Developmental GABA polarity switch and neuronal plasticity in Bioengineered Neuronal Organoids

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Brain organoids are promising tools for disease modeling and drug development. For proper neuronal network formation excitatory and inhibitory neurons as well as glia need to co-develop. Here, we report the directed self-organization of human induced pluripotent stem cells in a collagen hydrogel towards a highly interconnected neuronal network at a macro-scale tissue format. Bioengineered Neuronal Organoids (BENOs) comprise interconnected excitatory and inhibitory neurons with supportive astrocytes and oligodendrocytes. Giant depolarizing potential (GDP)-like events observed in early BENO cultures mimic early network activity of the fetal brain. The observed GABA polarity switch and reduced GDPs in >40 day BENO indicate progressive neuronal network maturation. BENOs demonstrate expedited complex network burst development after two months and evidence for long-term potentiation. The similarity of structural and functional properties to the fetal brain may allow for the application of BENOs in studies of neuronal plasticity and modeling of disease.
Brain organoids recapitulate several aspects of cortical development in vivo. Applications in disease modeling and drug screening have been demonstrated. Patterning protocols have been developed to generate fore-, mid-, and ventral-brain organoids. Although complex anatomical layering and activity of individual neurons has been elegantly demonstrated, little is known about network function and plasticity of established brain organoid models.

During early corticogenesis, bursts of action potentials cause spreading of giant waves of calcium influxes through the developing cortex. This synchronized activity, described as giant depolarizing potentials (GDPs), depends both on excitatory glutamatergic and GABA inputs. GDPs are initiated by so-called hub or pioneer neurons, which are known to have long axons and to connect with many other neurons in parallel. In humans, GABA and glutamate-sensitive GDPs have been observed in fetal cortex.

A number of studies have shown the importance of supporting glia for neuronal network function and plasticity. Moreover, a recent study on network function of brain organoids elegantly demonstrated the importance of inhibitory neurons for complex network development, both coinciding after 6 months of differentiation.

Aiming to recapitulate human brain network function, we develop Bioengineered Neuronal Organoids (BENOs) from human-induced pluripotent stem cells (iPSCs). BENOs consist of functionally integrated excitatory (glutamatergic) and inhibitory (GABAergic) neurons as well as supporting glia. Collectively, this cellular diversity demonstrates neuronal network function classically found in the developing brain, such as GDP and a GABA polarity switch, and within the more mature human brain, such as neuronal plasticity.

Results

BENO generation protocol. BENOs are generated from human iPSCs dispersed in a collagen type I environment and are subjected to a staged directed differentiation and maturation protocol. The introduced BENO protocol differs from previously established neuronal organoid cultures by (1) a 1-step culture, directed differentiation approach and (2) the use of fully defined components, such as a pluripotent stem cell source, collagen, small molecules, and growth factors. We initially tested five different directed differentiation protocols (Fig. 1a, Supplementary Fig. 1a–c), starting with a 10 day induction of neuroectodermal commitment in the presence of SB/noggin (dual smad inhibition) and retinoic acid (RA) (Fig. 1b, Supplementary Fig. 1b). This synchronized activity, described as giant depolarizing potentials (GDPs), depends both on excitatory glutamatergic and GABA inputs. GDPs are initiated by so-called hub or pioneer neurons, which are known to have long axons and to connect with many other neurons in parallel. In humans, GABA and glutamate-sensitive GDPs have been observed in fetal cortex.

BENO cellular composition. Next, we utilized whole-mount immunofluorescence (WmiF) analyses to investigate the spatio-temporal development of multiple cell populations in BENOs. On d15, we observed concentric expansion of highly proliferative PAX6+ Ki67+ NPCs (Fig. 3a). On d40, evidence for the development of a subventricular zone (TBR2+ cells) adjacent to an outer self-organizing cortical plate (CTIP2+ cells) was obtained (Fig. 3b). On d40, BENOs contained excitatory catecholaminergic (TH+) and glutamatergic (vGLUT+) neurons as well as the respective target cells expressing typical cortical glutamatergic receptors such as GLUR1 (Fig. 3c, d). In parallel, an extensive network of GABA+ cells as well as GABBR2+ cells were present in d40 BENOs (Fig. 3e). On d60, in line with the transcriptome data, BENOs were enriched with astrocytes marked by GFAP/S100beta expression (Fig. 3f), while first oligodendrocyte progenitors (OLIG2+ cells) appeared (Supplementary Fig. 5). By a further extension of BENO culture for up to 150 days, we observed OLIG2+ cells as well as MBP and CNP expression and the presence of the first myelinated axons after 90 days in culture (Fig. 3g). By day d150 the number of CNP+ OLIG2+ cells as well as myelinated axons increased markedly (Fig. 3h, Supplementary Fig. 5b–f).

