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Human iPSC-derived neurons with reliable synapses and large presynaptic action potentials

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Abstract

Understanding the function of the human brain requires determining basic properties of synaptic transmission in human neurons. One of the most fundamental parameters controlling neurotransmitter release is the presynaptic action potential, but its amplitude and duration remain controversial. Presynaptic action potentials have so far been measured with high temporal resolution only in a limited number of vertebrate but not in human neurons. To uncover properties of human presynaptic action potentials, we exploited recently developed tools to generate human glutamatergic neurons by transient expression of Neurogenin 2 (Ngn2) in pluripotent stem cells. During maturation for 3 to 9 weeks of culturing in different established media, the proportion of cells with multiple axon initial segments decreased, while the amount of axonal tau protein and neuronal excitability increased. Super-resolution microscopy revealed the alignment of the pre- and postsynaptic proteins, Bassoon and Homer. Synaptic transmission was surprisingly reliable at frequencies of 20, 50, and 100 Hz. The synchronicity of synaptic transmission during high-frequency transmission increased during 9 weeks of neuronal maturation. To analyze the mechanisms of synchronous high-frequency glutamate release, we developed direct presynaptic patch-clamp recordings from human neurons. The presynaptic action potentials had large overshoots to ~25 mV and short durations of ~0.5 ms. Our findings show that Ngn2-induced neurons represent an elegant model system allowing for functional, structural, and molecular analyses of glutamatergic synaptic transmission with high spatio-temporal resolution in human neurons. Furthermore, our data predict that glutamatergic transmission is mediated by large and rapid presynaptic action potentials in the human brain.

Significance statement

Presynaptic physiology remains poorly understood despite its relevance to neurological and psychiatric diseases. Studying presynaptic functions in human iPSC-derived neurons offers the important advantage of characterizing molecular mechanisms of neurotransmitter release in neurons derived from diseased patients. As a first step towards this goal, we established direct presynaptic whole-cell patch-clamp recordings from human glutamatergic neurons induced by transient Neurogenin 2 overexpression. We furthermore analyzed the structure of the synapses with super-resolution light microscopy and the synaptic short-term plasticity during high-frequency transmission. Our findings show that synchronous high-frequency transmission is mediated by rapid and large presynaptic action potentials in human neurons, similar to small conventional nerve terminals of rodent neurons.

Introduction

Neuronal communication relies on precisely-timed neurotransmitter release at presynaptic nerve terminals (Südhof, 2012). Despite intense efforts, several fundamental questions regarding presynaptic function and plasticity remain unresolved (Neher and Sakaba, 2008; Debanne et al., 2011; Südhof, 2013; Kaeser and Regehr, 2014). Further research is needed because presynaptic function and plasticity play important roles in learning, memory, and development of neurological and psychiatric diseases (Hawkins et al., 1993; Monday et al., 2018; Verhage and Sørensen, 2020). The presynaptic mechanisms are particularly poorly understood in human neurons.

Analysis of presynaptic function in human neurons is warranted as recent studies have suggested fundamental differences in the biophysical properties, the function, and the morphology of human neurons compared to the widely-studied neurons of mice and rats (Eyal et al., 2016; Beaulieu-Laroche et al., 2018; Fişek and Häusser, 2020; Gidon et al., 2020). Furthermore, animal models often fail to recapitulate human brain disease phenotypes or to predict treatment outcomes (Miller, 2010). Recently available induced pluripotent stem cells (iPSC)-derived neurons therefore hold promise for a better understanding of presynaptic function in human neurons in health and disease. In addition, iPSC-derived human neurons have the potential to replace some of the animal experiments in biomedical research representing an efficient 3R-strategy (Russell and Burch, 1959).

To study synaptic mechanisms in human neurons, several approaches have been used, including acute slices from surgically resected brain tissue as well as organoid and cell cultures derived from iPSCs or fibroblasts. Each of these model systems has complementary advantages and disadvantages. Ex vivo brain tissue best represents the morphological and functional organization of the adult human brain (Molnár et al., 2016; Hodge et al., 2019; Peng et al., 2019). However, brain tissue from patients is limited in its availability, the physiology might be compromised by the pathology, and genetic manipulations are complicated (Ting et al., 2018). A more accessible source are human fibroblasts and iPSCs that can be converted into neurons. The most common model system are cultured neurons derived from fibroblasts or iPSCs by pharmacological treatments or transient expression of pro-neural transcription factors.

An important milestone has been the finding that transient expression of the transcription factor Ngn2 suffices to rapidly induce the development of glutamatergic neurons from human iPSCs

(Zhang et al., 2013). Ngn2 overexpression has been proven as a valuable tool to produce glutamatergic human neurons (Hulme et al., 2022). By now, there are several human iPSC lines with endogenous inducible Ngn2 available, including some cell lines with mutations linked to neurodegenerative diseases (Ghatak et al., 2019; Karch et al., 2019; Nakamura et al., 2019; Sohn et al., 2019; Tracy et al., 2022; Zhou et al., 2022). Human neurons produced by Ngn2 expression exhibit markers of pyramidal neurons of the neocortex (Zhang et al., 2013) but several types of neurons might be present depending on the exact culture condition and the Ngn2 dosage (Lin et al., 2021). Recently, it was shown that human neurons induced by both pharmacological treatment and overexpressing Ngn2 can be cultured in islands to form 'autapses' with reproducible neuronal and synaptic properties (Fenske et al., 2019; Meijer et al., 2019; Rhee et al., 2019).

Here, we generated cultured human neurons derived from iPSCs by transient forced expression of Ngn2 (Zhang et al., 2013; Patzke et al., 2019). In general, cultured neurons offer ideal accessibility for high-resolution presynaptic electrophysiology (Kawaguchi and Sakaba, 2017; Ritzau-Jost et al., 2023) and for super-resolution imaging (Werner et al., 2021). Therefore, we compared various culturing media and analyzed the structural, molecular, and functional maturation of iPSCs-derived human neurons. Furthermore, we analyzed glutamatergic synaptic transmission. We found high-fidelity synaptic transmission with synchronous release up to 100 Hz mediated by large and rapid presynaptic action potentials.

Material and methods

Laboratory animals

Cortical astrocytes were prepared using C57BL/6N mice of either sex at postnatal day 0-3. Mixed cultures of rat and mouse cortical neurons and astrocytes were prepared using rat of either sex at postnatal day 1. Adult animals were kept in individually ventilated cages and received water and food ad libitum. All animal procedures were in accordance with the European (EU Directive 2010/63/EU, Annex IV for animal experiments), national and Leipzig University guidelines. All animal procedures were approved in advance by the institutional Leipzig University Ethics Committees and the federal Saxonian Animal Welfare Committee (mouse: T01/21, rat: T29/21). In order to reduce the number of animals we tried to use remaining cells from other neuron culture preparations in our laboratory whenever possible.

Human induced neuron (hiN) culture

Human induced pluripotent stem cells (hiPSCs) were differentiated into Glutamatergic neurons of cortical subtype by the transient overexpression of the transcription factor Neurogenin 2 (Ngn2) (Zhang et al., 2013). The hiPSC line BIHi005-A-24 (hPSCreg link: https://hpscreg.eu/cell-line/BIHi005-A-24) stably overexpressing the TetO-NGN2-T2A-PURO cassette was obtained from Berlin Institute of Health (BIH), Germany. This cell line constitutively expresses a Tet-ON activator which in turn allows induction of Ngn2 expression and the puromycin resistance gene by addition of Doxycycline to the culture medium (Rizalar et al., 2023). For capacitance measurements we used the iPSC line NWTC11.G3.0036 (Karch et al., 2019) derived from WTC11 (hPSCreg link: https://hpscreg.eu/cell-line/UCSFi001-A) , which also constitutively

expresses a Tet-ON activator and was differentiated into Glutamatergic neurons using the same protocol. Since the morphological apparency of the neurons and the functional essays did not reveal any differences between the two cell lines, we expect similar fundamental biophysical properties of presynaptic boutons in these cells. In both cell lines HIV, HBV, HCV and mycoplasma were not detected by PCR.

Prior differentiation hiPSCs were maintained in feeder-free culture. They were grown on plastic dishes coated with MatriGel (Corning). Medium was replaced every second day by fresh StemFlex medium (StemCell technologies) without antibiotics. Upon reaching 50~70% confluence cells were harvested with StemPro Accutase (ThermoFisher) and split. With each passage StemFlex medium was supplemented with 5 µM ROCK inhibitor Y-27632 (Stem Cell Technologies) to inhibit apoptosis. Frozen stocks were prepared in Bambanker HRM cryopreservation media (StemCell technologies) and stored over liquid nitrogen.

