAP2 and doublecortin (DCX), a marker for immature neurons (Figures S2A and S2B). DCX levels dropped significantly after 2 weeks, while MAP2 levels increased (Figures S2C–S2E). DCX levels remained constant in neurite tips (Figures S2F and S2G), reflecting ongoing growth (Spampanato et al., 2012).

**Human Cortical GlutNs Show Proper Membrane Properties**

Autaptic GlutNs showed evoked postsynaptic potential (PSP) and action potential (AP) firing patterns upon depolarization (Johnson et al., 2007): abortive (no APs), phasic I (single AP), phasic II (multiple APs during the early phase of depolarization), tonic (APs throughout depolarization), and spontaneous (irregular APs even without depolarization) (Figure S3). Input resistances ($R_{\text{input}}$) of the different neuron groups were similar, except that phasic I neurons had a lower $R_{\text{input}}$ (Figures S3Bi and S3Bii).

At 8 weeks, 90% of GlutM+FBSNs exhibited a tonic firing pattern (Figures 2A and 2B), while 90% of GlutM+FBSNs showed a tonic firing pattern at 6 weeks (Figures 2C and 2D). GlutR+FBSNs and GluRt+FBSNs exhibited properties similar to those of cells cultured with mouse astrocytes (Figures S3F–S3I).

Resting membrane potentials (RMPs) of GlutM+FBSNs were $-37.4 ± 2.5$ mV at 2 weeks and decreased until 4 weeks to then stabilize (4 weeks, $-51.6 ± 1.6$ mV; 6 weeks, $-55.5 ± 1.7$ mV; and 8 weeks, $-54.9 ± 1.5$ mV) similar to autaptic mouse cortical neurons ($-50.0 ± 1.5$ mV) (Figures 2E and S6B). RMPs of GlutM+FBSNs were stable at 2 weeks onward (2 weeks, $-44.5 ± 5.6$ mV; 4 weeks, $-51.5 ± 8.4$ mV; 6 weeks, $-46.4 ± 6.1$ mV; and 8 weeks, $-48.7 ± 4.9$ mV) (Figure 2E). RMPs of GlutRt+FBSNs and GluRt+FBSNs were similar to those of GlutM+FBSNs and GlutM+FBSNs (Figure S3J), indicating few RMP differences between cells cultured on mouse or rat astrocytes.

The capacitance, a measure of cell size, of GlutM+FBSNs and GlutM+FBSNs increased from 2 to 10 weeks (Figure 2F), as with GlutM+FBSNs and GlutRt+FBSNs (Figure S3K). The $R_{\text{input}}$ of GlutM+FBSNs and GlutM+FBSNs and of GlutM+FBSNs and GlutM+FBSNs decreased steadily and similarly over time (Figures 2G and S3L).

Active electrical properties of GlutNs were analyzed by recording threshold, overshoot, amplitude, and half-width of APs, and the afterhyperpolarization (AHP) amplitude. Overshoot and amplitude of APs in GlutM+FBSNs and GlutM+FBSNs increased from 2 to 8 weeks and then stabilized, while AP half-width decreased progressively and AHP amplitude remained stable (Figures 2H–2L). Similar data were obtained for GlutR+FBSNs and GlutRt+FBSNs (Figures S3M–S3Q). Thus, active and passive electrical properties of GlutNs are not affected by FBS or astrocyte type.

The density and activity of voltage-gated Na⁺ channels are the main determinants of AP overshoot and amplitude. The magnitudes of AP overshoot and amplitude increased with time in all tested cells (Figures 2I, 2J, S3N, and S3O), indicating that the density of voltage-gated sodium currents $(I_{\text{Na}})$ increased. $I_{\text{Na}}$ of GlutM+FBSNs was evoked by 500 ms test pulses at 10 mV intervals between −80 and +70 mV from a holding potential of −70 mV, with or without 300 nM tetrodotoxin (TTX) (Figure S3D). The densities of peak $I_{\text{Na}}$ in GlutM+FBSNs were not significantly different between 2 and 4 weeks (2 weeks, 185.8 ± 29.9 pA/pF; 4 weeks, 179.5 ± 21.1 pA/pF) but larger at 6 weeks (255.0 ± 26.7 pA/pF) and 8 weeks (240.7 ± 25.3 pA/pF) (Figure 2P). The peak $I_{\text{Na}}$ densities at 6 and 8 weeks in GlutM+FBSNs were similar to those of mouse cortical neurons (249.1 ± 41.6 pA/pF).

Voltage-gated K⁺ currents $(I_{\text{K}})$ determine width, repolarization, and frequency of APs. Voltage-clamp recordings were used to test whether delayed rectifier K⁺ currents $(I_{\text{DR}})$ and the transient A-type K⁺ currents $(I_{\text{A}})$ are correlated with AP maturation in GlutM+FBSNs and GlutM+FBSNs. Total K⁺ currents $(I_{\text{K}})$ were evoked by 500 ms depolarizing test pulses at +10 mV intervals between −80 and +70 mV from a holding potential of −70 mV; $I_{\text{DR}}$ was recorded using the same protocol in the presence of 1 mM 4-aminopyridine (AP4). $I_{\text{A}}$ traces were obtained by subtracting $I_{\text{DR}}$ from $I_{\text{K}}$ (Figure S3E). The density of $I_{\text{A}}$ in GlutM+FBSNs...
and GlutM:FBSNs increased between 2 weeks (124.2 ± 10.6 pA/pF) and 10 weeks (167.9 ± 23.2 pA/pF), as did the densities of I_Na and I_R (Figures 2M–2O). Instead, the density of I_dr and I_K in GlutRNs remained unchanged with time in culture (Figures S3R–S3T). AP maturation in GlutRNs and GlutMNs was similar at 8 weeks, but the densities of I_dr and I_K were lower in GlutRNs, indicating that mouse astrocytes support neuronal maturation slightly better than rat astrocytes.

**Medium Composition and Astrocyte Origin Affect Synaptic Activity of Human Cortical GlutNs**

Synaptic properties of GlutM:FBSNs and GlutM:FBSNs under different culturing conditions were studied using whole-cell voltage-clamp recordings. Evoked excitatory postsynaptic current (EPSC) amplitudes of GlutM:FBSNs gradually increased until 10 weeks (2.66 ± 0.26 nA). Beyond 10 weeks, neurons were scarce, likely due to astrocyte death. GlutM:FBSNs developed synaptic currents at 2 weeks, similar to GlutM:FBSNs, but EPSC amplitudes then increased less prominently to only 0.82 ± 0.34 nA at 10 weeks (Figure 3A), indicating that FBS affects synapse number and function. Similar findings were obtained in assays of the readily releasable pool (RRP) of synaptic vesicles, as estimated by integrating charge transfer during hypertonc 0.5 M sucrose application for 6 s. While RRP sizes of GlutM:FBSNs gradually increased over time, leveling off at 8 weeks (0.14 ± 0.01 nC), RRP sizes of GlutR:FBSNs were smaller (Figure 3E). The vesicular release probabilities (P_v), calculated by dividing the charge transfer of a single EPSC by the charge transfer of RRP release, were similar between GlutM:FBSNs and GlutM:FBSNs, although the P_v of GlutM:FBSNs fluctuated with time (Figure 3F). Thus, although the strength of synaptic transmission was higher in GlutM:FBSNs than in GlutM:FBSNs, the P_v was similar. EPSC amplitudes, RRP, and P_v in GlutR:FBSNs and GlutR:FBSNs cells were only measured between 7 and 10 weeks. During this period, the corresponding values did not change substantially, but they were consistently smaller in the absence of FBS (Figures 3C, 3G, and 3H).