GABA- and glutamate-dependent GDP-like events in BENO. We next confirmed the presence of functional neurons by whole-cell patch-clamp recordings of BENO (d35–d65) cultures, showing tetradotoxin (TTX)-sensitive excitatory postsynaptic potentials (TTX, 1 nM) and functional sodium and potassium channels (Supplementary Fig. 6a). To further characterize neuronal...
network development, we analyzed spontaneous and stimulation-induced calcium activity in BENOs between culture day 20 and 98. Interestingly, the BENO network analysis showed pronounced differences during BENO development, indicating three distinct stages of network development: (1) early stage (ES) prior to d25, (2) intermediate stage (IS) between d25–d40, and (3) late stage (LS) d40 and onwards.

During the ES, calcium signal analysis demonstrated a high abundance of neurons with TTX-sensitive spontaneous activity (Fig. 4a, Supplementary Fig. 6b). In the next couple of days, the appearance of highly organized, spontaneous, synchronous calcium bursts, resembling GDPs, suggested early network formation similar to processes identified in the developing brain (Fig. 4b, Supplementary Movie 1). At this stage, local electrical stimulation (single pulses, 300 µA) induced TTX-sensitive synchronized calcium bursts at up to 200 µm distance from the electrode, but failed to propagate to remote regions. In contrast, high frequency stimulations (HFS) in ES BENOs evoked...
GDP-like events throughout the organoid (Supplementary Fig. 6c).

During the IS, spontaneous GDP-like event frequency was the highest ($3.6 \pm 0.6$ events per 5 min, $n = 8$; Fig. 4c, Supplementary Movies 2 and 3). To understand which neurotransmitter contributed to network synchronization, we measured the spontaneous activity of BENOs in the presence and absence of selective inhibitors against GABAergic and glutamatergic receptors. GABAergic inhibition, by the non-selective antagonist for GABA-A/-C, Picrotoxin (PTX: 58 µM) and the selective antagonists for GABA-B, Saclofen (330 µM) reduced the occurrence of GDP-like events ($n = 4$ organoids; Fig. 4d, e, Supplementary Fig. 6d). The reduced activity suggested an existence of excitatory presynaptic GABA signals, which have been recognized to trigger GDPs in the developing brain.12 Inhibition of the glutamatergic network, by the combination of the non-competitive NMDA antagonist, Dizocilpine (MK-801: 0.2 µM) and the competitive AMPA/kainate receptor antagonist, Cyanquixaline (CNQX: 15 µM), strongly decreased the frequency of synchronous calcium activity ($n = 3$ organoids; Fig. 4d, f, Supplementary Fig. 6e). These data show that both glutamatergic and GABAergic neurotransmission contributes to the observed GDP-like events.

At the LS, no spontaneous GDP-like events could be recorded (Fig. 4c). To investigate whether the absence of GDPs in day 40

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**Fig. 2** Transcriptome analyses during BENO generation (from d-1 to d60) by RNA sequencing. a Heat map showing distinct developmental stages: d-1 to d0—pluripotent stem cell stage; d3–d8—neuroectodermal commitment stage; d8–d15—NPC stage; d15–d40—neurogenesis; d40 and onwards—neuronal maturation with concurrent gliogenesis. b Increase of the abundance of transcripts encoding for GABAergic, glutamatergic, dopaminergic, cholinergic, and serotonergic proteins from d28 onwards. c Heat map depicting the expression of transcripts encoding for proteins involved in synaptic transmission, including postsynaptic receptors, ion channels, and synaptic proteins.
Fig. 3 Cellular composition of BENOs in different times of differentiation. a Representative overview and higher magnification images of indicated region of a d15 BENO showing proliferating NPCs (PAX6+/Ki67+; refer to Supplementary Fig. 4a for the individual confocal planes). b Distinguishable concentric surrogate subventricular zone (TBR2+) and a cortical plate (CTIP2+) in d40 BENO. c-e Excitatory (vGLUT+, TH+) and inhibitory (GABBR2+) neurons as well as their respective receptors (GLUR1, GABBR2) in d40 BENOs. f Overview (left) of a d60 BENO stained for astrocyte markers (GFAP+/S100β+) and neuronal axons (NF+); close-up views (right) of GFAP+/S100β+ cells with typical astrocyte morphology. g Detection of oligodendrocyte progenitors (OLIG2+) and oligodendrocytes (MBP+/CNP+) in d90 BENO. Inserts 1 and 2 highlight MBP+ oligodendrocytes (*), which contribute to myelination of axons (<). h On d150 a higher number of mature oligodendrocytes and myelinated axons appeared. All data presented on this figure derive from at least three independent experiments with similar results. Scale bars: overviews 200 μm, close-up views 20 μm, unless indicated otherwise.
**Fig. 4 Development of neuronal networks during BENO generation.**