For differentiation into neurons, hiPSCs cultures where grown until reaching 50~70% confluence cells before being harvested with StemPro Accutase and plated onto Matrigel-coated 6 well plates at 200.000 cells/well in 2 ml/well StemFlex medium supplemented with 5 µM ROCK inhibitor Y-27632 to inhibit apoptosis. The following three days (day 0-2) the medium was replaced daily with 2ml/well fresh differentiation medium (DMEM/F12, 1:100 N2 supplement, 1:100 non-essential amino acids, 1:100 penicillin/streptomycin, all ThermoFisher; 10 ng/ml BDNF, 10 ng/ml NT3, both from StemCell technologies; 200 ng/ml Laminin, 1 µg/ml doxycycline, both from ThermoFisher). Puromycin selection was started by adding 10mg/ml puromycin (ThermoFisher) on day 2. hiPSCs rapidly changed morphology towards a bipolar shape with elongated neurites.

Because mouse astrocytes efficiently stimulate synaptogenesis in human induced neurons (Zhang et al., 2013), cultures from P1~3 mice were prepared two to three weeks earlier with Trypsin digestion followed by mechanical dissociation and maintained in astrocyte medium (DMEM with 4.5 g/l glucose, 1 mM pyruvate, 10% heat inactivated fetal calf serum, 1:100 penicillin/streptomycin). They were replated two times to remove any mouse neurons.

On day 3, neuron cultures were washed with Hanks' Balanced Salt Solution (HBSS) to remove Puromycin and harvested with Accutase. In some cases frozen stocks were prepared (Ishizuka and Bramham, 2020) and stored at -80 °C before use. Astrocyte cultures were harvested with Trypsin/EDTA. Co-cultures were then plated onto Poly-D-Lysine and Matrigel-coated coverslips in 24 well plates. For each coverslip 60000 neurons and 12000 astrocytes were allowed to settle in a 25 µl drop of neuron culture medium for about 30 min and then each well was filled with 500 µl culture medium (see Table 1) supplemented with 2% heat-inactivated fetal calf serum (ThermoFisher). Half of the medium was replaced with culture medium on day 6, 10, 14 and then twice a week. Doxycycline was added to the medium until day 10. Cytosine arabinoside (ara-C) was added at day 12 for 48 h to inhibit overgrowth of astrocytes. Due to osmolarity of the culture media (see Table 2) and possible evaporation, changing half of the media lead to measurable differences in osmolarity. To compensate for these differences, we adjusted the osmolarity to 300 mOsm using *Aqua dest.* for DMEM, BrainPhys and Neurobasal using the following approach. Half of the supernatant (250 µl/well) was collected from the cell cultures. The pooled supernatant was then mixed with equal volume of the fresh medium. The volume (V_{mix}) and the osmolarity (O_{mix}) of the mixture were measured. The osmolarity was then adjusted by adding a volume of *Aqua dest.* (V_{add}) according to the following formula: $V_{add} = (O_{mix} / 300\ mOsm - 1) \times V_{mix}$. The remaining supernatant was removed from the cell cultures and replaced by the mixture (500 µl/well). For Neurobasal Plus medium this approach was not possible because the measured osmolarity was

always below 300 mOsm (cf. Table 2). We therefore simply exchanged half of the Neurobasal Plus medium with fresh medium. Cultures were maintained for up to 10 weeks at 37°C and 5% CO_2. Concentration of electrolytes as well as glucose and lactate in all culture media as well as in ACSF were measured using an blood gas and electrolyte analyzer (ABL 90 flex, Radiometer, Willich, Germany; see Table 2).

Rat and mouse primary cortical cultures

Primary cultures from P1 rat and mouse cortex were prepared similar as previously described (Moutin et al., 2020). Animals from both sexes were used. After removal of meninges the neocortex was dissected and enzymatically digested with Papain (Sigma) in the presence of DNAse (Sigma), followed by mechanical dissociation and centrifugation through a cushion of 4% bovine serum albumin (Sigma). These steps were completed using Hibernate medium (Gibco). Cells were then plated onto Poly-D-Lysine (Sigma) coated coverslips in 24 well plates. For each coverslip 25,000 cells were allowed to settle in a 40 µl drop for about 30 min and then each well was filled with 500 µl growth medium: NeurobasalA/B27 (1:50, Invitrogen) supplemented with GlutaMax (1:400, Invitrogen), Glutamine (0.25-0.5 mM, Sigma), Penicillin/Streptomycin (1:100, ThermoFisher) and heat-inactivated fetal calf serum (10%, Sigma). Medium was partially exchanged on day 3 (480 µl) and day 7 (100 µl) with fresh maintenance medium: BrainPhys (StemCell), B27 (1:50, Invitrogen), GlutaMax (1:400, Invitrogen), Penicillin/Streptomycin (1:100, ThermoFisher). Cultures were maintained for up to 3 weeks without any further medium change at 37°C and 5% CO2.

Epifluorescence imaging

Immunocytochemistry was performed as previously described (Bullmann et al., 2009). Human neurons co-cultured with mouse astrocytes were grown on coverslips and fixed for 30min with 4% paraformaldehyde in PBS, washed twice with PBS+0.05% sodium azide and stored at 4°C. Fixed cultures were washed with Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.6) containing 0.05% Triton X-100 (TBS-Tx), unspecific binding was blocked for 30min with 2% normal donkey serum in TBS-Tx (TBS-Tx-NDS) and primary antibodies were applied in TBS-Tx-NDS overnight at 4°C. Next day, cultures were washed three times with TBS-Tx, and secondary antibodies were applied in TBS-Tx-NDS for 1~2 h at RT. Afterwards, cultures were washed three times TBS-Tx, one time with PBS+0.05% sodium azide and the coverslips were mounted upside down onto microscope slides using Mowiol (ThermoFisher). For axon initial segment counting (see Fig. 1B) primary antibodies were directed against MAP2 and ANK3 (see Table 3), imaging was performed using ZEISS epifluorescence microscope equipped with 10x air objective. For quantification of tau distribution (see Fig. 2C) primary antibodies were directed against tau, MAP2 and beta III Tubulin (see Table 3), imaging was performed using ZEISS epifluorescence microscope equipped with 40x water immersion objective. Using the MAP2 staining of soma and dendrites as a guidance, images from individual, “non-overlapping” neurons were taken and analyzed. Note that the image contains not only the axon of this individual neuron but most like the axons of other neurons as well.

STED imaging

Immunocytochemistry was performed as described for epifluorescence imaging. Briefly, human neurons grown in Neurobasal media for 3, 6 and 9 weeks were fixed and incubated with primary antibodies directed against Bassoon and Homer1 (see Table 3) which were then detected using StarRed and StarOrange conjugated secondary antibodies, respectively. Imaging (see Fig. 5A)

was performed using an Expert Line Abberior Confocal and STED microscope equipped with an Olympus (UPlanXApo) 60x oil objective with a numerical aperture of 1.42. The images were obtained with a 10nm pixel size (2D mode) and sequential line scanning by switching between the needed excitation lasers line-wise during each recording. Synapse labelled both for Bassoon and Homer1 were identified and for each channel a line profile perpendicular to the synaptic cleft was fitted with a Gaussian, and their peak-to-peak distance was measured (Fig. 5B). For comparison this assay was also performed on DIV14 rat cortical neurons.

Immunoblotting

Immunoblotting was performed as previously described (Bullmann et al., 2009). Briefly, human neurons were maintained in 6 well plates at an initial density of 240000 neurons/well. After 3, 6 and 9 weeks cultures were washed using HBSS and cells were extracted directly in Laemmli buffer. Samples were applied to 10% polyacrylamide gel electrophoresis (PAGE) and transferred to PDVS membranes using a tank blotter at 25V overnight. PDVS membranes were washed with TBS and 0.05% Tween-20 (TBS-Tw), unspecific binding was blocked for 30min with 2% bovine serum albumin (BSA) in TBS-Tw (TBS-Tw-BSA), and primary antibodies were applied in TBS-Tw-BSA overnight at 4°C. Next day, membranes were washed 3x TBS-Tw, and secondary antibodies were applied in TBS-Tw for 1 hour at RT. Afterwards, membranes were washed 3x TBS-Tx, equilibrated in bioluminescence substrate and imaged on Fusion FX (Vilber).

