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Physiological Normoxia and Absence of EGF Is Required for the Long-Term Propagation of Anterior Neural Precursors from Human Pluripotent Cells

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Abstract

Widespread use of human pluripotent stem cells (hPSCs) to study neuronal physiology and function is hindered by the ongoing need for specialist expertise in converting hPSCs to neural precursor cells (NPCs). Here, we describe a new methodology to generate cryo-preserved hPSC-derived NPCs that retain an anterior identity and are propagatable long-term prior to terminal differentiation, thus abrogating regular de novo neuralization. Key to achieving passagable NPCs without loss of identity is the combination of both absence of EGF and propagation in physiological levels (3%) of O2. NPCs generated in this way display a stable long-term anterior forebrain identity and importantly retain developmental competence to patterning signals. Moreover, compared to NPCs maintained at ambient O2 (21%), they exhibit enhanced uniformity and speed of functional maturation, yielding both deep and upper layer cortical excitatory neurons. These neurons display multiple attributes including the capability to form functional synapses and undergo activity-dependent gene regulation. The platform described achieves long-term maintenance of anterior neural precursors that can give rise to forebrain neurones in abundance, enabling standardised functional studies of neural stem cell maintenance, lineage choice and neuronal functional maturation for neurodevelopmental research and disease-modelling.

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Introduction

Recent advances in human pluripotent stem cell (hPSC) research is rapidly leading to the development of humanised cell culture models of developmental and degenerative neurological disorders. Although several neural conversion methods are available to generate neural precursors (NPCs) from hPSCs, many existing protocols describing long-term propagation result in the deregulation of spatial identity and differentiation potential [1,2]. Efficient neural conversion of human PSCs, that mimics default mammalian neurogenesis, in defined conditions that limit extrinsic signaling cues is well established [1–5]. Human PSCs undergo distinct formation of radially organized columnar neuroepithelia called ‘neural rosettes’ during neural conversion from the pluripotent state [6]. These neural rosettes assume an obligate primitive anterior identity by default in chemically-defined medium (CDM) [7] and can give rise to glutamatergic forebrain neurones with dorsal telencephalic identity in the absence of known morphogens [8,9]. Building on these observations a variety of methods are developed to generate cortical neurones, all of which notably require de novo neural conversion of hPSCs [10–14]. However, the ability to derive and, critically, maintain long-term human NPCs of anterior identity that predictably generate physiologically functional cortical neurones has not been reported.

To circumvent the need for de novo neural differentiation for every new experiment a number of attempts have been made to capture and propagate defined neural precursor populations, mostly relying on epidermal growth factor (EGF) and fibroblast growth factor (FGF) as mitogens [15,16]. Even though these methods provide a homogeneous neurogenic precursor population, the initial spatial identity remains subject to deregulation in long-term culture. For instance, long-term self-renewing rosette-type human embryonic stem derived neural stem cells (h-hESNSCs) established from neural rosettes with expansion in FGF2 and EGF lose anterior identity, marked by the loss of OTX2 expression that is required for the specification of neural precursors to become telencephalic, and predominantly differentiate into GABAergic neurons [16,17]. In the embryonic mouse telencephalon FGF-responsive multi-potential, self-renewing NSCs emerge before EGF-responsive NSCs and distinct populations proliferate in response to these mitogens [18]. However, whether combined
EGF and FGF2 treatment is permissive for the propagation of human PSC-derived NPCs with anterior identity is not established. Noting the importance of physiologically-relevant, low-O2 levels (3%) for embryogenesis, particularly regulating stem cell survival, fate, proliferation, genomic stability and differentiation [19–23] we have previously shown that NPCs can be derived de novo from human embryonic stem cells (hESC) in CDM at 3% O2 that show tri-lineage differentiation potential and are responsive to patterning cues [24].