We next recorded miniature EPSCs (mEPSCs) in 300 nM TTX. GlutM:FBSNs had higher mEPSC amplitudes and frequencies than GlutM:FBSNs, which increased with time in culture (Figures 3I and 3J). mEPSC amplitudes and frequencies in GlutR:FBSNs and GlutR:FBSNs peaked at 8 weeks and were smaller in GlutM:FBSNs at this time point (Figures 3K and 3L), again showing that FBS supports synaptic strength.

Diacylglycerol and phorbol esters stimulate transmitter release in rodent autapses via Munc13-family vesicle priming proteins and protein kinase C (PKC) (Rhee et al., 2002; Wierda et al., 2007). 3 μM phorbol 12,13-dibutyrate (PDBu) had a similar but less pronounced effect on GlutM:FBSNs and GlutR:FBSNs (Figures 3B and 3D), indicating that regulatory effects of Munc13s and PKC on transmitter release are similar in rodent and human neurons.

**Human Cortical GlutNs Thrive Best with Mouse Astrocytes and 0.5% FBS**

Because GlutR:FBSNs had lower survival rates than GlutM:FBSNs, we investigated whether the different culture conditions stress
cells differently. GlutM+FBSNs or GlutR+FBSNs were fixed and stained for Hsc70 and c-Fos, together with MAP2. Hsc70 is up-regulated in response to various stressors and plays a fundamental role in cell recovery (Klenke et al., 2013). c-Fos was used as an immediate early gene marker to estimate cellular activity (Okuno, 2011). Neurons were stressed by heat shock for 10 min at 45°C and then incubated at 37°C for 90 min to recover (Kotoglu et al., 2009). Hsc70 fluorescence intensity in GlutR+FBSNs at 8 weeks was 606.9 ± 75.2 a.u., 2-fold higher than in GlutM+FBSNs (309.4 ± 44.9 a.u.). c-Fos expression was always higher in GlutR+FBSNs, and c-Fos fluorescence intensity decreased from 532.4 ± 77.1 a.u. at 4 weeks to 124.4 ± 40.3 a.u. at 8 weeks. Decreased cellular activity and elevated stress markers in GlutR+FBSNs indicate that mouse astrocytes are slightly superior over rat astrocytes for the human neurons (Figure 4).

Furthermore, we assessed the effect of physiologically balanced BrainPhys medium on cultured human neurons (Figures S2H–S2J). Although BrainPhys had been shown to boost synaptic transmission in mass-cultured iPSC-derived human neurons (Bardy et al., 2015), we detected a negative effect on astrocytes in autaptic cultures, with consequent deleterious effects on GlutNs. Cultures were fixed at 2, 4, 6, and 8 weeks and labeled for GFAP and MAP2. Astrocyte areas cultured in BrainPhys were smaller and astrocytes developed abnormal pointy structures when compared to cells cultured with N3+FBS. Moreover, we noticed an increase of GFAP fluorescence intensity in astrocytes in BrainPhys (Figures S2H–S2J).

Once this phenotype had emerged, we cultured cells in N3 medium and replaced it with BrainPhys 5 days before electrophysiological recordings. Here, BrainPhys increased evoked EPSC amplitudes in GlutR+FBSNs from 0.58 ± 0.09 to 1.00 ± 0.1 nA from 8 to 9 weeks, without significant effects on RRP size, P vr, and mEPSC amplitude or frequency (Figures S2K–S2O). We repeated this experiment with the addition of FBS to the N3 medium and again replaced the media with BrainPhys 5 days before experimental recordings. Here, BrainPhys did not enhance synaptic transmission (Figures S2P–S2U). These data show that culturing neurons with mouse astrocytes in the presence of 0.5% FBS is the best choice.

**Rapid Maturation of NGN2-Induced Autaptic Human Neurons**

To circumvent the long-term culture required for the maturation of classically differentiated human neurons, we used our optimized culture conditions (i.e., mouse astrocytes, N3 medium, and 0.5% FBS) to generate autaptic cultures of human GlutNs after direct induction with NGN2 (iGlutNs) (Zhang et al., 2013). We first studied the morphology of iGlutNs after 2 and 4 weeks in culture by Sholl analysis (Figures 5A–5C). Cells at 2 weeks already showed pronounced processes, which had grown substantially at 4 weeks (intersection per neuron, 109.7 ± 8.9 at 2 weeks and 167.8 ± 18.0 at 4 weeks; enclosing radius, 131.3 ± 9.2 μm at 2 weeks and 165.8 ± 12.4 μm at 4 weeks). Similarly, the number of synapses, as determined by colabeling VGluT1, Shank2, and MAP2 and quantification as explained earlier, increased from 2 weeks (4.6 ± 1.3 per cell) to 4 weeks (69 ± 10.3 per cell) (Figures 5D and 5E).

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**Figure 3. Characterization of Synaptic Transmission in GlutNs**

(A) Example (top) and mean EPSC amplitudes. GlutM+FBSNs (black) at 2 weeks (n = 3), 3 weeks (n = 8), 4 weeks (n = 9), 5 weeks (n = 6), 6 weeks (n = 27), 7 weeks (n = 49), 8 weeks (n = 67), 9 weeks (n = 42), 10 weeks (n = 48), and 11 weeks (n = 7); GlutR+FBSNs (gray) at 2 weeks (n = 1), 4 weeks (n = 2), 6 weeks (n = 7), 7 weeks (n = 3), 8 weeks (n = 11), 9 weeks (n = 7), 10 weeks (n = 7), 11 weeks (n = 5), and 12 weeks (n = 6).

(B) Mean EPSC potentiation in GlutM+FBSNs by 3 μM PDBu (n = 11).

(C) Mean EPSC amplitudes. GlutR+FBSNs (black) at 6 weeks (n = 2), 7 weeks (n = 18), 8 weeks (n = 16), 9 weeks (n = 15), and 10 weeks (n = 11); GlutR+FBSNs (gray) at 7 weeks (n = 11), 8 weeks (n = 16), 9 weeks (n = 9), and 10 weeks (n = 6).

(D) Mean EPSC potentiation in GlutM+FBSNs by 3 μM PDBu (n = 12).

(E) Mean EPSC amplitudes. GlutM+FBSNs at 6 weeks (n = 7), 7 weeks (n = 21), 8 weeks (n = 29), 9 weeks (n = 18), and 10 weeks (n = 28); GlutR+FBSNs at 6 weeks (n = 3), 7 weeks (n = 2), 8 weeks (n = 6), 9 weeks (n = 9), 10 weeks (n = 4), 11 weeks (n = 4), 12 weeks (n = 3), and 12 weeks (n = 3).

(F) Mean P vr. GlutM+FBSNs at 6 weeks (n = 7), 7 weeks (n = 21), 8 weeks (n = 29), 9 weeks (n = 18), and 10 weeks (n = 28); GlutM+FBSNs at 6 weeks (n = 3), 7 weeks (n = 2), 8 weeks (n = 6), 9 weeks (n = 4), 10 weeks (n = 4), 11 weeks (n = 4), and 12 weeks (n = 3).