Electron microscopy

Neurons grown for 40 days in Neurobasal medium on coverslips, as done for light microscopy, were fixed with 2% glutaraldehyde in PBS for 1 hour, washed in PBS and postfixed in 1% osmium

tetroxide in 0,1M cacodylate buffer. After washing and staining *en bloc* by 1% aqueous uranyl acetate, cells were dehydrated in methanol gradient series, infiltrated by epoxy resin and polymerized. Coverslips were removed by liquid nitrogen and resin blocks were mounted for ultrathin sectioning. 60 nm sections were collected on grids and imaged at Zeiss 900 transmission electron microscope.

Patch clamp recordings

Solutions and recording conditions

Extracellular artificial cerebrospinal fluid (aCSF) contained (in mM): 125 NaCl, 3 KCl, 10 Glucose, 25 NaH_2CO_3, 1.25 Na_2HPO_4, 1.1 $CaCl_2$, 1 $MgCl_2$. The extracellular solution had an osmolarity of 298 mOsm and was continuously equilibrated with 5% CO_2 and 95% O_2 to pH 7.35. All recordings were performed at 35–37°C using a Peltier bath perfusion heater (Warner Instruments, Holliston, USA) with bath temperature sensing. Recording pipettes were filled with potassium gluconate–based (KGlu) internal solution containing (in mM): 150 K-gluconate, 3 Mg-ATP, 0.3 Na-GTP, 10 K-HEPES, 10 NaCl and 0.2 EGTA, pH adjusted to 7.2 by KOH, osmolarity 293 mOsm. For recordings from cultures grown in Neurobasal plus medium (250–270 mOsm), osmolarities of the internal and external solution were both adjusted to the culture osmolarity by adding *Aqua dest*.

Somatic current clamp recordings

Whole cell somatic current-clamp recordings were performed with pipettes pulled from borosilicate glass (Science Products, Hofheim, Germany) by a DMZ Universal Electrode Puller with filament heating (Zeitz Instruments, Martinsried, Germany). Recording pipettes were filled with potassium gluconate-based solution and had a resistance of 4–6.5 MΩ. Membrane potentials

were corrected for the 16 mV liquid junction potential calculated from the ion concentrations in the internal and external solutions according the stationary Nernst-Planck equation (Marino et al., 2014) using LJPcalc (https://swharden.com/LJPcalc/).

Pipettes were fixed on a custom-build pipette holder and mounted on a micromanipulator (Kleindiek Nanotechnik, Reutlingen, Germany). All recordings were sampled at 200 kHz with a 10-kHz-low-pass filter (Bessel, 8-pole) using a HEKA EPC10/2 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) controlled by the Patchmaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pipette capacitance was compensated with the internal capacitance compensation circuit of the EPC10. The circuit uses a capacitance value that is determined in voltage clamp mode in the cell-attached configuration and automatically subtracts 0.5 pF to prevent oscillations. The built-in bridge compensation was then adjusted to 100% of the series resistance determined in voltage-clamp. For recordings cultures were mounted on an upright microscope (Nikon FN-1, Nikon Instruments, Melville, USA) and visualized by a 60x water-immersion objective (NA 1.0) using difference interference contrast optics.

The voltage slope ($\dot{V}$) was calculated as the first time derivative of somatic membrane potential (dV/dt). Action potentials were identified as voltage inflections with an upward slope larger than 50 mV/ms. The current threshold (I_{th}) required for eliciting an action potential was denoted as rheobase. The membrane potential at which the slope reached 25 mV/ms was denoted voltage threshold (V_{th}). For each action potential the peak voltage (V_{max}) and peak slope ($\dot{V}_{max}$) was measured. The half-width was calculated using the first action potential at rheobase as the action potential duration at the membrane voltage halfway between threshold and peak voltage ($(V_{th}+V_{max})/2$).

297

298 *Postsynaptic voltage clamp recordings*

299 Extracellular artificial cerebrospinal fluid (aCSF) had the same composition as described above,
300 except using 5 mM Glucose and 5 mM Sucrose instead of 10 mM Glucose. Internal solution was
301 used as described above, except for using 0.05 mM instead of 0.2 mM EGTA. Pipettes had a
302 resistance of 2.5–6.5 MΩ. Excitatory postsynaptic currents (EPSCs) triggered by presynaptic
303 action potentials that were induced by extracellular stimulation were recorded as previously
304 reported (Maximov et al., 2007; Hallermann et al., 2010; Ritzau-Jost et al., 2014). Briefly, somatic
305 recordings were performed as described above from human neurons grown in NB medium. The
306 aCSF contained the selective NMDA receptor antagonist AP-5 (Tocris, 0.05 mM) to isolate AMPA
307 receptor currents recorded in voltage-clamp mode at a holding potential of -70mV using an EPC-
308 10 (HEKA Elektronik GmbH, Reutlingen, Germany) while correcting for a calculated liquid junction
309 potential of 16 mV. Recordings were sampled at 50 kHz. QX-314 (5 mM) was added to the
310 intracellular solution to block sodium channels to avoid sodium channel activation in incomplete
311 clamped cellular compartments. The access resistance of the somatic recordings was estimated
312 with the build-in whole-cell capacitance compensation circuit of the EPC10 and ranged between
313 4 and 15 MΩ. The whole-cell capacitance compensation was then deactivated during the
314 recordings.

315

316 Stimulation pipettes were also pulled from borosilicate glass, but were slightly larger and had an
317 open-tip resistance of 2–3 MΩ when filled with aCSF. Both recording and stimulation pipette were
318 mounted on uMp-3 micromanipulators (SensaPex, Oulu, Finland). After obtaining a whole cell
319 recording configuration, the stimulation pipette was placed at a distance of 50–200 µm and 0.1 ms
320 square pulses were delivered while scanning the area until mono-phasic EPSCs could be reliably

recorded at a minimally required stimulation intensity for reliable stimulation of 20–60 V. High frequency stimulations with 0.1 ms duration and frequency of 10, 50 and 100 Hz were delivered in combination with a consecutive series of 6 recovery-stimuli at an interval of 75, 125, 250, 500, 1000, and 3000 ms. The measurements were repeated several times at an interval of 20 s for 10 Hz and 50 Hz, and at an interval of 30 s for 100 Hz. All recordings were made at a temperature of 35–37°C.

EPSCs during high-frequency transmission were analyzed as follows: The baseline of each trace was subtracted and an average of all trace from one neuron recording with the same train-frequency was calculated and filtered (low-pass 5 kHz Butterworth filter). In these average trains, the stimulation artefacts were removed using blanking window with a total length of 1.8 ms (0.6 ms before the onset of stimulation until 1.2 ms after the end of a given stimulus). For every mean evoked EPSCs the baseline (0.4–2 ms before onset of the stimulation artefact) and the maximal negative peak (0–4 ms after the onset of the stimulation artefact) was calculated. The paired-pulse ratio (PPR) was calculated as the ratio of the amplitude of the second to the first EPSC during high-frequency transmission. The steady-state amplitude (SS) was calculated as the average of the peaks of the last five EPSCs. The time course of the amplitude of the EPSCs in the recovery phase was fitted using a mono-exponential function (constraining for a start at the steady-state value and a recovery to amplitude of the 1st EPSC) and the subsequent τ value was extracted. Average trains with an estimated τ value greater than 20 s were excluded for subsequent analysis (n = 7 out of 85 average trains in 3 out of 58 neurons). Inclusion of these data led to similar results. The normalized cumulative charge transfer was calculated by integrating the first EPSC of each train for 10 ms from the end of the stimulation artefact (1.2 ms after onset of stimulation) as described before (Uzay et al., 2023). The synaptic jitter was calculated as standard deviation of the trace-to-trace variation in the time of the peak of the EPCS

for each stimulation during high-frequency transmission. The decay of the EPSC was fitted with a monoexponential function over a time course over which the signal showed 80% to 20% of the negative peak of a given EPSC. Fits with $R^2<0.85$ were not used (1 out of 15 and 1 out of 13 neurons for 6 and 9 weeks, respectively).