Here, we address the combined effects of EGF signaling and O2 tension on long-term stability and identity of human NPCs isolated from neural rosettes and report the derivation of anterior NPCs (aNPCs) that can be propagated long-term as a monolayer and cryo-preserved, thus eliminating the need for de novo neural conversion from PSCs. Critically, long-term propagation of aNPCs relies on physiological O2 levels (3%) for culture stability and the absence of EGF for the maintenance of anterior identity. aNPCs derived at 3% O2 give rise to both deep- and upper-layer cortical excitatory neurons upon terminal differentiation and also retain responsiveness to developmental patterning cues. Furthermore, compared to NPCs differentiated at ambient O2 (21%), neurons derived from aNPCs at physiological O2 levels display enhanced uniformity and speed of functional maturation. These findings enable reliable generation of scalable and stable NPCs with anterior identity from human PSCs that predictably generate phenotype-relevant functional neuronal subtypes necessary for modeling developmental and neurological disorders.

Materials and Methods

Generation and Cryopreservation of aNPCs

hESC line H9 was obtained from WiCell (Madison, WI) under full ethical/IRB approval of the University of Edinburgh. iPSC lines used in this study were either previously described [25] or derived with the ethics permission obtained from the NHS Lothian Research Ethics Committee (REC/10/S1103/10). Written informed consent was obtained from each individual participant. aNPCs were subsequently derived from parental pluripotent stem cell lines. Human ESCs and iPSCs (reprogrammed with pseudotyped retroviral vectors expressing coding sequences of genes OCT4, c-MYC, SOX2, KLF4) were maintained on CF-1 irradiated mouse embryonic fibroblasts, with Advanced DMEM/F12 (A-DMEM/F12), 20% Knockout Serum Replacement, 10 ng/mL basic FGF2, 1 mL L-glutamine, 100 mM 2-mercaptoethanol and 1% penicillin/streptomycin (P/S). Human PSCs were neurally converted in suspension in CDM as described in [26]. The media was changed to base media (A-DMEM/F12, 1% P/S, 1% Glutamax, 1% N2), 0.4% B27, 2.5 ng/mL FGF2 upon observation of radially organised structures in neurospheres (10–21 days) and plated on Laminin (Sigma) coated tissue culture plates (Nunc) a week later. Neural rosettes were mechanically isolated, dissociated with Accutase (Sigma) and 20–40 k cells were plated in one Laminin-coated well of a 96-well plate in proliferation media (Base media, 0.1% B27, 10 ng/mL FGF2 and 10 ng/mL EGF where stated). aNPCs were grown to high density before passing 1:2 with Accutase on laminin coated plates until passage 5–6 and maintained on 1:100 Reduced-growth factor Matrigel (BD Biosciences). aNPCs were re-suspended in proliferation media supplemented with 10% DMSO (Sigma). Cryovials were placed in a CoolCell cryovial were re-suspended in proliferation media with 0.3 mM RA and then plated down on poly-D-lysine (Sigma), laminin (Sigma) and fibronectin (Sigma) coated coverslips in Neurobasal, 0.1 µM RA, 2 µM Purmorphamine (Calbiochem), 1% N2, 1% P/S, 1% Glutamax, 5 mM bFGF and 21% CO2 for 7–10 days to generate motor neuron precursors. Motor neuron precursors were replated and media was gradually switched to Neurobasal, 0.5% N2, 0.2% B27, 1% P/S, 0.5% Glutamax, 10 ng/ml BDNF, 10 ng/ml GDNF.

PCR & qRT-PCR

RNA was isolated using the RNeasy kit (Qiagen) and genomic DNA was removed with the DNA-Free kit (Ambion). cDNA was synthesised using 0.5 µg total RNA with the DyNAme™ cDNA Synthesis Kit (Thermo). Technical replicates as well as no template and no RT negative controls were included and at least three biological replicates were studied in each case. Real-time quantitative PCR reactions were set up with DyNAmo ColorFlash SYBR Green qPCR kit (Thermo) or TaqMan Universal PCR Master Mix (Applied Biosystems) and run on a CFX96 System (BioRad) or 7300 Real Time PCR System (Applied Biosystems). The data were analysed using the iCycler software (BioRad) or the MxPro QPCR analysis software (Stratagene) and the qbase PLUS software (Biogazelle) for statistical comparisons. Primer sequences are provided in Table S1. Human foetal brain total RNA (21 weeks old) sample was purchased from Stratagene. BDNF induction was assessed by qRT-PCR after membrane depolarization of five week old neurons with 25 mM KCl and 5 mM CaCl2. Induction was assessed by qRT-PCR after membrane depolarization of five week old neurons with 25 mM KCl and 5 mM CaCl2.