(G) Mean RRP sizes. GlutM+FBSNs at 7 weeks (n = 5), 8 weeks (n = 11), 9 weeks (n = 6), and 10 weeks (n = 10); GlutR+FBSNs, 7 weeks (n = 4), 8 weeks (n = 5), 9 weeks (n = 4), and 10 weeks (n = 3).

(H) Mean P vr. GlutR+FBSNs at 7 weeks (n = 5), 8 weeks (n = 11), 9 weeks (n = 6), and 10 weeks (n = 10); GlutR+FBSNs at 7 weeks (n = 4), 8 weeks (n = 5), 9 weeks (n = 4), and 10 weeks (n = 3).

(I) Mean mEPSC amplitudes. GlutM+FBSNs at 6 weeks (n = 6), 7 weeks (n = 18), 8 weeks (n = 28), 9 weeks (n = 13), and 10 weeks (n = 29); GlutR+FBSNs at 6 weeks (n = 3), 8 weeks (n = 4), 9 weeks (n = 2), 10 weeks (n = 3), 11 weeks (n = 4), and 12 weeks (n = 2).

(J) Mean mEPSC frequencies. GlutM+FBSNs at 6 weeks (n = 6), 7 weeks (n = 18), 8 weeks (n = 28), 9 weeks (n = 13), and 10 weeks (n = 29); GlutR+FBSNs at 6 weeks (n = 3), 8 weeks (n = 4), 9 weeks (n = 4), 10 weeks (n = 3), 11 weeks (n = 4), and 12 weeks (n = 2).

(K) Mean mEPSC amplitudes. GlutM+FBSNs at 7 weeks (n = 5), 8 weeks (n = 6), 9 weeks (n = 5), and 10 weeks (n = 9); GlutR+FBSNs at 7 weeks (n = 2), 8 weeks (n = 5), 9 weeks (n = 2), and 10 weeks (n = 2).

(L) Mean mEPSC frequencies. GlutM+FBSNs at 7 weeks (n = 5), 8 weeks (n = 6), 9 weeks (n = 5), and 10 weeks (n = 9); GlutR+FBSNs at 7 weeks (n = 2), 8 weeks (n = 5), 9 weeks (n = 2), and 10 weeks (n = 2).

(M) STP of GlutM+FBSNs at 5 Hz (n = 36), 10 Hz (n = 87), and 40 Hz (n = 61) and of GlutR+FBSNs at 10 Hz (n = 15) and 40 Hz (n = 7).

(N) STP of GlutM+FBSNs at 5 Hz (n = 29), 10 Hz (n = 27), and 40 Hz (n = 27) and of GlutR+FBSNs at 5 Hz (n = 9), 10 Hz (n = 7), and 40 Hz (n = 7).

(O) Diagram and representative traces of double 40 Hz stimulation with different time intervals, and plot of the cumulative charge transfer curve (arrowhead indicates the y intercept).

(P) EPSC (black) and RRP (gray) recovery ratios of GlutM+FBSNs (all n = 10).

Insets in (A), (C), (E), and (G) show representative traces (GlutM+FBSNs: 4 weeks, red; 6 weeks, blue; 8 weeks black; GlutR+FBSNs: 8 weeks, gray). Insets in (B) and (D) show representative traces before (black) and during PDBu (blue) application. Insets in (I) and (J) show representative mEPSC traces.
At 2 weeks, iGlutNs showed robust EPSCs (0.67 ± 0.16 nA), which increased to ~1.5 nA at 4 weeks (Figure 5F). The parallel increase of the RRP over development was more uniformly continuous (0.03 ± 0.01 nC at 2 weeks to 0.12 ± 0.03 nC at 4 weeks) (Figure 5H), so the calculated P_r was 8.5% ± 2.1% at 2 weeks versus 6.6% ± 0.8% at 4 weeks (Figure 5I). mEPSC amplitudes were stable around 33 pA over development, while mEPSC frequencies increased continuously from 1.3 Hz at 2 weeks to 3.1 Hz at 4 weeks (Figures 5J and 5K). PDBu increased EPSCs by ~1.4-fold (Figure 5G), similar to GlutNs. Thus, basic synaptic features of iGlutNs were similar to those of GlutNs.

Furthermore, features of STP, paired-pulse ratio (PPR), and EPSC and RRP recovery in iGlutNs (Figures 5L and 5M) were qualitatively similar to those of GlutNs (Figures 3M–3P) and...
Figure 5. Characterization of Morphological and Functional Properties of iGlutNs

(A) Number of dendritic intersections of iGlutNs (2 weeks and 4 weeks) plotted as a function of distance from the soma. Binarized images in insets show iGlutNs at 2 weeks (left) and 4 weeks (right).

(B) Average number of dendrites intersecting Sholl circles 5–250 μm from the soma as a function of time.

(C) Enclosing radius of dendrites measured from the soma. iGlutNs at 2 weeks (n = 27) and 4 weeks (n = 25).

(D) Confocal images of iGlutNs at 2 and 4 weeks (top and bottom panels, respectively). Antigens and stains are indicated.

(E) Average number of synapses per neuron at 2 weeks (n = 36) and 4 weeks (n = 29).

(F) Mean EPSC amplitudes. iGlut+DoxNs (black) at 2 weeks (n = 18), 3 weeks (n = 18), and 4 weeks (n = 42); iGlut/C0 DoxNs (gray) at 2 weeks (n = 15), 3 weeks (n = 15), and 4 weeks (n = 33).

(G) Mean EPSC potentiation in iGlut+DoxNs by 3 μM PDBu (n = 10).

(H) Mean RRP sizes. iGlut+DoxNs at 2 weeks (n = 6), 3 weeks (n = 9), and 4 weeks (n = 20); iGlut−DoxNs at 2 weeks (n = 7), 3 weeks (n = 6), and 4 weeks (n = 12).

(I) Mean mEPSC frequency. iGlut+DoxNs at 2 weeks (n = 6), 3 weeks (n = 9), and 4 weeks (n = 12); iGlut−DoxNs at 2 weeks (n = 10), 3 weeks (n = 6), and 4 weeks (n = 10).

(K) Mean mEPSC amplitudes. iGlut+DoxNs at 2 weeks (n = 4), 3 weeks (n = 11), and 4 weeks (n = 12); iGlut−DoxNs at 2 weeks (n = 5), 3 weeks (n = 12), and 4 weeks (n = 10).

(L) STP of iGlut+DoxNs at 5 Hz (n = 12), 10 Hz (n = 21), and 40 Hz (n = 21) and of iGlut−DoxNs at 5 Hz (n = 13), 10 Hz (n = 21), and 40 Hz (n = 16).

(M) EPSC (black) and RRP (gray) recovery ratios of iGlut+DoxNs (all n = 10).

Insets in (F)–(H) show representative traces (+Dox/control, black; −Dox/PDBu, blue). Mann-Whitney test was applied (*p < 0.05, ****p < 0.0001).
mouse cortical neurons (Figures S6S and S6T). However, STD and paired pulse depression were more pronounced in iGlutNs.

Because doxycycline (Dox) is used to induce NGN2 expression, we assessed whether continued Dox treatment affects iGlutNs. None of the tested parameters were different between GlutM+FBSNs and iGlutM+DoxNs (Figures 5F–5M), indicating that Dox presence does not affect the maturation and function of iGlutNs after initial induction.

We tested an additional iGlutN line (C35) and compared key features with the routinely used C14 line. Passive membrane properties, AP characteristics, EPSC amplitudes, and STD were strikingly similar between the two lines (Figures S4A–S4E), demonstrating that our culture system leads to reliable and reproducible readouts even from two different cell lines.