Presynaptic recordings

Direct presynaptic current-clamp recordings were performed as previously described (Ritzau-Jost et al., 2021, 2023) from human neurons grown in BrainPhys (9 weeks, n = 9) or Neurobasal medium (4 and 10 weeks, n = 5 and 4, respectively). Briefly, current-clamp recordings were performed with pipettes pulled from quartz glass (without filament; Heraeus Quartzglas, Kleinostheim, Germany) with a DMZ Universal Electrode Puller with oxygen-hydrogen burner (Zeitz Instruments, Martinsried, Germany) (Dudel et al., 2000). Pipettes had tip resistances of 6–15 MΩ and were filled with the same potassium gluconate–based internal solution used for somatic recordings but with 100 µM Atto-594 added (ATTO-TEC, Siegen, Germany) for confirmation of the axonal recording (Fig. 7A). Voltages were recorded using a Multiclamp 700A patch-clamp amplifier (Molecular Devices, San Jose, USA), filtered with the internal 10 kHz 4-pole Bessel filter and subsequently digitized (200 kHz) with the HEKA EPC10/2 using Patchmaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) or digitized (100kHz) with the Digidata 1550B using Clampex software 11.2 (Molecular Devices, San Jose, USA). Pipette capacitance compensation was performed with the hybrid voltage-/current-clamp approach (Ritzau-Jost et al., 2021, 2023), which uses the value of the pipette capacitance determined in the voltage-clamp mode for the pipette capacitance neutralization in the current-clamp mode. To minimize the impact of pipette capacitance on voltage recordings, shortest possible pipettes were fixed on a custom-build pipette holder that enabled a steep pipette immersion angle and mounted

on a micromanipulator (Kleindiek Nanotechnik, Reutlingen, Germany). Low bath perfusion levels were used and silicone grease was applied onto the recording electrode wire to prevent internal solution from being pulled up by adhesive forces and onto the electrode shank under binocular guidance. During recordings cultures were mounted on an inverted microscope (Nikon TI-U, Nikon Instruments, Melville, USA) and visualized by a 100x water-immersion objective (NA 1.1) using difference interference contrast optics.

Capacitance measurements were performed as previously described (Ritzau-Jost et al., 2014) at a holding potential of −100 mV and a sine-wave stimulation at 8.3 kHz frequency with an amplitude of ±50 mV. The “sine+DC” method of the HEKA EPC10/2 amplifier controlled by Patchmaster software was used (HEKA Elektronik, Lambrecht, Germany). The intracellular solution contained (in mM): CsCl 130, $MgCl_2$ 0.5, TEA-Cl 20, HEPES 20, Na_2ATP 5, NaGTP 0.3. The extracellular solution contained (in mM): NaCl 105, $NaHCO_3$ 25, glucose 25, TEA 20, 4-AP 5, KCl 2.5, $CaCl_2$ 2, NaH_2PO4 1.25, $MgCl_2$ 1, and tetrodotoxin (TTX) 0.001, equilibrated with 95% O_2 and 5% CO_2. The sine wave stimulation was interrupted with brief depolarizing pulses to 0 mV for 10 ms duration. The calcium current amplitude was measured in a 2-ms-window at the end of the depolarizing pulse (i.e. before the tail current). The change in capacitance was measured in a 100-ms-window beginning 50 ms after the end of the depolarizing pulse.

Experimental Design and Statistical Analysis

Data analysis was performed with custom made scripts in Python (3.11.2) using the package seaborn (0.12.2) for plotting. Statistical testing was performed with the packages scipy (1.10.1) and statsmodels (0.13.5). Line plots show mean and its standard error. For Fig. 1D (proportion of multiple AIS), the line plot shows mean and its confidence interval calculated by Wilson’s method

and a 68% confidence interval was chosen because this corresponds approximately to the standard error used in the other plots. Box plots (Fig. 5B, Fig. 7C) show median and quantile range.

To test for dependence on culture time and medium a two-way ANOVA with interactions was performed with post-Hoc Tukey's HSD. All-positive data were log transformed which lead to normal distributed residuals. We also analyzed our data without transformation using the non-parametric Scheirer-Ray-Hare test (using R version 4.3.1; (R Core Team, 2023)) which led to the same results. Culture time and medium were treated as ordinal variables.

To test the dependence of the recovery time constant, steady state depression and paired pulse interval (Fig. 6D, E, F) on culture time and stimulation interval a two-way ANOVA with interactions was performed with post-Hoc Tukey's HSD as described above. Culture time and medium were treated as ordinal variables.

If only one factor was considered (Fig. 5C) a Kruskal-Wallis H test was used. A Mann-Whitney U test was used to compare two groups (Fig. 5C, pooled human vs. rat).

The proportions of neurons possessing multiple AIS (Fig. 1D) were compared by the Chi squared test. In all cases, first, dependency on medium was tested for 3, 6, 9 weeks separately. Second, data were pooled for medium and tested for dependency on culture time. This was followed by multiple pairwise tests (3 vs. 3, 3 vs. 6, 6 vs. 9 weeks) with p-value correction according to the Benjamin-Hochberg procedure.

Western blots (Fig. 2B) were quantified using FIJI/Image by measuring the optical density of the band and subtracting the optical density of the background. For each combination of the culture media (DMEM, BP, NB, NB+ medium) and culture age (3, 6, 9 weeks) we obtained one measurements (total of n = 12 measurements). We then performed two-way-ANOVA without interactions (degrees of freedom, df = 6).

For presynaptic recordings, data for 9 and 10 week old neurons were pooled after no significant differences were observed for action potential overshoot (V_{max}, Mann-Whitney U test, p = 0.11) as well as half-width (Mann-Whitney U test, p = 0.43).

Results

The proportion of cells with multiple axon initial segments decreases to low levels during maturation

To identify suitable conditions for the structural and functional maturation of human iPSC-derived neurons, we studied neurons cultured with mouse astrocytes for 3, 6, and 9 weeks in four different commonly used media based on Dulbecco's Modified Eagle Medium (DMEM), BrainPhys (BP), Neurobasal (NB) and Neurobasal Plus (NB+). First, we confirmed that these culture do not contain GABAergic neurons by immunolabeling for GAD65/67, which is present in the presynaptic compartment of GABAergic neurons only. Indeed, we hardly detected any GAD65/67 immunolabelling in the human cultures compared to mouse neocortical neuron cultures (Fig. 1B). Second, we checked the specification of the axon initial segment and the maturation of the axon proper. About 30% of human neurons generated by NGN2-overexpression previously had multiple axons when grown in island (autaptic) cultures after 4 to 6 weeks *in vitro* (Meijer et al., 2019; Rhee et al., 2019). We therefore investigated the age- and medium-dependence of the occurrence of multiple axons in human iPSC-derived neurons in dissociated (mass) cultures forming 2D networks. Similar to autaptic neurons, the proportion of neurons with multiple axons in our cultures was ~30% after three weeks in culture and across the four tested media (Fig. 1C, D). However, the proportion of neurons with multiple axons decreased during maturation (Chi square test: $p < 0.001$). Pairwise chi square tests with Benjamini-Hochberg correction revealed a significant difference between 3 and 9 weeks ($p < 0.001$) and 6 and 9 weeks ($p = 0.02$), but not between 3 and 6 weeks (Tukey's HSD: $p = 0.23$). At 9 weeks, about 10% of the neurons possessed two instead of one AIS. There was no statistically significant dependence on the growth medium (Chi square test: $p = 0.65$). These data indicate a medium-independent structural maturation of the human iPSC-derived neurons up to 9 weeks in culture.

The amount of tau protein in the axon increases during maturation

A hallmark of axonal maturation is the increasing axonal content of tau protein throughout development *in vitro* (Kempf et al., 1996) and in vivo (Fiock et al., 2020). We therefore quantified total tau protein levels by immunoblotting (Fig. 2A) for 3 different time points (3, 6, or 9 weeks of culture age) and 4 different media. Two-way ANOVA for Tau levels showed a significant increased during development (Fig. 2B; $p = 0.047$) but showed no significant influence of culture medium ($p = 0.976$). Next, we investigated the molecular maturation of axons focusing on the localization of tau protein. To this end, we imaged cultures immunolabeled for tau protein, pan-neuronally expressed βIII-tubulin, and dendritically-expressed MAP2 by confocal microscopy (Fig. 2C). Images were then segmented and axons were identified based on the presence of βIII-tubulin and the absence of MAP2 (Fig. 2D). Human neurons showed extensive axonal arborizations and large dendritic trees. Axonal tau immunofluorescence was then compared to axonal βIII-tubulin immunofluorescence. During development the ratio of tau bound to axonal microtubules strongly increased (Fig. 2E, ANOVA: $p < 0.001$). Post-hoc test revealed a significant difference between 3, 6 and 9 weeks (Tukey's HSD: $p < 0.001$) but not between different media (Tukey's HSD: $p>0.46$). These data indicate a continuous maturation throughout development from 3 to 9 weeks.