**a** Calcium activity in d21 BENO. Upper-left: overview of the complete organoid loaded with the calcium indicator Fluo-8-AM. Upper-middle: close-up view of the boxed area. Upper-right: active correlation map of different regions of interest (ROI) showing calcium activity; each ROI is representative for a specific neuronal activity. Bottom: heat map of calcium activity of 52 neurons as a function of time.

**b** Active correlation map of d35 BENO and color-coded calcium traces (ΔF/F0) showing GDP-like events (refer also to Supplementary Videos).

**c** GDP-like event frequency at early stage (ES, d < 25), intermediate stage (IS, 25 < d < 40), late stage (LS, 40 < d < 65); n = 15 organoids, 2 independent experiments, *P* values of 10 and 100 µmol/L GABA ES vs LS were 0.0035 and <0.0001, respectively, one-way ANOVA with Sidak’s multiple comparisons post hoc test.

**d** GDP-like event inhibition by PTX, CNQX, and MK-801; note that upon washout synchronicity was restored.

**e** Overview of calcium activity propagation induced by single pulses (100 µA) in the same fused BENO on d40 (1 mm signal propagation relative to stimulation electrode) and on d45 (2 mm signal propagation relative to stimulation electrode).

**f** In ES and IS BENOs, GDP-like events were evoked by 10 and 100 µmol/L GABA or 10 and 100 µmol/L glutamic acid (calcium signals recorded from 3 independent organoids; symbols indicate data from 15 ROIs). In LS BENOs GDP-like events were evoked by glutamate, but not GABA (calcium signals recorded from 3 organoids of 1 experiment; symbols indicate data from 15 ROIs). Bar graphs summarize calcium trace area (left) and signal strength in response area (right), indicating the extent of the neuronal activity in the investigated BENO. Values were normalized to 100 µmol/L glutamate responses in ES/IS and LS organoids; For relative trace area the p values of 10 and 100 µmol/L GABA ES vs LS were 0.002 and <0.0001, respectively. For relative response area the p values of 10 and 100 µmol/L GABA ES vs LS were 0.027 and 0.0011, respectively; one-way ANOVA with Tukey’s multiple comparisons post hoc test.

**g** Representative traces of spontaneous calcium activity under GABAergic and glutamatergic inhibition in LS BENOs (representative image from ROIs recorded in 3 BENOs). j Electrical stimulation-induced calcium activity under GABAergic and glutamatergic inhibition in LS BENOs (representative image from ROIs recorded in 1 of 3 BENOs, n = 3 organoids, 2 independent experiments). Spontaneous and induced activity was enhanced under GABA receptor blockade, while induced activity under glutamatergic inhibition was reduced. Upon washout the network balance was restored.

Scale bars: Overview 200 µm; high magnification 50 µm. All time scale bars on calcium traces are 30 s. Data are presented as mean values ± SEM.
BENOS indicated further maturation of the neuronal networks, we again applied electrical point stimulation. Single pulses of low intensity (100 µA) evoked TTX-sensitive calcium activity at up to 1 mm distance to the stimulation electrode (Fig. 4g). Continuous neuronal network development was demonstrated by a further increase in signal propagation up to 2 mm after stimulation from the same location (n = 6 BENOS tested); in some cases, propagation between two BENOS after fusion could be observed (Fig. 4g).

Given that, GDP-like events were suppressed in ES and IS stage BENOS by GABAergic and glutamatergic inhibitors, we assessed whether these neurotransmitters can induce GDP-like events in more mature LS stage BENOS. We found that 10 and 100 µM glutamate could evoke GDP-like events in ES, IS, and LS BENOS (n = 3 organoids and 14 ROIs/group; Fig. 4h). In contrast, 10 and 100 µM GABA-evoked GDP-like events only in ES and IS BENOS, but not LS BENOS. These data suggested a developmental shift of GABA from excitatory to inhibitory around differentiation day 40 (n = 3 organoids and 14 ROIs/group; Fig. 4h). To verify the inhibitory role of GABA in LS BENOS we measured spontaneous and electrically induced calcium activity under GABA inhibition, while glutamatergic network inhibition (CNQX/MK-801) resulted in a marked decrease of signal intensity (n = 3 organoids; Fig. 4i). The reduced incidence of GDPs and the characteristic shift from excitatory to inhibitory GABA activity suggest LS BENOS undergo similar staged developmental maturation as observed in the human brain.