Immunofluorescence

Cells were fixed with 4% PFA (+0.1% glutaraldehyde for Rb staining), permeabilised with 0.2% Triton X-100 at room temperature, and then blocked in 3% goat or donkey serum, followed by incubation with primary (Table S2) and secondary antibodies (Alexa Fluors, Invitrogen). The nuclei were counterstained with 80 ng/mL Hoechst 33342 (Sigma) and 40 ng/mL DAPI staining (Sigma) and the images were processed and using Axiovision v. 4.8.1 (Zeiss). Fields based on uniform DAPI staining were selected and imaged in four channels for cell counts between weeks 3–5. On average more than 400 cells were analysed for each marker from at least three independent experiments. Cryosectioning of neurospheres was performed as previously described [26].
Karyotyping

Standard G-banding chromosome analysis was performed by the Addenbrooke’s Hospital, Cytogenetics Unit, Cambridge, UK.

Calcium Imaging

Ca²⁺ imaging was performed as described [27] at 37°C in aCSF (in mM; 150 NaCl, 3 KCl, 10 HEPES, 2 CaCl₂, 1 MgCl₂, 1 glucose, pH 7.2). Briefly, cells were loaded with Fluo-3 AM (11 μM; from a stock solution of Fluo-3 (2.2 mM) dissolved in anhydrous DMSO containing 20% (v/v) Pluronic detergent) for 30 min at 37°C. Fluo-3 fluorescence images (excitation 472±15 nm, emission 520±15 nm) were taken at one frame per 5 s using a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. Cells were depolarized using an elevated K⁺ solution (in mM; HEPES 10, KCl 170, MgCl₂ 1 and CaCl₂ 2, pH 7.2) added to the medium to achieve a final [K⁺] of 50 mM and the L-type VGCC agonist FPL 64176 (5 μM). To calibrate images, Fluo-3 was saturated by adding ionomycin (50 μM) to the perfusion chamber (to obtain Fmax) and quenched with MnCl₂ (10 mM)+ionomycin (50 μM) to levels corresponding to 100 nM Ca²⁺ [28], which was in turn used to calculate Fmax. Free Ca²⁺ concentrations were calculated from fluorescence signal (F) according to the equation [Ca²⁺] = Kd(F – F_min)/F_max – F), and expressed as a multiple of the Kd of Fluo-3 (which is approximately 315 nM). Approximately 350 cells were analysed expressed as a multiple of the Kd of Fluo-3 (which is approximately 315 nM). Approximately 350 cells were analysed within 7 independent experiments.

Electrophysiology

The whole-cell patch-clamp configuration was used to record macroscopic currents from human ES-cell derived cortical neurones using an Axon Multiclamp 700B amplifier ( Molecular Devices, Union City, CA). Patch electrodes were filled with a solution comprising (in mM): NaCl 132, KCl 155, MgCl₂ 2, Na-HEPES 10, Na-PG-creatine 10, Mg-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MO. Coverslips containing cultured cortical neurones were placed in the recording chamber, which was super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, Na-PiCreatine 10, Mg₂-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MΩ. Coverslips containing cultured cortical neurones were placed in the recording chamber, which was super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, Na-PiCreatine 10, Mg₂-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MΩ. Coverslips containing cultured cortical neurones were placed in the recording chamber, which was super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, Na-PiCreatine 10, Mg₂-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MΩ. Coverslips containing cultured cortical neurones were placed in the recording chamber, which was super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, Na-PiCreatine 10, Mg₂-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MΩ. Coverslips containing cultured cortical neurones were placed in the recording chamber, which was super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, Na-PiCreatine 10, Mg₂-ATP 2 and Na₃-GTP 0.3, pH 7.3 (300 mOsm) and possessed resistances of 4–7 MΩ.