Passive properties indicate continuous cell growth and a stable resting membrane potential after 6 weeks

After having found structural and molecular axonal development in human iPSC-derived neurons, we set out to determine changes in the passive membrane properties and action potential firing as a measure of functional maturation. We recorded the currents elicited by depolarizing voltage steps of 5 mV in somatic whole-cell recordings in four different media and at three developmental

ages (3, 6, and 9 weeks; Fig. 3A and B). From the passive transients, we calculated the input resistance (R_{in}) and the cell capacitance (C_m, a measure of total neuronal surface area). During development, R_{in} decreased (Fig. 3C; ANOVA: $p < 0.001$) irrespective of the medium, except for a difference between BP and DMEM (Tukey's HSD: $p_{adj} = 0.022$). Since we did not determine the seal resistance systematically (knowing only that it was >1 GΩ), the input resistance of the neurons could have been underestimated. In contrast to the decrease of R_{in}, C_m increased ~4-fold (Fig. 3D; ANOVA: $p < 0.001$) suggesting continuous cell growth throughout maturation from 3 to 9 weeks. In addition, we measured the membrane time constant (τ_m) by fitting the membrane voltage responses to a series of current injections (Fig. 3E). During development, τ_m decreased (Fig. 3F; ANOVA: $p < 0.001$) irrespective of the medium (ANOVA: $p = 0.401$). Finally, we measured the resting membrane potential (V_{rest}), which decreased during development (Fig 3G; ANOVA: $p < 0.001$). Interestingly, V_{rest} stabilized after 6 weeks (Tukey's HSD for 6 vs. 9 week: $p_{adj} = 0.55$) at about –64 mV across all four tested media (ANOVA: $p = 0.45$). These data indicate that despite the continuous growth from 3 to 9 weeks, the functional maturity assessed by the resting membrane potential reached a plateau after 6 weeks.

Somatic action potential duration decreased and excitability increased during maturation

As a more relevant parameter of functional maturity, we analyzed action potential firing. We recorded action potentials in the different media and developmental stages (Fig. 4A). To quantify the basic excitability we measured the rheobase as the current threshold (I_{th}) required to elicit an action potential during 50 ms of current injection. I_{th} increased developmentally in all media (Fig. 4B, ANOVA: $p < 0.001$), with similar ~8-fold increases in I_{th} in neurons grown in all media except from BP which showed a ~12-fold I_{th} increase (Tukey's HSD: $p_{adj} = 0.0004$, 0.011, 0.076 for BP vs. DMEM, NB, NB+, respectively). When normalized by the neuronal surface (C_m; see Fig. 3C),

threshold currents appeared fairly stable during maturation except from BP (Fig. 4C; ANOVA: $p = 0.013$; Tukey's HSD: $p_{adj} < 0.002$ for BP vs other media). Next, the impact of cultivation age and media on action potential shape were determined (Fig. 4D, also see methods section). The half-duration of action potentials measured at near-physiological recording temperature (34°C) decreased developmentally (Fig. 4E, ANOVA: $p < 0.001$). The voltage threshold (V_{th}) of action potentials decreased by ~10 mV from 3 to 9 weeks in culture across all media (Fig. 4F; ANOVA: $p < 0.001$; ~8 mV for NB medium) and the peak voltage (V_{max}) slightly increased (Fig. 4G; ANOVA: $p < 0.001$; e.g. ~12 mV for NB medium). Finally, the maximum upward voltage slope ($\dot{V}_{max}$) was measured (Fig. 4H) which showed a developmental increase from ~200 mV/ms to ~400 mV/ms (ANOVA, $p < 0.001$; Tukey's HSD, $p_{adj} = 0.001$ between all age groups). Furthermore, it depended on culture medium (ANOVA, $p = 0.024$) and at 9 weeks $\dot{V}_{max}$ was lower in DMEM (~250 mV/ms) when compared to NB medium (Tukey's HSD, $p_{adj} = 0.008$; ~380 mV/ms) or NB+ medium ($p_{adj} = 0.001$; ~450 mV/ms).

Finally, we determined developmental changes in repetitive firing of action potentials in all four media evoked by incremental current injections (Fig. 4I). To account for the variability in cell size under the experimental conditions, the current injection amplitude was normalized to the cell capacitance. The number of action potentials fired at current injections of 3 pA/pF significantly increased from 3 to 6 weeks (two-way ANOVA, $p < 0.001$; Tukey's HSD, $p_{adj} = 0.001$) but not afterwards ($p_{adj} = 0.693$; Fig. 4J). The number of action potentials obtained after 6 to 9 weeks approximately corresponds to a frequency of 50 Hz. These data indicate that the capability for high-frequency action potential firing reached maturity upon 6 weeks, independent of the culture medium.

Human induced neurons develop synapses with narrow apposition of pre- and postsynaptic scaffolding proteins

Structural synapse development was determined by super-resolution stimulated emission depletion (STED) microscopy and electron microscopy. Double immunolabelling of the presynaptic marker Bassoon and the postsynaptic marker for glutamatergic synapses Homer1 revealed synaptic contacts as early as 3 weeks in any of the four culture media (Fig. 5A). For neurons grown in Neurobasal medium, the distance between the maximum intensities of Bassoon and Homer1 was measured for synapses oriented perpendicular to the optical plane (Fig. 5B). This distance did not change during development (Fig. 5C, Kruskal-Wallis H-test: $p = 0.252$) and was similar to synapses in rat cortical neuron cultures (Mann-Whitney U test: $p = 0.125$). This distance (~130 nm) was only slightly smaller than the Bassoon-Homer1 distances measured from synapses in the accessory olfactory bulb (~155 nm) and cortex of adult mice (~150 nm) using STORM microscopy (Dani et al., 2010). Furthermore, electron microscopic images (3 individual coverslips, n = 50 boutons observed) revealed presynaptic vesicles, a synaptic cleft, and a postsynaptic density, reminiscent of mature vertebrate synapses (representative example shown in Fig. 5D). These data indicate that induced human neurons form proper glutamatergic synapses with characteristic molecular and ultrastructural features.

High-frequency transmission with synchronous release up to 100 Hz

To analyze the function of these synapses, we measured short-term plasticity during high-frequency synaptic transmission and the recovery from synaptic depression at 3, 6, and 9 weeks *in vitro*. To this end, we recorded excitatory postsynaptic currents (EPSCs) evoked by extracellular stimulation with a second pipette at a frequency of 10, 50, and 100 Hz at near-physiological recording temperature (35–37°C). Evoked EPSCs could be reliably measured

already after 3 weeks *in vitro* (Fig. 6A). Furthermore, there was a substantial developmental change over the course of several weeks *in vitro*. We analyzed the time constant of recovery from short-term depression, the steady state EPSC amplitude of the last 5 EPSCs, and the paired-pulse ratio (PPR) of the first two EPSCs as illustrated in Fig. 6C for EPSCs elicited by a 100 Hz stimulation. The recovery from depression accelerated with higher stimulation frequencies (two-way ANOVA, $p = 0.013$) but there was no significant dependency on age (two-way ANOVA, $p = 0.09$; Fig. 6D; n = 78 trains from n = 55 neurons; each train being the average of 1 to 19 traces from one neuron with the same train-frequency). The amount of short-term depression assessed by the normalized steady-state amplitude increased with the stimulation frequency in all age groups (2-way ANOVA, $p < 0.001$; n = 85 trains from 58 neurons, Fig. 6E). However, short-term depression decreased during maturation from 3 to 9 weeks (2-way ANOVA, $p < 0.001$). This decrease was significant between 3 and 6 weeks but not between 6 and 9 weeks (post-hoc Tukey's HSD, $p = 0.018$ and $p = 0.38$, respectively, n = 26, 29, and 30 trains for 3, 6, and 9 weeks, respectively; Fig. 6E). Consistently, the PPR increased with frequency and decreased during maturation (2-way ANOVA, $p < 0.001$ for both). This decrease was significant between 3 and 6 weeks but not between 6 and 9 weeks (Tukey's HSD, $p < 0.001$ and $p = 0.62$, respectively, Fig. 6F). At 50 Hz stimulation the proportion of facilitating synapses (i.e. PPR > 1) was 11% (1 out of 9 neurons), 27% (4 out of 15 neurons) and 54% (7 out of 13 neurons) for 3, 6 and 9 weeks in culture, respectively. These data indicate an increase in the fidelity of synaptic transmission during maturation from 3 to 9 weeks, reaching reliable synaptic transmission at frequencies up to 100 Hz at 6 and 9 weeks .