**BENOS develop complex network bursts.** Spatiotemporal neuronal network organization was assessed in d20 BENOS (n = 4) over 40 days using multi-electrode arrays (MEAs) to monitor individual network activity (Fig. 5a). ES-BENOS showed sparse electrical activity, which slowly organized in neuronal bursts as well as synchronous activity between neurons in different areas of the organoids, known as network bursts (NB). During the IS, the number of individual bursts and NB increased; in LS BENOS bursts organized in complex synchronous events (Fig. 5b, Supplementary Fig. 7). A heat map of spike amplitude (µV) and spike rate (spike/sec) revealed the regional connectivity of the neurons (Fig. 5c). The mean firing rate and the number of active electrodes significantly increased during the different developmental network stages (LS: 1.5 ± 0.18 Hz and 33 ± 2%; Fig. 5d, Supplementary Fig. 8a). Moreover, there was a dramatic increase in synchronous firing in different regions of the BENO depicted by the area under the normalized cross-correlation graph (ES: 0; IS: 0.04 ± 0.01; LS: 0.12 ± 0.01; Fig. 5e). This was in line with the concomitant increase of the number of spikes participating in...
bursts and NB (Fig. 5f, Supplementary Fig. 8b,c). Since GABA in BENOs switched from excitatory to inhibitory between IS and LS, we compared other properties at these two stages. Although the burst and NB frequency was unchanged (0.1 Hz) the mean interspike interval (ISI) between burst or NB significantly decreased in LS BENOs to 20 ms and 2 ms, respectively (Fig. 5f). In addition, the NB duration significantly increased with time (Supplementary Fig. 8e). These data demonstrate accelerated formation of complex networks of excitatory and inhibitory neurons in BENOs.

**BENOs present electrically induced neuronal plasticity.** Since three of the five most significant GO groups upregulated in BENOs were associated with synaptic plasticity and long-term synaptic potentiation (GO:0048167; GO:0060291 GO:0048168, Supplementary Data 1), we next assessed BENO neuronal plasticity by investigating paired-pulse depression (PPD) as well as short- and long-term potentiation and depression (STP/D and LTD/P). To test for PPD, we stimulated LS BENOS by a bipolar electrode and observed calcium activity of neurons located 200 μm away from the stimulation electrode (Fig. 6a). Paired-pulse stimulation resulted in depression of calcium activity of every second pulse (Fig. 6b–d). Since one of the mechanisms underlying PPD observed in the hippocampus is mediated by presynaptic GABA release21, we repeated the stimulation under GABA-receptor blockade with PTX. PPD was alleviated by PTX and reappeared upon washout, providing evidence for presynaptic GABA release involvement in the observed PPD (Fig. 6b–d, Supplementary Fig. 9a, b depicts detailed traces from two independent BENOs). Another typical form of plasticity observed in the hippocampus and associated with learning is potentiation or depression of neuronal signal in response to HFS22. To test whether BENOs contain neurons which can demonstrate LTD/P, STP/D as signs for plasticity, 300 μm slices (from n = 6 BENOS) were placed on MEAs for monitoring of electrical activity (Supplementary Fig. 9c). BENOs were stimulated with single pulses (100 μA, 100 μs pulse width, 30 s inter-pulse interval) until signals were stable and then three pulses of HFS (60 μA, 100 Hz) were delivered. After HFS, electrical signals were recorded for 1 h. Electrodes with >15% signal decrease from baseline indicated potentiation; electrodes with >15% signal decrease from baseline indicated depression (representative traces in Fig. 6f). Evidence for potentiation and depression could be obtained in all investigated BENOs (n = 6; Fig. 6g), demonstrating that plasticity is a fundamental property of maturing BENOs.

**Discussion**

Until recently, studies on human brain development were limited to rare human fetal brain material23. Emerging organoid technologies provide human neuronal cell culture models, which recapitulate in some aspects embryonic brain with cortical layer development24. These 3-dimensional cultures typically rely on neural induction of pluripotent stem cell cultures, i.e., typically human embryonic or induced pluripotent stem cell cultures, and subsequent embedding of the differentiated neuronal progenitors in laminin-rich Matrigel1–3,3,6,8, which is well known for being supportive in neuronal cell culture25. Alternative cell aggregation models, referred to as cortical spheroids, are developed without the need for the addition of a matrix4,7,9. Organoid and spheroid models have been extensively studied as to cell content1–8 and directed to develop into distinct brain structures1–6. Electrophysiological analyses documented consistently that individual neurons in organoids/spheroids maintain their autonomous function7,8. Network formation, being essential for complex brain function, has not yet been characterized in detail in brain organoids, but appears to occur in some models after 9 months8. In a recent elegant spheroid study, network oscillations were demonstrated after 7 months9. Here, we demonstrate complex network function of the developing (GDP) and the more mature (LTP) human brain after 2 months of BENO culture. Evidence for plasticity underlying learning and memory are in our view particularly exciting as the basis for future exploitation of BENOs in drug development targeting the underlying mechanisms.