To address the effects of mitogens FGF2 and EGF (Fig. 1A) on the regional identity of long-term propagated NPCs, studies were first undertaken on the H9 hESC line and subsequently replicated on multiple independently derived human induced PSC lines (hiPSCs). H9 hESCs were first neurally converted under substrate-free conditions in CDM at 21% O₂ as described previously [24], kept in suspension culture until internal neural- rosette like structures began to form from 7–14 days in culture and were either processed for cryosectioning to determine cellular composition or plated onto laminin-coated substrate prior to mechanical isolation of neural rosettes (Fig. S1A). Immunofluorescence analysis of these 3D neural aggregates revealed radially organized cells around a central lumen that were positive for the telencephalic transcription factor FOXG1 [29] in agreement with previous studies showing self-organisation of cortical tissues from hPSCs (Fig. 1B) [11,13,16]. Cells within the radially-organised structures also expressed early neuroepithelial markers PAX6, NESTIN and OTX2, a homeodomain transcription factor expressed in the anterior neuroectoderm [30] (Fig. 1C and Fig. S1B). Propagation of neural rosette-derived NPCs in the presence of EGF and FGF2 at 21% O₂ has been previously reported, termed long-term self-renewing neuroepithelial-like stem cells [lt-NES cells] [16,17]. lt-NES cells in culture progressively lose the initial anterior identity marked by loss of OTX2 expression, assume a transcriptional factor expression profile consistent with hindbrain identity and generate predominantly GABAergic neurons upon differentiation. Therefore, EGF and FGF2 propagation of rosette-derived NPCs at 21% O₂ was not investigated further in this study. To address the effect of EGF and FGF signaling on anNPCs propagated at 3% O₂, noting widespread expression of EGFR on neural- rosette like columnar cells (Fig. 1D), isolated neural- rosettes were enzymatically dissociated and cultured either in EGF/FGF2 or EGF/FGF2 containing conditions. No difference in growth rates of anNPCs in FGF2 or EGF/FGF2/PD treatment was observed (Fig. 1E). To confirm that regulation of anterior identity was dependent on EGF signaling, early passage anNPCs established with FGF2 were swapped into EGF/FGF2 or EGF/FGF2 treatment with a selective inhibitor of EGFR tyrosine kinase activity PD168393 (EGF/FGF2/PD) and propagated for five passages. Analysis by qRT-PCR revealed that FGF2 or EGF/FGF2/PD treatment significantly down regulated OTX2 by passage 15, whilst the levels of neural progenitor marker NESTIN were unaffected (Fig. 1F). To confirm that deregulation of anterior identity was dependent on EGF signaling, early passage anNPCs established with FGF2 were swapped into EGF/FGF2 or EGF/FGF2/PD treatment with a selective inhibitor of EGFR tyrosine kinase activity PD168393 (EGF/FGF2/PD) and propagated for five passages. Analysis by qRT-PCR revealed that FGF2 or EGF/FGF2/PD treatment significantly down regulated OTX2 by passage 15, whilst the levels of neural progenitor marker NESTIN were unaffected (Fig. 1F).

FGF2-propagated anNPCs Maintain a Long-term Anterior Identity When Propagated in 3% O₂

anNPCs established in the presence of FGF2 showed complete loss of pluripotency markers NANOG and OCT4 by passage 5 and continued to express high levels of OTX2 up to passage 30 (Fig. 2A). anNPCs propagated long-term as a monolayer displayed rosette-like patterns in culture and quantitative immunofluorescence analysis revealed that proliferating cells uniformly expressed NESTIN with high and low levels of OTX2 expression (84.6±6.4%; n = 4 independent derivations, passage >20; regard-

Statistical Analysis

Data are presented as mean ± s.e.m. Statistical analysis for parametric data was conducted using, as appropriate, unpaired t-test and one-way analysis of variance (ANOVA) with the post hoc Tukey’s test. For non-parametric data, ANOVA analysis was performed using the Kruskal-Wallis test with the post hoc Dunn’s test.
FGF2-derived Early Passage aNPCs Propagated in Either 21% or 3% O2 Have Comparable Regional Identity but give Rise to Neurons with Different Functional Potential

Routinely, human PSC-derived NPCs are propagated only a few passages in order to increase their yield before differentiation. Passage 5 was chosen as FGF2-only treated aNPCs grown at 21% O2 displayed spontaneous differentiation, morphological changes and notable cell death beyond passage 6–7, and indeed only one of six derivations was maintained to passage 10 (Fig. 3A). For aNPCs propagated in 3% O2 the expression of anterior markers OTX1, OTX2, and LHX2 were comparable (Fig. 3B) however these aNPCs had significantly higher levels of VEGF expression as expected [23] compared to 21%. Furthermore at 4 weeks post differentiation, both 3% and 21% populations gave rise to similar proportions of neurons positive for cortical deep-layer marker, CTIP2 (29.6 ± 1.9% versus 30.7 ± 2.5%, respectively, Fig. 3C), suggesting comparable differentiation potential.