Temporal precision of synaptic transmission increases during development

570 To analyze the temporal precision of synaptic transmission, we first analyzed the synchronicity of
571 synaptic transmission. In contrast to a study that reported an age-dependent desynchronization
572 of postsynaptic currents in iPSC-derived human neurons (Uzay et al., 2023), we found little
573 change in the synchronicity between 3 and 9 weeks. If anything, the grand-average of all trains
574 showed a higher peak and less asynchronous release at 9 weeks, particularly during the last two
575 EPSCs (#20 and #21 in Fig. 7A). Moreover, the normalized cumulative charge transfer (NCCT)
576 for first 10 ms starting from the peak of the first EPSC during the high-frequency stimulations (Fig.
577 7B) did not show a prominent right-shift as observed by Uzay et al. (2023). Correspondingly, the
578 time to half-maximal charge transfer ($t_{50\%}$) did not change significantly during development ($p =$
579 0.664, Kruskal-Wallis test, n = 9, 15, and 13 neurons for 3, 6, and 9 weeks, respectively; Fig. 7C).
580
581 As another measure of temporal precision of synaptic transmission, we calculated the standard
582 deviation of the trace-to-trace variation (i.e. the jitter) in the time of the peak of the EPCSs during
583 high-frequency transmission for each neuron and averaged these standard deviations across all
584 neurons. Repetitive stimulation lead to a pronounced increase of the EPSC jitter, but with notable
585 differences at 3, 6 and 9 weeks (Fig. 7D). The initial EPSC jitter did not change significantly during
586 development ($p = 0.309$, Kruskal-Wallis test, n = 45 neurons, using the 1st EPSC of either
587 stimulation frequency; Fig. 7E). In contrast, the jitter of the last EPCSs during high-frequency
588 transmission decrease significantly during development ($p = 0.050$, Kruskal-Wallis test, n = 28
589 cells, using the 19th to 21st EPCS from 50 Hz stimulations; $p = 0.012$ between 3 and 9 weeks,
590 Mann-Whitney test; Fig. 7F). Finally, we analyzed the EPSC decay time constant, which depends
591 on several parameters but also on the amount of asynchronous release. However, also the decay
592 time constant did not change during development ($p = 0.181$, Kruskal-Wallis, n = 9, 14, and 12
593 neurons for 3, 6, and 9 weeks, respectively; Fig. 7G). These data did not reveal signs of

desynchronization of release during maturation and indicate an increased temporal precision of synaptic transmission during maturation.

Presynaptic action potentials are large and rapid

The high fidelity and precision of synaptic transmission indicates that induced human neurons can serve as a model to study the mechanisms of synaptic transmission. To analyze the presynaptic mechanisms in more detail, we aimed to establish presynaptic whole-cell patch-clamp recordings in human neurons, similar to the method previously described for cultured mouse neurons (Ritzau-Jost et al., 2021, 2023). Specifically, we visually identified presynaptic boutons in difference interference contrast (DIC) microscopy. Moreover, in selected experiments we used co-staining with FM™ 1-43 dye (Gaffield and Betz, 2007) to confirm that the visually-identified structures indeed represent presynaptic boutons capable of vesicle cycling. In each recording, the recorded structure was filled with the fluorescent dye Atto-488 contained in the intracellular pipette solution. The diameter of the boutons was ~1 µm and additional *en passant* boutons along the axon were visible (Fig. 8A).

To determine central parameters controlling the precision of synaptic transmission, we measured presynaptic action potentials with current-clamp recordings. As previously shown (Ritzau-Jost et al., 2021), the pipette capacitance limits the temporal resolution and the amplitude of the measured action potentials. We therefore lowered the height of the solution in the bath chamber to a minimum, used short quartz-glass pipettes (Dudel et al., 2000), and pipettes minimally filled with intracellular solution (Ritzau-Jost et al., 2021, 2023). Brief current injections (1 ms) were applied to elicit presynaptic action potentials (Fig. 8B). Quantification of the maximum peak potential (overshoot) and the action potential half-duration reveal large and rapid action potentials

(Fig. 8C) at near-physiological recording temperature (35–37°C). At 4 weeks the peak potential was +16 [12, 20] mV and the half-duration was 0.60 [0.52, 0.73] ms (median [interquartile range]; n = 6). At 9–10 weeks the peak potential was +22 [15, 42] mV and the half-duration was 0.53 [0.46, 0.76] ms (median [interquartile range]; n = 14). There were no significant differences for amplitude (Mann-Whitney U-test, p = 0.27) and half-duration (Mann-Whitney U-test, p = 0.56). The amplitude and half-duration were similar to corresponding recordings of presynaptic action potentials from conventional small boutons in cultured neocortical neurons of mice (Ritzau-Jost et al., 2021).

To test whether we recorded from functional presynaptic nerve terminals, we performed capacitance measurements (Fig. 8D), which allow direct monitoring of exo- and endocytosis. Depolarizations to 0 mV for 10 ms elicited calcium currents of 16.40 [9.70, 30.15] pA amplitude (median [interquartile range]; n = 6) and an increase in the membrane capacitance of 0.83 [0.24, 1.00] fF (median [interquartile range]; n = 6). These properties are similar to previous corresponding recordings from small conventional boutons of vertebrate neurons (Kawaguchi and Sakaba, 2017). Therefore, the structures we recorded from represent functional presynaptic terminals. Because action potential recordings and capacitance measurements cannot be made simultaneously from the same boutons, we cannot completely exclude the possibility that some of the action potentials were recorded from neuronal structures that are not functional presynaptic terminals. Taken together, however, these data suggest that the presynaptic action potentials of human glutamatergic neurons are large and fast, with properties that are similar to those that have previously been measured in vertebrate neurons.

Discussion

Here we investigated the mechanisms of synaptic transmission in human neurons derived from iPSCs by transient expression of Ngn2. We show (1) that the maturation of morphological, molecular and electrophysiological properties of these neurons is similar in different commonly used culture media, (2) that the fidelity and the temporal precision during high-frequency synaptic transmission increases during maturation, (3) that 9-week-old neurons exhibit reliable synchronous synaptic transmission up to a frequency of 100 Hz, and (4) that the presynaptic action potentials have a large amplitude and short duration with no change during maturation from 4 to 9–10 weeks. Our results establish Ngn2-induced iPSC-derived neurons as a model for high-resolution structure-function analyses of synaptic transmission and uncover fundamental parameters of glutamatergic signaling between human neurons.

Synchronous high-frequency transmission

The here-used induced human neurons exhibit structural (one AIS; Fig. 1), molecular (tau in axon; Fig. 2) and functional properties (resting membrane potential and action potential firing; Fig. 3 and 4) similar to mature murine neurons. Furthermore, synapses showed characteristic structural signs of maturation (Fig. 5) and surprisingly reliable transmission up to 100 Hz (Fig. 6 and 7). The fidelity and synchronicity of neurotransmitter release seemed higher compared to autaptic human neurons (Fenske et al., 2019; Meijer et al., 2019; Rhee et al., 2019), but this might be due to the physiological recording temperatures used here (Pyott and Rosenmund, 2002). In a recent study analyzing 3- to 7-week-old cultures of Ngn2-induced neurons, the absence of inhibitory neurons was found to cause endoplasmic reticulum stress and augmented asynchronous release in neurons older than 5 weeks *in vitro* (Uzay et al., 2023). However, we did not observe such a decrease in synchronicity of release during maturation from 3 to 9 weeks *in vitro* (Fig. 7C). Instead, we observed a developmental increase in the temporal precision of synaptic transmission during

high frequency synaptic transmission (Fig. 7D-F). There are several factors that could have contributed to this different finding: First, although we use the same Ngn2 overexpression method to differentiate glutamatergic neurons from iPSCs, our iPSC line is different from the one used in the previous study. Second, we use 2% fetal calf serum throughout the culture, which might increase viability of the neuron cultures and prevent the premature aging phenotype. Independent of the varying onset of aging processes in this *in vitro* model, the here-observed surprisingly synchronous release up to a transmission frequency of 100 Hz seems consistent with the emerging view that human neurons are particularly tuned to high frequency signaling (Testa-Silva et al., 2014; Goriounova et al., 2018; Galakhova et al., 2022).

Presynaptic patch-clamp recordings in human neurons

The reliability and high frequency-bandwidth of synaptic transmission indicates that transiently expressing Ngn2 in iPSC-derived neurons leads to rather mature glutamatergic transmission. To decipher the mechanisms of synchronous release during high-frequency transmission in humans, we established direct presynaptic patch-clamp recordings from boutons of 4- to 10-week-old human neurons. Presynaptic recordings represent a valuable tool to analyze presynaptic function with an exceptionally high temporal resolution (Neher, 1998; Vandael et al., 2021). The technique has been applied to large synapses in the vertebrate brain, such as the calyx of Held in the brain stem and mossy fiber boutons in the hippocampus and cerebellum (reviewed by von Gersdorff and Borst, 2002; Bischofberger et al., 2006; Delvendahl and Hallermann, 2016). Here, we extended the applicability of this technique from vertebrate to human synapses.