BENO development can be divided in four distinct stages: (1) d3–15 commitment to NPC, (2) d15–28 neurogenesis, (3) from d28 onwards neuronal maturation, which is (4) supported by gliogenesis from day 50 onwards. These stages appear to resemble fetal brain development, where neurogenesis precedes gliogenesis. During gliogenesis astrocytes, outer radial glia, and oligodendrocytes developed, with concomitant synaptic maturation, in line with in vivo data26. It is important to note that BENOs after d90 contained increasing numbers of myelinated axons comparable to published protocols designed to support cortical spheroid myelination27.

Synaptogenesis coincides during development with neuronal connectivity and network formation. To test the hypothesis that BENOs may serve as a model to study and understand neuronal network formation and maturation, we utilized calcium imaging and MEA analyses for longitudinal studies and found similar network activity dynamics in BENOs as reported for the fetal brain. Studies in mouse and human fetal brain, showed that a hallmark of neuronal network development is the presence of GDPs11. The TTX-sensitive GDPs are GABA and glutamate dependent11 and have been shown to be initiated by pioneer GABAergic somatostatin (SST) positive cells of the hippocampus known as hub neurons12. During fetal development GDP frequency initially increases, then decreases and then it ceases completely in early postnatal life13–15,28. Similar to the fetal brain, BENOs present TTX-sensitive GDP-like events, which are GABA and glutamate dependent. The GDP-like event frequency increases between day 25 and 40, but significantly decreases in later stages. After culture day 40, no GDP-like events were recorded. However, we cannot exclude that GDPs may still occur at very low frequency. BENO transcriptome profiling showed enrichment of hippocampal neuronal markers, including SST, between days 28 and 40. Future experiments based on genetic tracing of SSTpos neurons in combination with calcium imaging may help to identify the specific mechanisms underlying GDP, which includes the investigation whether GABApos SSTpos hub neurons12 initiate the GDP-like events. Although we did not systematically examine BENOs for parvalbumin positive GABAergic neurons, the transcriptomic data show a small increase at d60, thus suggesting that it is a matter of time for this population to emerge. A more systematic analysis, including single cell sequencing in d90 organoids should be performed in the future in order to fully appreciate the dynamics of BENO cellular complexity.

Another hallmark of neuronal network maturation is the switch of excitatory to inhibitory GABAergic neurotransmission29. Interestingly, the disappearance of GDP-like events in BENOs coincided with the switch of GABA from excitatory to inhibitory. In contrast to BENOs, 2D cultures of iPSC-derived forebrain neurons show GDP-like events at low frequency only after prolonged culture (d58 in 2D vs d22 in ES BENOs) and a peak of GDP activity by d70 in 2D culture (vs d35 in IS BENO culture). Reduction in GDP was reported after d100 in 2D (vs d40 LS BENOs)17. The 2D forebrain cultures contained few GABAergic neurons and thus their contribution to GDP-like events could not be demonstrated17. Although no GDP events were previously demonstrated in an organoid level, Sakaguchi et al. showed that 109d-old neurons (deriving from dissociated brain organoids) exhibit local calcium networks bursts80. They further revealed that
these areas contained both glutamatergic and GABAergic neurons. These data are in agreement with our findings and underline the importance of inhibitory neurons for comprehensive network formation. The expedited neuronal development in BENOs was further evidenced by the observation of features indicating network complexity after 2 months in culture, whereas similar features have been reported recently in other brain organoid models after 7 months in culture\(^9\). In both studies the network complexity increased in the presence of inhibitory GABAergic neurons (2 months in BENOs vs 6-10 months in cortical spheroids\(^9\)). Our
data, in line with the recent data from the Muotri group under-
score the requirement of inhibitory GABAergic neurotrans-
mission for neuronal network maturation in brain organoids.