Unusual Morphological and Functional Features of NGN2-Induced Neurons
NGN2-induced neurons have the clear advantage of rapid maturation. However, this rapid maturation may lead to unusual developmental trajectories, although earlier studies did not detect abnormalities (Zhang et al., 2013).

We consistently detected unusual multi-peak EPSCs in a subset of single autaptic iGlutNs (Figure 6A). We first used hypertonic sucrose stimulation to test whether multi-peak EPSCs might be due to more than one neuron present, expecting sucrose responses combined with an AP-like spike response, which was never observed (Figure 6Ai). To test whether multi-peak EPSCs might originate from cells with multiple axons, we filled patched neurons with 0.2% biocytin, stained them using streptavidin and antibodies to MAP2 and Ankyrin G (marker of axon initial segments) (Figure 6Aiv), and found that double EPSC peaks corresponded to cells with two or more axons. Regardless of Dox presence, this phenotype was found in about a quarter of all iGlutNs (GlutM+Dox, 28% ± 2%; and iGlutM+Dox, 29% ± 4%), while only 8% GlutM+FBSNs showed multiple axons (Figure 6B). Moreover, axons of iGlutNs were longer than those of GlutM+FBSN axons, as assessed by staining iGlutNs at 4 weeks and GlutM+FBSNs at 4 and 8 weeks for SMI-312 (axons) and MAP2 (iGlut at 4 weeks, 3.7 ± 0.1 mm; GlutM+FBS at 4 weeks, 0.53 ± 0.04 mm; and GlutM+FBS at 8 weeks, 1.3 ± 0.1 mm) (Figures 6C and 6D). The length of iGlutN axons and their small diameter, multi-peak EPSCs with inter-peak intervals of 2–5 ms are conceivable. We further tested whether the long iGlutNs axons also cause longer delays between AP and EPSC onsets (Figure 6Ei). GlutM+FBSNs showed slightly longer synaptic delays compared with mouse cortical neurons, and the delay was more pronounced in iGlutM+Dox/DoxNs (3.47 ± 0.11, 5.18 ± 0.3, and 4.54 ± 0.2 ms, respectively) (Figures 6Eii and S4). The presence of Dox did not affect the synaptic delay (Figure 6Eii).

Apart from multi-peak EPSCs, some iGlutNs showed unusual EPSC kinetics with a pronounced slow component indicative of a GABAergic contribution. When we applied the GABA_A receptor antagonist bicuculline or the z-AMPA receptor antagonist N-methyl-4-isoxazoleproionic acid (AMPA) receptor antagonist NBQX, the former blocked the slow EPSC component while the latter blocked the fast one (Figures 6F–6I), indicating corelease of GABA and glutamate by individual iGlutNs. Accordingly, staining of VGAT (a GABAergic presynapse marker) and VGlut1 (a glutamatergic presynapse marker), along with labeling of Shank2 and MAP2, showed that some iGlutNs coexpress VGAT and VGluT1 at the same synapses (Figures 6J–6L).

Human Inhibitory Neurons in Autaptic Culture
Because GABAergic dysfunction is implicated in many brain diseases, we devised autaptic culture methods for iPSC-derived GABAergic neurons, comparing rat versus mouse astrocytes and the presence versus the absence of FBS in the media. We started with GABANs and analyzed a subset of electrophysiological parameters. Evoked inhibitory postsynaptic currents (IPSCs) were found in GABA_M+FBSNs by 3 weeks and increased to ~1.2 nA by 7 to 9 weeks. GABA_M+FBSNs behaved similarly, with further increases of IPSC amplitudes until 13 weeks.
Figure 7. Characterization of Synaptic Transmission in GABANs and iGABANs

(A) Mean IPSC amplitudes. GABA_{M+FBSNs} (black) at 3 weeks (n = 6), 5 weeks (n = 8), 7 weeks (n = 7), and 9 weeks (n = 10); GABA_{M/C0+FBSNs} (gray) at 5 weeks (n = 11), 7 weeks (n = 3), 9 weeks (n = 23), 11 weeks (n = 28), and 13 weeks (n = 3).

(B) Mean IPSC amplitudes. GABA_{R+FBSNs} (black) at 9 weeks (n = 12), 11 weeks (n = 3), and 13 weeks (n = 5); GABA_{R/C0+FBSNs} (gray) at 7 weeks (n = 14), 9 weeks (n = 37), 11 weeks (n = 24), and 13 weeks (n = 17).

(C) EPSC amplitudes in a small subset of all tested GABANs (n = 4).

(D) Mean IPSC potentiation in GABA_{M+FBSNs} (n = 6) and GABA_{M/C0+FBSNs} (n = 9) by 3 μM PDBu.

(E) Representative trace of STD in GABA_{M+FBSNs} (black) and GABA_{M/C0+FBSNs} (gray) during 10 Hz stimulation (left), and STP (at 7–9 weeks, GABA_{M+FBSNs}, n = 6; GABA_{M/C0+FBSNs}, n = 8) (right). The inset shows the first three episodes of the train.

(F) Representative trace of STD in GABA_{R+FBSNs} (black) and GABA_{R/C0+FBSNs} (gray) during 10 Hz stimulation (left), and STP (at 7–9 weeks, GABA_{R+FBSNs}, n = 9; GABA_{R/C0+FBSNs}, n = 13). The inset shows the first three episodes of the train.

(G) Mean IPSC amplitudes. iGABANs at 3 weeks (n = 16), 4 weeks (n = 14), 5 weeks (n = 17), 6 weeks (n = 9), 7 weeks (n = 17), and 8 weeks (n = 23).

(H) Mean IPSC potentiation in iGABANs by 3 μM PDBu (n = 9).

(I) Percentages of iGABANs with different types of evoked release. Single evoked, black (n = 86); delayed evoked, navy (n = 11); corelease, pink (n = 4); glutamate release, red (n = 2); non-responders, gray (n = 50).

(J) Mean RRP sizes. iGABANs at 3 weeks (n = 12), 4 weeks (n = 10), 5 weeks (n = 12), 6 weeks (n = 6), 7 weeks (n = 14), and 8 weeks (n = 17).

(K) Mean P_{pr}. iGABANs at 3 weeks (n = 12), 4 weeks (n = 10), 5 weeks (n = 12), 6 weeks (n = 6), 7 weeks (n = 14), and 8 weeks (n = 17).