To determine whether O2 levels altered the functional potential of NPCs we next compared the maturation profile of paired passage 5 cultures propagated at 3% or 21% O2. Functional properties of 21% vs 3% O2-derived neurons differentiated from passage five FGF2-derived aNPCs concurrently generated from three PSC batches were investigated. The activity of week 5 neurons was assessed by their ability to fire action potentials. Neurons were classified as being ‘active’ if they were able to either fire single or trains of APs in response to depolarising current injection. For all batches, 21% O2-derived neurons were significantly less active than those derived in 3% O2 (Fig. 3D–F). Importantly, neurones differentiated from hiPSC-derived aNPCs in 3% O2 also showed robust AP firing (data not shown). Neuronal development and adaptive functions also require activity-dependent gene regulation and thus we investigated the transcriptional activation of the BDNF gene [31] by KCl-induced membrane depolarization. 3% O2-derived neurons had ~2-fold higher transcriptional induction of the BDNF exon IV compared to 21% O2 counterparts (Fig. 3G). Collectively, these observations suggest that 21% O2 is not permissive to long-term propagation of aNPCs under current culture conditions and derivation of neurons at physiological O2 also provides a functional improvement compared to 21% O2.

FGF2-propagated aNPCs in 3% O2 Generate Cortical Neurons and Retain Developmental Competence to Patterning Cues

Having established that aNPCs propagated with FGF2 at 3% O2 maintain a stable anterior identity we next assessed the telencephalic marker profile of aNPC-derived neurons together with their developmental competence to patterning cues. Following withdrawal of FGF2 and subsequent culture in differentiation medium the expression of EAIX2, a transcription factor essential for the specification of cortical neuroblasts and the formation of
Figure 2. Characterisation of aNPCs in long-term culture. (A): Expression of pluripotency markers NANOG and OCT4 are not detectable by RT-PCR in 3% O2 aNPCs while anterior neuroectoderm marker OTX2 expression is maintained (p:passage). (B): Proliferating aNPCs display uniform NESTIN expression and mosaic OTX2 expression (scale bar 50 μm). (C): Immunohistochemical staining against p75 (green), NESTIN (red) and DNA (blue) in proliferating 3% O2 aNPCs (scale bar 20 μm). (D): RT-PCR analysis of rostral markers DACH1, LH2X, OTX1, OTX2 and caudal markers HOX2, HOXB4 and HOXC4 in passage 15 and 30 aNPCs maintained in FGF compared to isolated neural-rosettes (Ros) or RA-patterned aNPCs (cont), respectively. (E): Representative chromosome analysis of a H9 hESC-derived aNPC line (passage 25) by G-banding showed that long-term propagating NPCs maintained a normal karyotype. doi:10.1371/journal.pone.0085932.g002