Another hallmark of network function is neuronal plasticity. Our study identified phenomena associated with neuronal plas-
 ticity being present in BENOs, such as PPD and STP/D and LTP/
D. PPD, a typical form of plasticity observed in the hippocampus,
is explained by presynaptic mechanisms, such as neuro-
 transmitter depletion or postsynaptic mechanisms. In BENOs, the
observed PPD was mediated by postsynaptic inhibitory
GABAergic neurons since GABA-A receptor blockade could
completely abrogate this phenomenon. These data demonstrate
the amount of interneurons in plasticity and complex network
function. Interestingly, evolutionary studies have shown that the
importance of interneurons in plasticity and complex network
function are considered key caveats. Transcriptional and protein
levels in BENOs suggested the presence of glutamatergic,
GABAergic, catecholaminergic, and serotonergic neurons as well
as myelinating (oligodendrocytes) and non-myelinating (astro-
cytes) glia. BENOs traverse through distinct phases of develop-
ment with primitive network function (GDP) and more advanced
network function (PPD and LTP/D), which requires the interplay
of excitatory (glutamatergic) and inhibitory (GABAergic) neu-ons. Our data suggest that the use of brain organoids in de-
velopmental or drug discovery studies has to carefully consider their
developmental stage to address specific pathophysiological
relevant questions. The development of a natural cellular com-
position (e.g., excitatory and inhibitory neurons) and the defini-
tion of the stage of electrophysiological maturation (day 25 vs
50 in BENOs with little and consistent network activity, respectively)
are important factors when designing drug discovery experi-
ments, for example in the context of epilepsy.

Methods

**BENO generation (protocol 5).** The hMPS hiPSC line (TC1133, Lonza) and an in-
house generated line (hiPSC-G1) were used for BENO generation. The BENO differen-
tiation protocol was established using the hiPSC-G1 reference line. Immuno-
nfluorescence analyses and the functional data were replicated in both lines to test
for protocol robustness. One day prior to neuronal differentiation, a 1:1 mixture of
acid-solubilized bovine collagen I (Collagen Solutions) and serum free 2x DMEM
(Temecoscientific) was prepared and neutralized by 0.1 M NaOH. iPSCs were
suspended at 250 µl/ml in StemMACS™ iPS-Brew XF medium (Millipore, 20 ng/ml of
laminin), with 10 µg/ml of FGF-2 and 10 ng/ml of RA (Sigma). Y27632 (Stemgent). iPSCs
were added to the collagen/DMEM mixture to achieve a 1:1 ratio. Neuronal
differentiation medium was added every day for 7 days. Neuronal differentiation
medium consisted of StemMACS™ iPS-Brew XF supplemented with 10 ng/ml FGF and
10 ng/ml of Y27632 (Stemgent). iPSCs were added to the collagen/DMEM mixture to
achieve a 1:1 ratio.

**Calcium imaging.** For calcium analysis, whole BENOs were loaded for 15–30
min with 1 µg/ml Fluo-8-AM in carboxgenated artificial cerebral fluid (ACSF)
buffer (mM: 126 NaHCO3, 10 dextrose, 1 MgSO4·7H2O, 1.2 NaH2PO4, 2.5 KCl,
126 NaCl, 2 CaCl2) pH 7.3. Calcium imaging was performed on a confocal
microscope (Zeiss LSM 780 equipped with ZEN 2010 software), while BENOs were
continuously perfused with ACSF and the temperature was maintained at 37 °C.
Fluorescence was recorded at 2.5–5 Hz whilst electrical stimulation was given at a
fixed time with five pulses (50 µs, 50–100 µA) with ISIs 75 ms every 2 s. Action
potential mediated calcium activity was blocked by 1 µmol/L TTX (final concen-
tration) for 1 min and reperfusion with ACSF. Similarly, GABA-B receptors
were blocked by 330 µmol/L Saclofen (Tocris), GABA-A receptors were
blocked by 58 µmol/L Picrotoxin (PTX; Sigma), NMNDR by 0.2 µmol/L (+)MK-
801 hydrogen maleate (Sigma) and AMPAR by 15 µmol/L CNQX (Sigma). Activi-
ty of the GABAergic or the glutamatergic network was achieved by 10 µmol/L
GABA (Sigma) or 10 µmol/L Glutamatic acid (Sigma) respectively. Automated
synchronization detection was performed using Matlab R2012a (The Math
Works, USA).