(legend continued on next page)
iGABANs were mostly of the tonic AP firing type (Figures S5A and SSB). They had a stable RMP of about -60 mV at 6 weeks (Figures 7C). Furthermore, IPSCs in iGABA_{R+FBSNs} (7–8 weeks) and GABA_{R+FBSNs} (8 weeks) increased similarly in response to PDBu treatment (1.75- to 1.97-fold) (Figure 7D), and GABA_{R} and GABA_{B} showed similar STD time courses during high-frequency stimulation, irrespective of the presence or absence of FBS (Figures 7E and 7F). Overall, these data show that GABANs in autaptic culture, unlike GlutNs, are rather insensitive to the astrocyte type and to FBS in the culture medium.

iGABANs induced by ASCL1 and DLX2 (Yang et al., 2011) on mouse astrocytes (in N3 medium with 0.5% FBS) developed faster than GlutNs, much like iGlutNs. From 2 to 8 weeks in culture, iGABANs were mostly of the tonic AP firing type (Figures S5A and SSB). They had a stable RMP of about -50 mV over time in culture, increased in size as assessed by cell capacitance from 2 weeks (~18 pF) to a plateau at 6 weeks (~36 pF) (about half the size of corresponding GlutNs and iGlutNs), and showed a progressive decrease in R_{input} from 2 weeks (~1,200 MΩ) to stabilize at 6 weeks (~700 MΩ) (Figures S5G–S5E). AP threshold (~17 to -24 mV), overshoot (38 to 50 mV), amplitude (60 to 67 mV), and width (1.5 to 2.4 ms) remained rather stable over time in culture, while AHP increased from ~16 mV at 2 weeks to ~24 mV at 8 weeks (Figures S5F–S5J). Like iGlutNs (Figures S4R–S4T), iGABANs had higher k_{c}, I_{acc}, and I_{an} densities (Figures S5G–S5M) than GlutNs (Figures 2M–2O), indicating that the degree of differentiation correlates with k_{c}, I_{acc}, and I_{an} density. iGABANs grew rapidly from 2 to 4 weeks and then gradually increased in size and complexity until 8 weeks (Figures S5G–S5Q). Synaptic currents in iGABANs first appeared at 3 weeks, after which IPSC amplitudes gradually increased to 4.95 ± 1.38 nA at 6 weeks; beyond 7 weeks, IPSC amplitudes decreased again (Figure 7G). In accordance with changes in IPSC amplitudes with time, the number of inhibitory synaptic puncta also increased (Figure S5R).

PDBu caused a slight (1.34-fold) increase in IPSC amplitudes (Figure 7H). More than 50% of cells showed single IPSCs in response to single APs, about 30% of cells did not show synaptic responses, 7.2% displayed delayed synaptic responses, and 3% showed GABA and glutamate corelease (Figure 7I). The RRP size increased from ~0.5 nC at 3 weeks to 1.87 ± 0.44 nC at 6 weeks and then remained steady (Figure 7J). Correspondingly, the P_{o} of R_{input} remained relatively stable over time (Figure 7K). Miniature inhibitory post synaptic current (mIPSC) amplitudes were generally stable throughout the culturing period, with some fluctuation at 7 weeks (30–42 pA), while mIPSC frequencies fluctuated substantially during culture time (Figures 7L and 7M). Finally, iGABANs showed strong STD at stimulation frequencies of 5–40 Hz (Figure 7N).

**DISCUSSION**

Cell cultures are commonplace in all areas of biomedical research. They are used as reductionist experimental systems despite certain disadvantages, such as loss of tissue context, because of their low complexity, reproducibility, amenability to manipulations, and high-throughput potential. In neuroscience, thousands of studies have employed primary neuron cultures, mostly from rodents, to discover new biology—from basic principles to disease mechanisms. However, the usefulness of cultured rodent neurons reaches its limits in the context of neurological and psychiatric brain diseases. Many of these have a substantial genetic etiology, but modeling corresponding genetic disease causes is often impossible because of their multigenic nature. Furthermore, intrinsic differences between rodent and human neurons may limit the interpretability of data. Here, the advent of methods to generate human neurons from iPSCs of probands (Okita and Yamanaka, 2011; Takahashi and Yamanaka, 2016; Yang et al., 2017) and to rapidly manipulate them genetically (Doudna and Charpentier, 2014) heralds a revolution. Systematic analyses of patient-derived neurons are possible and hold great promise, because the genetic disease causes manifest at the cellular level.

In terms of standardized, systematic analyses of morphological, biophysical, and synaptic properties of wild-type and mutant neurons with efficient throughput, the autaptic culture system with single neurons on small astrocyte feeder islands is particularly useful, because it combines easy experimental access to cells and the potential to read out a plethora of cellular features with standardization, reproducibility, and medium-throughput potential (Bekkers and Stevens, 1991; Pyott and Rosenmund, 2002; Burgalossi et al., 2012). Even single experimenters can electrophysiologically characterize dozens of autaptic cells in a few days, and inter-laboratory differences are typically so small that datasets can be combined to deduce biological principles (Schotten et al., 2015).

We optimized the autaptic culture system for human iPSC-derived neurons, i.e., classically differentiated GlutNs and GABANs, as well as iGlutNs and iGABANs, both induced by forced transcription factor expression. GlutNs and GABANs were used to determine optimal media compositions, astrocyte origin, and FBS supplementation. N3 medium with 0.5% FBS and mouse astrocytes turned out to be optimal. In BrainPhys, micro-island astrocytes were smaller and showed unusual morphology and increased GFAP immunofluorescence, with deleterious effects on cultured neurons. Furthermore, it did not improve functional readouts when used after an initial culturing phase in N3 medium with 0.5% FBS. Our medium, however, improved cell survival, maturation, and functionality under
almost all conditions tested. FBS had the strongest positive effect on the maturation of synaptic functionality, while cell sizes and membrane properties were less affected or unaffected, indicating that these parameters are under strong cell-intrinsic control. The human astrocyte cell line we used was unsuitable for human autapses, because cells died beyond 4 weeks. This may be different with altered culture conditions (or human primary astrocytes), but further optimization was not deemed necessary, because mouse and rat astrocytes supported the growth, maturation, and analysis of human autapses in long-term cultures. Because rat astrocytes were slightly less supportive, e.g., regarding neuronal survival and AP maturation, and caused higher neuronal stress, we propose that mouse astrocytes are optimal for human autaptic cultures. By using N3 media with 0.5% FBS and mouse astrocytes, we found that interexperimental variations of cellular parameters were small, indicating that we developed consistent and reproducible autaptic culture conditions.

GlutNs and GABANs were pure in terms of transmitter type but took at least 8 weeks (GlutNs) and up to 13 weeks (GABANs) in culture to mature. They showed robust PSCs and STP features that are comparable to those of iGlutNs and mouse cortical neurons, as well as to iGABANs and mouse striatal GABAergic neurons (Nair et al., 2013). Furthermore, GlutNs were mostly of the tonic AP firing type at 8 weeks and showed EPSC and RRP recovery features that were similar to those of mouse cortical neurons. The latter finding indicates that activity-dependent synaptic vesicle recycling operates similarly in synapses of human and rodent neurons.

iGlutNs and iGABANs have the clear advantage of faster maturation, and our gene editing approach enables highly controlled neuronal induction via standardized inducible transcription factor expression from the AAVS1 safe-harbor locus. Key synaptic parameters in iGlutNs and mouse cortical neurons, as well as to iGABANs and mouse striatal GABAergic neurons (Nair et al., 2013). Furthermore, GlutNs were mostly of the tonic AP firing type at 8 weeks and showed EPSC and RRP recovery features that were similar to those of mouse cortical neurons. The latter finding indicates that activity-dependent synaptic vesicle recycling operates similarly in synapses of human and rodent neurons.

All human neuron types we tested can be used for standardized and systematic functional studies in autaptic cultures, e.g., in the context of patient-derived cells or human cells with targeted mutations. Overall, the tested cell types were pure with regard to transmitter type, and surprisingly large fractions of cells showed a tonic AP firing type, indicating that the autapse culture system may promote neuronal maturation or even select for mature cells. As far as direct comparisons of parameters are possible (e.g., with normalized readouts of STP and PSC/RRP recovery), the different related cell types (i.e., GlutNs versus iGlutNs, GABANs versus iGABANs, Glut/iGlutNs versus mouse cortical neurons, and GABA and iGABANs versus mouse striatal neurons) showed similar features, which was also true for a comparison of two iGlutN lines (C14m versus C35m). These findings indicate that the autaptic culture system may eliminate some sources of heterogeneity among iPSC-derived neurons and may thus be useful for future analyses of disease-relevant neurons.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2019.04.059.