Human presynaptic action potentials

689 Presynaptic patch-clamp recordings allowed measuring large and rapid presynaptic action
690 potentials at the calyx of Held and the hippocampal and cerebellar mossy fiber bouton (overshoot
691 potential ~30 to 50 mV, half duration ~0.1 to 0.4 ms at 35-37°C; Geiger and Jonas, 2000;
692 Taschenberger and Von Gersdorff, 2000; Ishikawa et al., 2003; Alle et al., 2011; Ritzau-Jost et
693 al., 2014). However, there is still some uncertainty regarding the properties of presynaptic action
694 potentials at small conventional synapses in the vertebrate brain. For example, genetically-
695 encoded voltage sensors indicate that the amplitudes of presynaptic action potentials in small
696 conventional boutons of cultured hippocampal neurons is only 70% of the amplitude at the soma
697 (0 mV overshoot; Hoppa et al., 2014). Furthermore, the extracellular voltage decreased while
698 propagating along the axon (Radivojevic et al., 2017), suggesting a decreased action potential
699 amplitude in more distal axons. It was also reported that the duration slightly decreased in distal
700 compared to proximal axons (Gonzalez Sabater et al., 2021). In addition to these optical and
701 extracellular techniques, high-resolution current clamp recordings from small conventional
702 boutons of cultured neurons of mice and pyramidal neurons in acute brain slices of mice were
703 performed indicating large and rapid presynaptic action potentials (overshoot ~+30 to +50 mV,
704 half duration ~0.3 to 0.4 ms at 35-37°C; (Oláh et al., 2021; Ritzau-Jost et al., 2021)). We here
705 establish this technique for small conventional boutons of human iPSC-derived neurons and
706 measured comparable parameters (Fig. 8C). Thus, despite fundamental differences between
707 human and rodent neurons, our data indicated that glutamatergic synaptic transmission is
708 mediated by large and rapid presynaptic action potentials in both rodents and humans. The rapid
709 and large presynaptic action potentials enable fast activation and deactivation of presynaptic
710 calcium channels (Sabatini and Regehr, 1996; Borst and Sakmann, 1998; Bischofberger et al.,
711 2002), which contributes to here-observed reliable and synchronous neurotransmitter release
712 during high-frequency transmission in human neurons. The presynaptic action potentials
713 exhibited no change during development (Fig. 8C). Therefore, the increase in the fidelity (Fig. 6)

and temporal precision of synaptic transmission (Fig. 7) during development is most likely caused by other factors including, e.g., the developmental acceleration of vesicle recruitment (Taschenberger and Von Gersdorff, 2000; Yamashita et al., 2010) or decrease of the calcium-channel-to-vesicle coupling distance (Wang et al., 2008; Bornschein et al., 2019; Chen et al., 2024).

In vitro maturation of human neurons

The commonly used DMEM based media have not been developed for culturing neurons. Therefore, the family of Neurobasal media (Brewer et al., 1993) and most notably the BrainPhys medium (Bardy et al., 2015) have been promoted as more suitable for neuronal cultures (Satir et al., 2020; Zabolocki et al., 2020). The latter has an ionic composition close to the artificial cerebrospinal fluid widely used for electrophysiological recordings. We systematically tested different cell culture media but detected only slight differences in the morphological, molecular, and electrophysiological properties, except for a significantly stronger increase in threshold current in BrainPhys medium. This was surprising because there are notable differences in the ionic composition between different media (see Table 1 and 2). Previous reports with chemically differentiated neurons showed that BrainPhys accelerates maturation compared to a mixture of DMEM and Neurobasal-A medium (Satir et al., 2020). However, in autaptic cultures of human neurons induced by Ngn2 expression, BrainPhys seemed to have a negative effect on astrocytes and did not enhance synaptic transmission (Rhee et al., 2019).

To accelerate the maturation of stem cells to neurons, we adopted transient Ngn2 over-expression (Zhang et al., 2013). Ngn2 encodes a neural-specific basic helix-loop-helix (bHLH) transcription factor that is a strong driver of pro-neural genes (Hulme et al., 2022) and was shown

738 to generate excitatory glutamatergic neurons from murine embryonic stem cells (Thoma et al.,
739 2012). The formation of super-numerous axons was observed in autaptic cultures (Meijer et al.,
740 2019; Rhee et al., 2019) but primary vertebrate neurons can also have multiple AISs (Guo et al.,
741 2017; Huang and Rasband, 2018). It seems plausible to assume that the Ngn2-induced forced
742 maturation causes the super-numerous axons but Ngn2 expression does not affect the proportion
743 of neurons with multiple axons (Rhee et al., 2019). Independent of the mechanistic reasons for
744 super-numerous axons, our data indicate that within 9 weeks of maturation 90% of all induced
745 neurons harbor only a single axon.

746

747 **Perspectives**

748 Our results demonstrate that Ngn2-induced iPSC-derived neurons represent a convenient and
749 simple-to-establish model to study synaptic function at high spatial and temporal resolution in
750 human neurons. The here-established presynaptic patch-clamp recordings have the potential to
751 uncover the physiological and pathophysiological mechanisms of neuronal communication in
752 humans (Patzke et al., 2019; Verhage and Sørensen, 2020). Indeed, several iPSC cell lines
753 harboring mutations in the tau gene associated with familial frontotemporal dementia and
754 parkinsonism (FTDP-tau) are already available (Karch et al., 2019) and more will be provided by
755 the recently announced *iPSC Neurodegenerative Disease Initiative* (Ramos et al., 2021).

756

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Figures legends

Figure 1. The proportion of cells with multiple axon initial segments decreases to low levels during maturation

(A) Bright field images of cultures of human neurons with mouse astrocytes at 3 weeks.

(B) Example confocal fluorescence images of neurons stained for GAD65 (magenta) and MAP2 (green) at 6 weeks in human and mouse neuron cultures.

(C) Example confocal fluorescence images of neurons stained for ANK3 (magenta) and MAP2 (green) at 3 weeks showing neurons with one or two axon initial segments.

(D) Percentage of neurons with multiple axon initial segments decreases during maturation (n = 16–45 neurons per group)

Figure 2. The amount of tau protein in the axon increases during maturation

(A) Western blots of total tau in human neurons during maturation in different media. Beta-Actin was used as a loading control.

(B) Tau expression increases during maturation of human neurons, quantified from first 12 lanes of the tau Western blot shown in (A).

(C) Example confocal fluorescence images of neurons stained for microtubules (βIII-tubulin, blue) as well as the microtubule-associated proteins tau (red) and MAP2 (green).

(D) Mask for microtubules in dendrites (MAP2-positive) and axons (MAP2-negative).

(E) Ratio of tau to tubulin immunofluorescence signals in the axon increases during maturation. Each point represents mean ± standard error from n = 5 neurons per age and medium group from 12 independent cultures.

Figure 3. Passive properties indicate continuous cell growth and a stable resting membrane potential after 6 weeks

(A) Example DIC image of a whole-cell patch-clamp recording from a 6-week-old neuron.

(B) Example of a square-pulse stimulus (with 100 ms duration and 5 mV amplitude). The input resistance (R_{in}) was calculated from the steady-state current (I_{ss}).

(C) The input resistance R_{in}

(D) The cell capacitance C_m

(E) Example of membrane voltage responses to a series of current injections. The membrane time constant (τ_m) was estimated by fitting a monoexponential function to each voltage response.

(F) The membrane time constant (τ_m).

(G) The resting membrane potential V_{rest}

Recorded neurons per group: n = 8–26, except (C) with n = 6–19 and (F) with n = 3–12.

Figure 4. Somatic action potential duration decreases and excitability increases during maturation

(A) Examples of somatic action potentials recorded in ACSF after development in different culture media (dashed lines indicate 0 mV potentials)

(B) The current threshold I_{th}

(C) The current threshold divided by the cell capacitance C_m (current density threshold)

(D) Example showing the parameters used to describe an action potential, including half-duration, threshold voltage V_{th} and peak voltage V_{max}.

(E) Action potential half-duration

(F) Action potential threshold voltage V_{th}

(G) Action potential peak voltage V_{max}

(H) Action potential maximum slope $\dot{V}_{max}$

(I) Examples of action potentials elicited by square pulse stimuli (with 100 ms duration and a current density of 3 pA/pF) in ACSF after development in different culture media (dashed lines indicate 0 mV potentials).

(J) The number of action potentials in response to a current injection (current density of 3 pA/pF)

Recorded neurons per group: n = 8–31, except (I) with n = 7–14.