**Electrophysiology analysis.** NA and K currents were recorded by conventional
whole-cell patch-clamping. BENOs were stained with Fluo-8-AM as described
above. Ca-activity was visualized with an upright microscope (Zeiss LSM 780
equipped with ZEN 2010 software). Patch pipettes (2–4 MΩ) were pulled from
borosilicate glass (1.6 mm outside diameter) and filled with intracellular solution
containing (in mmol/L): 135 HMeSO4, 10 KCl, 1 EGTA, 10 HEPES, 2 MgCl2, 2
ATP-Na, 0.1 GTP-Na, 6.6 Phosphocreatine-Na2, with pH adjusted to 7.2–7.4 by
KOH. Intracellular solution was adjusted to 290–295 mmol/L. Membrane
potentials were depolarized between –60 mV and +50 mV, in 10 mV increments,
from a holding potential of –70 mV. An EPC-9 patch-clamp amplifier equipped
with Patchmaster software (HEKA Electronics, Germany) was used for
data acquisition. Liquid-junction potentials, leak currents (Pin protocol), fast and
slow capacitances, and series resistances were corrected on-line. The data were
sampled at 20 kHz and filtered at 10 kHz (four-pole Bessel) and 5.9 kHz (three-
pole Bessel). Data were stored and exported to Matlab (The Math Works, USA)
for subsequent analyses.

**MEA recording.** Six-well plates with 64 platinum microelectrodes arrays per well
(MEA; 0.04 MΩ/microwire, 30 µm microelectrode diameter, 200 µm spacing)
were coated with Matrigel® 1:120 diluted in PBS for 1 h at room temperature prior
seeding. One or two slices (thickness: 300 µm) were placed into a MEA well and
immobilized by a concentrically coiled tungsten ring. 10–15 min recordings/2 h
were performed every day for up to 60 days using the AxioMoro MEA system
(Axon Biosystems). Data recordings were automatically scheduled with the Axio
Software (AxIS Navigator) using the manufacturer’s Spontaneous Neural
Configuration. Data analysis was performed using the manufacturer’s standalone tools,
Neural Metric Tool, and AxIS Metric Plotting Tool (Axion Biosystems). The
spike detecting threshold was set to 5.5 standard deviations, and the electrodes
that detected at least 5 spikes per min were classified as active. Spike bursts
were identified using an ISI threshold requiring a minimum number of five spikes with
a maximum ISI of 100 ms. NB were identified by an envelope algorithm with
a threshold of 1.25, minimum ISI interval of 100 ms, 75% burst inclusion with
a minimum of 10% active electrodes. Firing statistics were corrected for
the area under the normalized synchrogram cross-correlogram for a time window of
20 ms. For potentiation measurements, multi-channel fEPSP recordings were
performed with a MEA2100 device (MC_Rack 3.2.1.0 software, Multi Channel
Systems, Reutlingen, FRG). To reduce the background noise, the bath was
supplemented with an extra-custom-made Ag/AgCl electrode attached to the MEA
amplifier ground socket. LTG was induced by 3 trains of HFS (20 pulses 100 Hz, 50 µs, 100 µA) in a 20 s
interval. Prior to HFS, neurons were stimulated by single pulses (100 µA, 50 µs
pulse width, 30 s inter-pulse interval). After stimulation the same protocol
was performed for 1 h in order to quantify differences in synaptic strength.

**Wholemount immunofluorescence (WmIF).** BENOs were fixed with 4% for-
maldehyde solution (Histoxy, Carlrot) for 2 h at 4°C. Subsequently, they were
washed twice with PBS and blocked for 30 min at 4°C with staining buffer (SB; 3%
FBS, 1% BSA, 0.5% Triton X-100 in PBS). BENOs were incubated with primary
antibodies diluted in SB for 2 days at 4°C (100 µL/BENO). Upon washing with SB
for 6–8 h, BENOs were incubated with secondary antibodies and Hoechst 33342
(Sigma) for another 2 days at 4°C. After SB washings for a total of 6–8 h BENOs
were mounted on glass coverslips. An antibody list with respective dilutions is
published in a Supplementary Table. Calcium imaging performed on a Zeiss LSM 710
confocal microscope equipped with ZEN 2010 software.
RNA extraction and quantitative PCR. RNA was extracted from BENOs with the Nucleospin RNA isolation kit (Macherey-Nagel), according to the manufacturer’s instructions. Reverse transcription was performed using oligo(dT)~18 primer (Eurofins Genomics), dNTP mix and M-MLV reverse transcriptase (Promega). For qPCR analysis SYBR Green (Promega and Eurogentec) and a 7900 HT Fast real-time PCR system (Applied Biosystems) were used. qPCR data were collected and analyzed by SDS2.4 software (Applied Biosystems). Primer sequences are provided in Supplementary Data 3.