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AUTHOR CONTRIBUTIONS

J.S.R., O.B., and N.B. conceived the study. K.R., T.K., and M.H. generated and analyzed human iPSC-derived neurons; H.J.R. and C.L. performed and analyzed electrophysiology experiments; A.H.S. performed and analyzed morphological analyses of iPSC-derived neurons; P.S. wrote analysis software and performed data analysis; C.T. wrote analysis software; E.G. developed and optimized analysis protocols; A.G. prepared autaptic cultures; M.P.; and O.B. supervised human iPSC experiments; J.S.R. supervised autaptic culture experiments; J.S.R., N.B., A.H.S., E.G., K.R., M.P., and O.B. wrote the paper. All authors contributed to the final editing of the paper.

DECLARATION OF INTERESTS

O.B. is cofounder of and has stock in LIFE & BRAIN GmbH.

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REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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#### Experimental Models: Organisms/Strains

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(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for resources, reagents, and further information should be directed to the Lead Contact, JeongSeop Rhee (rhee@em.mpg.de). Questions relating to cell generation can be directly addressed to Michael Peitz (peitz@uni-bonn.de). Protocols, information, and unique tools and reagents regarding autaptic cultures and their electrophysiological and morphological analysis can be obtained from JeongSeop Rhee (rhee@em.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

iPSCs - source and generation
The use of all iPSC lines was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (approval 275/08), and informed written consent was obtained from the donors. All experiments were performed in accordance with German guidelines and regulations. The following numbers of cell lines and in vitro differentiations were performed: GlutNs, 5 sets of in vitro differentiations; GABANs, 2 lines, 3 sets of in vitro differentiations per line; iGlutNs, 2 lines, 5 and 3 sets of in vitro differentiations, respectively; iGABANs, 2 sets of in vitro differentiations.

Mouse maintenance
Animal experiments were done in compliance with applicable animal care guidelines and performed as approved (Lower Saxony State Office for Consumer Protection and Food Safety; permits 33.9-42502-04-13/1359 and 33.19-42502-04-15/1921). Animals were kept in groups according to European Union Directive 63/2010/EU and ETS 123 (individually ventilated cages, specific pathogen-free conditions, 21 ± 1°C, 55% relative humidity, 12 h/12 h light/dark cycle). Mice received food and tap water ad libitum and were provided with bedding and nesting material. Cages were changed once a week. Animal health was controlled daily by caretakers and a veterinarian. Health monitoring (serological analyses; microbiological, parasitological, and pathological examinations) was done quarterly according to FELASA recommendations with either NMRI sentinel mice or animals from the colony. The mouse colony used for experiments did not show signs of pathogens. The sex of animals used for experimentation was not checked because all previous studies had indicated that the sex does not affect the parameters studied here.

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**METHOD DETAILS**

**Differentiation of iPSCs into glutamatergic cortical neurons**

iPSCs were cultured in mTeSR (StemCell Technologies) or StemMACS iPSC-Brew (Miltenyi Biotec) and split with EDTA during maintenance (Beers et al., 2012). Undifferentiated iPSCs were seeded as single cells at high density (1x10^6 cells per cm^2) in iPSC medium with 10 μM ROCK inhibitor (RI) Y-27632 (Tocris). The next day, the medium was switched to GLUT neural induction medium (3N medium with 1 μM Dorosomorphin/200 nM LDN-193189, 10 μM SB431542). 3N medium consists of 49% DMEM/F12, 49% Neurobasal medium, 1% B27 supplement, 0.5% NEAA, 0.5 mM GlutaMax, 50 μM β-mercaptoethanol, 10 nM progesterone, 50 μM putrescine, 30 nM sodium selenite, 50 ng/ml apo-transferrin, 12.5 μg/ml insulin, 0.8 mg/ml glucose (ThermoFisher Scientific or Sigma). On day 11, the medium was switched to 3N with 20 ng/ml FGF2 to promote neural rosette formation. On day 12, cultures were split using accutase (ThermoFisher Scientific). Obtained cells were propagated in 3N medium with 20 ng/ml FGF2 and 10 μM RI at a split ratio of 1:3 in Matrigel (MG)-coated (Corning) 6-well plates. Starting on day 14, FGF2 was reduced to 10 ng/ml and 100 ng/ml heparin was added. On day 18 and day 22, cultures were dissociated as clumps and seeded at a split ratio of 1:2 on fresh MG-coated plates for further propagation. On day 32, cortical progenitors were dissociated with accutase in the presence of 10 μM RI for 20 minutes at 37°C and frozen down as one batch in ice-cold freezing medium (90% KOSR, 10% DMSO). For further maturation after thawing, cryopreserved progenitors were seeded in 3N medium supplemented with 10 μM RI on MG-coated plates (0.5x10^6 cells per cm^2). On day 44, cultures contained a large fraction of neurons and were dissociated and seeded for final maturation. On the next day, the medium was changed to 3N medium with 10 ng/ml BDNF, 10 μM PD0325901, and 10 μM DAPT to promote differentiation of remaining precursors. The same medium was refreshed on day 47. On day 49, the cultures were mitotically inactivated by 5 μM AraC (cytosine β-D-arabinofuranoside hydrochloride), to prevent further proliferation, and treated with 10 ng/ml BDNF. On day 50, AraC-containing medium was removed carefully and replaced by 3N with 10 ng/ml BDNF. On day 51, neurons were frozen as a large batch as described below.

**Differentiation of iPSCs into GABAergic forebrain neurons**

To generate GABAergic forebrain differentiation, iPSCs were cultured in E8 medium and split with EDTA during maintenance (Beers et al., 2012). Undifferentiated iPSCs were seeded as single cells at a density of 0.2x10^6 per cm^2 in E8 medium supplemented with 10 μM RI. The next day, the medium was switched to GABA neural induction medium (N2/B27, 500 nM LDN-193189, 15 μM SB431542). N2/B27 consists of 49% DMEM/F12, 0.5% N2 supplement, 49% neurobasal medium, 1% B27 supplement, 0.5% NEAA, 0.5 mM L-glutamine, 50 μM β-mercaptoethanol. On day 9, the neural induction medium was supplemented with 20 ng/ml FGF2 to promote neural rosette formation. On day 10, the cultures were split using accutase. Obtained cell clumps were seeded in N2/B27 medium with 20 ng/ml FGF2 and 10 μM RI at a split ratio of 1:3 onto MG-coated 6-well plates. The cultures were cultivated in N2/B27 medium supplemented with 20ng/ml FGF2 until day 12. Cells were further propagated in N2/B27 medium which was changed every other day. On day 20, progenitors were dissociated and frozen as one batch in ice-cold freezing medium. For maturation of cryopreserved progenitors, cells were seeded in N2/B27 medium with 10 μM RI (0.5x10^6 cells per cm^2). On day 25, cultures were mitotically inactivated with 5 μM AraC to prevent further proliferation and carefully removed after 24 h. On day 27, neurons were frozen as a large batch.