Figure 5. Human induced neurons develop synapses with narrow apposition of pre- and postsynaptic scaffolding proteins

(A) Examples of STED images showing the ultrastructure of synapses with distinct pre- and postsynaptic labeling of Bassoon (green) and Homer1 (magenta) at 3, 6 and 9 weeks.

(B) For measurement of peak-to-peak distance between presynaptic Bassoon and postsynaptic Homer1, synaptic contacts were aligned (small insert) and line profiles (white box) were fitted to Gaussians (gray line).

(C) During development a non-significant change in the distance of presynaptic Bassoon and postsynaptic Homer1 was observed. For comparison measurements are shown for rat cortical neurons at DIV14. Number of synapses per group: n = 29, 19, 24, 23.

(D) Example electron microscopic image of a synaptic contact between human neurons cultured for 7 weeks.

Figure 6. High-frequency transmission with synchronous release up to 100 Hz

(A) Example traces of evoked EPSCs elicited by high-frequency stimulation at 50 Hz and 6 pulses of recovery (with exponentially increasing time difference) at different developmental stages (3, 6, and 9 weeks). The average (black) and individual consecutive traces of a recording from an individual cell (grey) are superimposed (stimulation artefacts removed).

(B) Average amplitudes normalized to first EPSC of a series of EPSCs with stimulation at 10 Hz, 50 Hz and 100 Hz at the age of 9 weeks.

(C) Example traces of evoked EPSCs in a 6-week-old neuron elicited by high-frequency stimulation at 100 Hz and 6 pulses of recovery at different time scales illustrating the analysis of recovery from depression, the steady-state amplitude and the paired-pulse ratio (RRP; stimulation artefacts removed).

(D) Steady-state amplitude (normalized amplitude of the last 5 EPSCs during high-frequency transmission).

(E) Paired-pulse ratio (PPR) calculated from the amplitude of first two EPSCs in a trace.

(F) Time constant of a mono-exponential fit (τ_{rec}) of the EPSC amplitudes during the recovery. Recordings without recovery due to strong facilitation were excluded from the analysis of the recovery kinetics.

Number of neurons per group are 20, 29 and 21 for 3, 6 and 9 weeks, respectively. Except for (B) with 11, 13 and 6 neurons for 10, 50, and 100 Hz, respectively.

Figure 7. Temporal precision of synaptic transmission increases during development

(A) Grand averages of the first and last EPSCs of a 50 Hz stimulation at 3 and 9 weeks shown as mean ± standard error.

(B) The normalized cumulative charge transfer for the first EPSC of a 50 Hz stimulation (median and interquartile range, for n = 9 and 13 neurons for 3 and 9 weeks, respectively).

(C) The time to the half of the normalized maximal charge transfer for the first EPSC during 50 Hz stimulation (n = 9, 15, and 13 neurons for 3, 6, and 9 weeks, respectively).

(D) Average jitter of the time of the peak of the EPSC during 50 Hz stimulation (n = 8, 10, and 10 neurons for 3, 6, and 9 weeks, respectively; only including neurons with at least 3 traces to calculate the jitter)

(E) Jitter of the first EPSC (n = 18, 15, and 12 neurons for 3, 6, and 9 weeks, respectively; using the first EPSC from the 20, 50 and 100 Hz stimulation; only including neurons with at least 3 traces to calculate the jitter).

(F) Average jitter of the last three EPSCs during the 50 Hz stimulation as shown in panel D (n = 8, 10, and 10 neurons for 3, 6, and 9 weeks, respectively).

(G) Decay time constant for the first EPSC during 50 Hz stimulation (n = 9, 14, and 12 neurons for 3, 6, and 9 weeks, respectively; only including monoexponential fits with $R^2>0.85$).

Figure 8. Presynaptic axon potentials are large and rapid

(A) Example image of a whole-cell patch-clamp recording from a bouton of an 9-week-old iPSC derived neuron showing the fluorescence of the ATTO 488 dye in the pipette solution (*top left*), the super-position of the fluorescence and the DIC image (*bottom left*), and the florescence due to filling of the axon with the dye after withdrawal of the patch pipette (*right*).

(B) Example of a presynaptic action potential at 9 weeks.

(C) Peak amplitude and half-duration of the presynaptic action potentials (n = 6 and14 bouton recordings at 4 and 9–10 weeks, respectively).

(D) Example of presynaptic capacitance recording showing voltage command (V_m), the pharmacologically isolated calcium current (I_{Ca}), and the membrane capacitance (C_m). The elicited increase in Cm is indicated (ΔC_m). Grey marked inset shows a magnification of V_m and I_{Ca} (scale bar 100 mV and 25 pA).

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		Culture medium			
Component	**Amount**	**BP**	**DMEM**	**NB**	**NB+**
Base medium		BrainPhys	DMEM/F12	Neurobasal-A	Neurobasal-Plus
Penicillin/streptomycin	1:100	+	+	+	+
GlutaMax	1:100	+		+	+
Non-essential amino acids	1:100		+		
B27 supplement	1:50	+		+	
B27 plus supplement	1:50				+
Serum	2%	+	+	+	+
BDNF	10 ng/ml	+	+	+	+
NT3	10 ng/ml	+	+	+	+

Laminin	200 ng/ml	+	+	+	+

Table 1. Composition of culture media.

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		Culture medium				
Parameter	**Unit**	**BP**	**DMEM**	**NB**	**NB+**	**ACSF**
Osmolarity	mOsm	320	310	270	230	300
K^+	mM	3.9	4.0	5.0	5.0	2.9
Na^+	mM	147	151	102	84	148
Ca^{2+}	mM	0.86	0.82	1.46	1.46	0.93
Cl^-	mM	120	126	87	68	126
Glucose	mM	2.3	16.2	23.0	23.8	5.0
Lactate	mM	0.1	0.2	0.0	0.0	0.0

1102 **Table 2. Measured properties of culture media and ACSF.**

Antibody	Antigen	Dilution	Source	Identifier
Rabbit anti-GAD65/67 antibody	GAD65/67	1:500	Sigma	Cat.No.: G5163 RRID:AB_477019
Mouse anti-ANK3 antibody, clone N106/36	ANK3	1:1000	NeuroMAB	Cat.No.: 75_146 RRID:AB_2877524
Polyclonal rabbit anti-MAP2 antibody	MAP2	1:2000	Millipore	Cat.No.: AB5622 RRID:AB_91939
Polyclonal rabbit anti-beta III tubulin antibody	Tubulin	1:2000	Synaptic Systems	Cat.No.: 302302 RRID:AB_10637424
Polyclonal Rabbit Anti-Homer1 antibody	Homer1	1:500	Synaptic Systems	Cat.No.: 160003 RRID:AB_887730
Monoclonal mouse Anti-Bassoon antibody	Bassoon	1:500	Enzo Life Sciences	Cat.No.: ADI-VAM-PS003-F

				RRID:AB_11181058
Monoclonal mouse anti-tau antibody, clone	Tau	1:1000	DSHB	Cat.No.: 5A6 RRID:AB_528487
Polyclonal Rabbit Anti-Human Tau antibody	Tau	1:4000	Agilent	Cat.No.: A0024 RRID:AB_10013724
monoclonal rat anti-beta-actin antibody, clone W16197A	rat	1:1000	BioLegend	Cat.No.: 664804 RRID:AB_2728496
Anti-Rabbit-HRP	Rabbit IgG	1:20000	Dianova	Cat.No.: 711-035-152
Anti-mouse-HRP	Mouse and rat IgG	1:20000	Dianova	Cat.No.: 515-035-151
Donkey-anti-rat-AF647	Rat IgG	1:1000	Dianova	Cat.No.: 712-605-153

Goat-anti-mouse-StarOrange	Mouse IgG	1:400	Abberior	Cat.No.: STORANGE-1001-500UG
Goat-anti-rabbit-StarRed	Rabbit IgG	1:400	Abberior	Cat.No.: STRED-1002-500UG
Goat-anti-guinea pig-AF488	Guinea Pig IgG	1:500	Biozol	Cat.No.: 106-545-003
Donkey-anti-rabbit-AF488	Rabbit IgG	1:500	Biozol	Cat.No.: 711-545-152
Donkey-anti-rabbit-AF647	Rabbit IgG	1:500	Biozol	Cat.No.: 711-605-152
Donkey-anti-mouse-Cy3	Mouse IgG	1:500	Jackson	Cat.No.: 715-165-150

Table 3. Antibodies, dilutions used for immunocytochemistry and western blots. Further information can be found on https://www.antibodyregistry.org/ using the RRID provided for each primary antibody.