RNA sequencing. RNA was isolated using the Macherey-Nagel RNA isolation kit (cat. no. 740955). RNA integrity was verified by Agilent Bioanalyzer 2100. The cDNA library was prepared from 100 ng of total RNA, by TrueSeq Stranded Total RNA Sample Prep (Illumina) according to the manufacturer’s instructions. Briefly, total RNA was depleted from ribosomal RNA by magnetic bead separation. Ribodepleted RNA was fragmented and first strand synthesis was performed. Second strand synthesis was performed using dUTP so that at the PCR amplification step only the first strand was amplified. Prepared libraries were quantified using the Qubit High Sensitivity Assay (Invitrogen), the size distribution was controlled using Bioanalyzer 2100 and sequencing was performed using HiSeq2000 (SR 50 bp) according to the manufacturer’s instructions. Bead files were demultiplexed and converted to fastq using fastq2bcl. Fastq files were mapped using TopHat (v2.1.1) and fragments per kilobase of transcript per million (FPKM) calculated using Cufflinks (v2.2.1)38. Only protein coding transcripts were consiered for further analysis. Human genome annotation used was GRCh38.87. All genes with FPKM < 1 were omitted for the respective data sets for Fig. 2. Differential gene expression was performed using Cuffdiff39. GO analysis was performed done in R.

Statistical testing. All data are displayed as mean ± standard error of mean (SEM). The investigated sample number is provided as n. Statistical differences between two groups were tested by two-tailed unpaired Student’s t tests. In case of three or more groups, one-way or two-way ANOVA with appropriate post-hoc testing was performed. The performed statistical tests are specified in the respective figure legends. Statistical significance was assumed if p < 0.05. RNAseq data were corrected according to Benjamini and Hochberg42. First statistical analyses and graphical display of the data Graph Pad Prism (GraphPad Software) was used.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The gene array data sets generated in this work have been deposited in Gene Expression Omnibus under the accession number GSE139101. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 27 November 2019; Accepted: 2 July 2020; Published online: 29 July 2020

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Author contributions

M.P.Z. contributed to the conception, the design of the work; the acquisition, analysis, interpretation of functional data and revised the manuscript. J.E.H. contributed to early stages of the work. R.H. contributed to the acquisition, analysis, interpretation of RNA sequencing data, and drafted the associated method part. A.B. contributed to data acquisition and analysis. M.K-S contributed to the acquisition of immunofluorescence data. A.F. contributed to the transcriptome analysis and revised the manuscript. D.S. revised the manuscript. W.H.Z. contributed to the conception, the design of the work, drafted and revised the manuscript.

Competing interests

The University Medical Center Göttingen has filed a patent covering production and applications of the BENO technology (WO2018228948A1). M.P.Z. and W.H.Z are listed as inventors of the BENO technology, myriamed GmbH has licensed the BENO technology for applications in drug development. W.H.Z. is co-founder and equity holder of myriamed GmbH. The remaining authors declare no competing interests.

Acknowledgements

The authors would like to especially thank Reinhard Jahn and Laura Zehraian for the scientific discussions as well as Krasimiro Sharkova, Petra Tucholla, Chela Rocha, Kea Schmoll, and Lennart Schneider for the excellent technical assistance. M.P.Z., A.F., and W.H.Z. are supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under German's Excellence Strategy—EXC 2067/1-390729940. J.E.H. is funded by the NHMRC (National Health and Medical Research Council) of Australia. W.H.Z. is supported by the DZHK (German Center for Cardiovascular Research) and the German Research Foundation (DFG ZI 708/10-1, SFB 1002 C04, S01). A.F. is supported by the DZNE (German Center for Neurodegenerative Diseases), the ERC consolidator grant DEPIDCODE (648898), and the DFG (SFB1002 D04 and SFB1286 B06). Generation of the GMP line LiPSC-GR1.1 (also referred to as TC1133; lot number 50-001-21) was supported by the NIH Common Fund Regenerative Medicine Program, and reported in Stem Cell Reports. The NIH Common Fund and the National Center for Advancing Translational Sciences (NCATS) are joint stewards of the LiPSC-GR1.1 resource. We acknowledge support by the Open Access Publication Funds of the Göttingen University. All authors have approved the submitted version and agreed both to be personally accountable for the author’s own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Author contributions

M.P.Z. contributed to the conception, the design of the work; the acquisition, analysis, interpretation of data; and drafted the manuscript. G.B. contributed to the design of the work; the acquisition, analysis, interpretation of functional data and revised the manuscript. J.E.H. contributed to early stages of the work. R.H. contributed to the acquisition, analysis, interpretation of RNA sequencing data, and drafted the associated method part. A.B. contributed to data acquisition and analysis. M.K-S contributed to the acquisition of immunofluorescence data. A.F. contributed to the transcriptome analysis and revised the manuscript. D.S. revised the manuscript. W.H.Z. contributed to the conception, the design of the work, drafted and revised the manuscript.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17521-w.

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Peer review information Nature Communications thanks Mohammed Mostajo-Radj and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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