**Plasmid cloning for forward programming**

AAVS1-GFP plasmid and the corresponding TALEN pair were provided by Dr. Su-Chun Zhang (University of Wisconsin) (Qian et al., 2014). To establish the AAVS1-ASCL1-P2A-DLX2 cassette, which was designed in silico based on codon-optimized human coding sequences and synthesized (GeneArt). After cutting from the cloning plasmid, the cassette was inserted into the AAVS1-backbone via conventional ligation.

**iPSC nucleofection for AAVS1 targeting**

AAVS1-targeted iPSC lines were generated by nucleofecting 3-4 million cells with the corresponding targeting plasmid (4 μg) together with the TALEN pair (0.5 μg each) using the Amaxa nucleofection kit V (Lonza) with program B-023 after a preincubation step with 10 μM of RI for 1 h. Nucleofected iPSCs were seeded on Geltrex-coated dishes (ThermoFisher Scientific) at low density in StemMACS IPS Brew (Miltenyi Biotec) with 10 μM RI as well as 5 μM L755507. After 24 h, medium was replaced with StemMACS IPS Brew including 10 μM RI, and renewed every other day. RI was supplemented until the formation of small puromycin-resistant colonies and then discontinued. Puromycin selection (0.3 μg/ml) was started at 48 h post-nucleofection and maintained for at least 7 days. Resistant colonies were picked, expanded, and analyzed via genotyping PCR. Site-specific transgene insertion was validated by PCR using the following primer combination: fwd, ACCAACGCACGCTCCTCACG; rv1, CAGACCTTGCCCTGGTGT; rv2, CACCGGTACGTGAACGC.

**Forward programming of iPSCs into GABAergic forebrain neurons**

Before the start of differentiation, iPSCs were maintained in StemMACS IPS-Brew (Miltenyi Biotec) and split with EDTA 2-3 times. To start differentiation, iPSCs were seeded as single cell suspension in Geltrex-coated six-well plates at a density of 40,000 cells/cm^2.
and maintained in iPSC medium. Two days later, the medium was switched to N2 neural induction medium containing 2 μg/ml doxycycline. On day 2 after induction, cells were dissociated with accutase and plated in laminin-coated T175 flasks at a density of 100,000 cells/cm² in NB/B27 medium containing doxycycline. On day 3 after induction, 10 μM DAPT was added and kept until day 6. The cells were treated with 5 μM AraC on day 7 and cryopreserved as a large batch on day 9 as described below. Details of the generation of iGlutNs by expression of NGN2 are provided in the accompanying paper (Meijer et al., 2019).

Cryopreservation of post-mitotic neurons
Two days after AraC treatment, neurons were cryopreserved as a single batch. Neuronal cultures were dissociated slowly by incubating accutase supplemented with 10 μM RI for 60-90 min at 37°C. Cells were washed off with warm medium, dissociated further by pipetting, and passed through a cell strainer. The cell solution was divided into several Falcon tubes, and centrifuged at 300 x g for 5 min. Subsequently, the cells were resuspended in ice-cold freezing medium (70% KOSR, 20% 1 M trehalose, 10% DMSO) and deep frozen with a rate of approximately –1°C/min. Cells remaining in the cell strainer were collected for DNA analysis.

Immunostaining of immature neuronal batches
For QC, neurons were thawed, counted, and seeded on 96-well plates (ibidi, μ-plates) in NB/B27 medium supplemented with 10 μM RI. After 7 days, neurons were fixed with freshly thawed pre-warmed 4% PFA for 10-15 min. For the fixation of GABA, the PFA solution was supplemented with 0.04% glutaraldehyde. Cultures were blocked with 10% FBS in PBS + 0.1% Triton X-100 for one hour. The primary antibodies were diluted in 10% FBS in PBS + 0.1% Triton X-100 and incubated over night at 4°C. Afterward, adequate secondary antibodies were diluted in 10% FBS in PBS + 0.1% Triton X-100 and incubated for one hour. The following antibodies were used: BRN2 (ms IgG, Santa Cruz, sc-393324, 1:500; rb IgG, Abcam 94977, 1:500), CTIP2 (rat IgG, Abcam, ab18465, 1:500), GABA (ms IgG, Sigma, A0310, 1:1000; rb IgG, Sigma, A2052), TBR1 (rb IgG, Proteintech, 20932-1-AP, 1:1000), TUJ1/p3-tubulin (ms IgG, Covance, MMS-435P, 1:1000; rb IgG, Covance, PRB-435P, 1:2000; ck IgY, Milipore, AB9354), VGlut1 (rb IgG, Synaptic Systems, 135303, 1:1000), VGlut2 (rb IgG, Abcam ab84103), FOXG1 (rb IgG, Abcam, ab18259). Images were acquired using the INCell analyzer 2000 (GE Healthcare). Cellular markers were quantified automatically using the InCell Developer toolbox.

Preparation of astrocyte micro-island cultures
Astrocyte micro-island plates were prepared and cultured as previously reported (Burgalossi et al., 2012). Briefly, a human astrocyte cell line (ScienCell) was cultured and maintained until use in complete astrocyte medium (ScienCell) with 0.1% penicillin/streptomycin or DMEM (with GlutaMax, GIBCO), 1% N2 supplement, 10% FBS (GIBCO) and 20 ng/ml EGF recombinant human protein (ThermoFisher Scientific). Mouse and rat astrocytes for autaptic cultures were obtained from mouse and rat cortices dissected from P0 wild-type animals and enzymatically digested for 15 min at 37°C with 0.05% (w/v) trypsin-EDTA. Astrocytes were plated in T75 culture flasks in DMEM (containing 10% FBS, 20 U/ml penicillin, 20 μg/ml streptomycin, Mito Serum Extender (Corning)) and grown for 7-10 days in vitro (DIV). The different astrocyte species were trypsinized and plated at a density of 30,000 cells/well onto 32 mm²-diameter glass coverslips that had previously been coated with agarose (Sigma) and stamped using a custom-made stamp to generate 400 μm x 400 μm substrate islands with a coating solution containing poly-D-lysine (Sigma), acetic acid, and collagen (BD Biosciences).

Mouse forebrain cortical neuron culture
P0 mouse forebrains were isolated and digested for 80 min at 37°C in DMEM containing 25 U/ml papain (Worthington Biomedical Corporation), 0.2 mg/ml cysteine (Sigma), 1 mM CaCl₂, and 0.5 mM EDTA. After washing, the dissociated cortical neurons were seeded onto the micro-island plates in pre-warmed Neurobasal medium supplemented with B27, Glutamax, and penicillin (100 U/ml)/streptomycin (100 μg/ml) at a density of 4,000 cells/well of 6-well plates. The neurons were allowed to mature for 10-12 days before electrophysiological assays.

Recovery and culture of cryopreserved human iPSC-derived neurons
One ml of cryopreserved neurons were quickly thawed in a 37°C water bath and diluted into 9 ml of pre-warmed N3 medium. After passing through a 40 μm cell strainer (BD Falcon) into 50 ml tubes, viable cells were counted using Trypan Blue staining. Different numbers of cells (GlutNs, 3,000; iGlutNs, 80,000; GABANs, 3,000, iGABANs, 6,000 cells/well of 6-well plates) were plated onto the astrocyte micro-island plates with 2 ml of N3 medium supplemented with 5 μM RI (Tocris). Cells were placed into the 37°C incubator for 24 h. On the next day, the whole medium was changed with 2 ml of different conditioned fresh N3 medium without ROCK inhibitor, depending on the culture. Medium was changed continuously once per week until cells were used. N3 medium consists of 49% DMEM/F12, 49% Neurobasal medium, 1% B27 supplement, 1% N2 supplement, 50 μM β-mercaptoethanol, 1 mM L-Glutamine, 0.5% NEAA with or without 0.5% FBS or 2 μg/ml Doxycycline (Sigma). BrainPhys Neuronal medium (STEMCELL) consists of NeuroCult SM1, Neuronal Supplement (STEMCELL technologies), N2 Supplement-A (STEMCELL technologies), recombinant human BDNF (Peprotech), recombinant human GDNF (Peprotech), dibutyly cAMP (Sigma), and L-Ascorbic Acid (Sigma). All materials for cell cultures were purchased from Life Technology unless mentioned otherwise.
**Immunofluorescence staining and imaging**

Human neurons were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature and then washed with PBS, quenched with 50 mM Glycine for 10 min and washed with PBS. Samples were incubated with IT-FX image enhancer (Life Technologies) in PBS for 20 min. The permeabilization steps were performed either with 0.1, 0.2, or 0.3% Triton X-100 and 2.5% normal goat serum (NGS) in PBS for 30 or 45 min at room temperature. The neurons were then incubated with the primary antibodies for 1 h. After washing with 0.1% Triton X-100/2.5% NGS/PBS, they were incubated with the secondary antibodies for 45 min, followed by extensive washing steps with 0.1% Triton X-100/2.5% NGS/PBS and PBS. The samples were then mounted with Aqua-Poly Mount mounting medium. Confocal acquisition of images was done with Leica SP2 and SP5 microscopes, with 40X, 60X, or 100X objectives with numerical apertures of 1.25, 1.4, and 1.45, respectively. Ar 65 mW 488 nm, HeNe 1 mW 633 nm, and DPSS 20 mW 561 nm lasers were used. White light laser (WLL, 1.5 mW) with a range from 470 to 670 nm with 70% power was used when fluorescence intensity needed to be quantified. The generated images were analyzed by Fiji and ImageJ (NIH). Synapse counting was done by in-house written IJ1 macros that isolate presynaptic structures, apply thresholds, convert to a mask, and then create a selection that is multiplied by 1 pixel size. The same steps were repeated for postsynaptic structures without the selection enlargement step, and colocalizing punctae were counted as synapses. Sholl analysis was made as described (Ripamonti et al., 2017; Sholl, 1953). For quantifying fluorescence intensities, the data were acquired for all the different conditions of a given set of experiments at the same day. Background was subtracted and intensities at the cell bodies were quantified by drawing a selection and measuring the mean gray value. For quantification of intensities at the neurites, segmented line scans of thickness three were used. Graphs and analysis were mainly generated with GraphPad Prism versions 5 and 7 and in some cases with SigmaPlot 13.

**Electrophysiology**

For whole-cell voltage-clamp recordings, cells were held at −70 mV, and holding voltage was increased to 0 mV (2 ms) to depolarize cells (MultiClamp 700B amplifier, Axon Instruments, Molecular Devices) under the control of the Clampex program 10.1 (Molecular Devices). The flow of extracellular solution was maintained during recordings and pharmacological solutions were applied if required. Hypertonic sucrose solution (0.5 M in external solution) was used to trigger fusion of the readily releasable pool of vesicles (RRP). NBOX (HelloBio) and Bicuculline (Tocris) were used to inhibit AMPA and GABA receptors, respectively. Miniature PSCs were recorded in the presence of 300 nM TTX (Tocris). The extracellular solution contained 140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM Glucose, 4 mM CaCl2, 4 mM MgCl2 (320 mOsmol/liter), and the patch-pipette solution for recording contained 136 mM KCl, 17.8 mM HEPES, 1 mM EGTA, 4.6 mM MgCl2, 4 mM NaATP, 0.3 mM Na2GTP, 15 mM creatine phosphate, and 5 U/ml phosphocreatine kinase (315-320 mOsmol/liter), pH 7.4. Cells were visualized by an inverted microscope (Olympus or Zeiss). Custom-made manipulators controlled the movement of the microelectrode. All drugs were applied with a custom-built fast flow system consisting of an array of flow pipes controlled by a stepper motor that allows complete and rapid solution exchange with time constants of ~30 ms. Pressure was on for 20 ms. Measurements were performed and registered using an Axon 700B Amplifier (Axon Instruments) and signals were converted with an Axon Instrument digitizer. Recording rate was 10 kHz. Microelectrodes were made by using a Sutter 2000 filament-based horizontal puller and used only if they had pipette resistances of 2.5-4.5 MΩ. Serial resistance was compensated by 35%-60% and cells with serial resistances below 12 MΩ were analyzed. All chemicals were purchased from Sigma, unless mentioned otherwise. In the current clamp mode, whole-cell recordings were performed with a HEKA EPC-9USB amplifier and Patch-Master (Ver. 2X90.3) software (HEKA electronics). Measurements of Rinput were calculated from membrane potential changes in response to subsequent 1 s current injections from −100 to 0 pA at 20 pA intervals. APs were generated by progressive incremental depolarizing current injection of +5 pA starting at 0 pA. All active electrical properties were analyzed in the first trace in which APs were evoked. The external solution for measurement of potassium currents contained 150 mM choline-Cl, 1.5 mM MgCl2, 6 mM H2O, 0.25 mM EGTA, 4 mM ATP-Mg2+, 0.3 mM GTP–Na+ (solution adjusted to pH 7.4, ~320 mOsmol/liter). Voltage-gated sodium and potassium currents (INa,K) in neurons were evoked by 500 ms test pulses from a holding potential of −70 mV to set potentials between −80 mV and +70 mV (10 mV intervals). IK was recorded with same protocol in the presence of 300 mM TTX. INa was obtained by subtracting the traces of IK from the INa,K traces. External and internal solutions were the same as for the current clamp recordings described above. Total outward K+ currents (IK) were evoked by 500 ms depolarizing test pulses from a holding potential of −70 mV to set potentials between −80 mV and +70 mV (10 mV intervals), and IDR was measured with the same protocol in the presence of 1 mM 4AP. IK traces were obtained by subtracting the trace of IDR from IK. The external solution for the measurement of potassium currents contained 150 mM choline-Cl, 5 mM KCl, 1.5 mM CaCl2, 10 mM HEPES, 1 mM MgCl2, 0.2 mM CdCl2, 10 mM Glucose (solution adjusted to pH 7.3, ~310 mOsmol/liter). The internal solution contained 138 mM K-glucionate, 16.8 mM HEPES, 10 mM NaCl, 1 mM MgCl2, 6H2O, 0.25 mM EGTA, 4 mM ATP-Mg2+, 0.3 mM GTP-Na+ (solution adjusted to pH 7.4, ~320 mOsmol/liter). The densities of all voltage gated ion currents were normalized to cell membrane capacitance (pF). All electrophysiology data were analyzed using Axograph (Ver.1.5.4) software (Axograph Scientific).
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis of data was performed with GraphPad Prism (Versions 5 or 7) software (GraphPad Software Inc.). Statistical comparisons between two groups of data were made using two-tailed unpaired Student’s t test and Mann Whitney test. Multiple comparisons were determined using one-way analysis of variance (ANOVA) followed by post hoc testing (Tukey’s, Dunn’s and Kruskal-Wallis multiple comparison tests). p-values less than 0.05 were considered significant for single and multiple comparisons, respectively. Data are presented as mean ± SEM.