Figure 3. Cortical neurons derived at 3% O2 display uniform and enhanced functional maturation. (A): Phase-contrast images of aNPCs derived from the same rosette-isolation, propagated in FGF at 21% and 3%. aNPCs proliferated at 21% O2 with FGF2 show rosette-like morphology at early passages (21% O2 p5) but display increased differentiation and altered morphology with successive passaging (21% O2 p10). aNPCs propagated at 3% O2 with FGF2 show stable cellular morphology (3% O2 p10). Scale bars 200 μm. (B): Comparison of OTX1, OTX2, LH2X and VEGF relative expression levels between 3%- and 21%-O2 derived aNPCs at passage 5 as determined by qRT-PCR, n = 4, * P < 0.05, ns: non-significant, unpaired t-test. (C): Quantitative immunohistochemical analysis of CTIP2 expression after four weeks of differentiation of aNPCs derived at 3%- and 21%-O2 at passage 5. (D): Example current-clamp recordings of activity induced by a depolarising current pulse (+30 pA) from a potential of –74 mV. From left to right, the categorised responses depict; (top panels) no response, failed initialisation; (bottom panels) single AP, train of APs. (E): Bar graph showing the cumulative distribution of activity response of 3%- and 21%-O2 aNPC-derived week 5 neurons from three independent de novo aNPC derivations. (F): Bar graph summarising mean (± s.e.m.) percentage of active 3% and 21% O2 aNPC-derived week 5 neurons per de novo batch of aNPC paired derivations (n = 3 batches; P < 0.05; unpaired t-test). Mean input resistance measurements were not different between conditions, but a difference (p < 0.05) in whole-cell capacitance was observed (21%: 12.8 pF vs 3%; 16.3 pF). (G): Comparison of BDNF exon IV transcription induction between 5 week old neurons differentiated at 3%- and 21%-O2 in response to membrane depolarisation with K+ in the presence of FPL 64176 (5 μM) as determined of qRT-PCR. Expression is normalised to β-ACTIN and fold induction normalised to untreated respective control cultures is shown (n = 3, P < 0.05, unpaired t-test). doi:10.1371/journal.pone.0085932.g003
the dorsal telencephalon [32], was robustly upregulated in a time-
dependent fashion (Fig. 4A). At day 6 of differentiation, cultures
expressed high levels of dorsal telencephalic markers OTX1, PAX6
and EAH2 whereas the expression of ventral telencephalic gene
Nkx2.1 was not detected (Fig. 4B), suggesting that aNPCs assume
a dorsal identity by default. Over the course of 5 weeks aNPCs
generated neurons that were positive for RELN and CTIP2
[33] and upper-layer markers CUX1, BRN2 and SATB2 [34]
(Fig. 4 C–H). The differentiated cultures were highly enriched for
neurons (86.7±3.6%, β-3 tubulin) with less than 10% GFAP+
arborex staining (Fig. 4I and Fig. S1C). The neuronal
population displayed cortical marker expression of CTIP2+ 31.9±2.4%,
CUX1+ 38±4.3%, BRN2+ 34±1.4%, and SATB2+ 18.6±2.7% (Fig. 4I; n = 3–4 independent aNPC derivations,
started from cryo-preserved stocks between passages 20–30).
Importantly, hiPSCs neuralised in CDM also gave rise to
OTX2+ (71.2±4.9%) aNPCs that could be propagated long-term
with FGF2 at 3% O2 and that differentiated to cortical neurons by
default (CTIP2+ 29.9±2.5%, SATB2+ 18.9±1.9%, GFAP+
10.1±3.1%, n = 3 independent iPSC lines, passages 10–28,
Fig. 4I and Fig. S2 A–D).

Early rosette stage cells display a broad differentiation potential
and can be patterned to generate different neuronal subtypes
[3,35]. Previous studies have shown that the developmental
competence of NPCs to patterning signals is temporally deter-
mined with late or long-term propagated NPCs losing the ability
to respond predictably to morphogens [1,15,36]. Treatment of long-
term aNPCs with motor neuron-inducing signals retinoic acid
(RA) and puromorphamine upregulated HOXB4 expression and
resulted in subsequent expression of OLIG2, ISL1 and HB9,
indicators of motor neuron induction (Fig. 4J). Critically,
quantitative immunofluorescence analysis revealed that early
and late passage aNPCs had similar OLIG2 induction efficiencies
indicating that patterning potential is maintained in long-term
culture (Fig. 4K; passage 5 vs 25). Similarly, hiPSC-derived lines
also displayed predictable responsiveness to patterning cues RA
and puromorphamine (Fig. S2F).

Functional Characterization of Cortical Neurons
Differentiated from 3% O2 FGF2-propagated aNPCs

Week 5 aNPC-derived neurons were subjected to Ca2+ imaging
before and during treatment with elevated K+ in the presence of
the L-type voltage-gated Ca2+ channel agonist FPL64176 which
promotes strong Ca2+ influx in forebrain neuronal cultures [37].
Treatment with K+ +FPL64176 resulted in a uniformly strong
increase in [Ca2+] (Fig. 5A and Fig. S3A) that also resulted in
robust transcriptional upregulation of the immediate early gene
FOS, the prototypical activity-dependent gene (Fig. 5B) [38].

The passive membrane properties of the 3% O2-derived neurons were consistent with, and indicative of, cells undergoing
developmental maturation (Fig. S4 A–C). This maturing profile
was confirmed by the fact that at week 5, >95% of cells fired APs
(Fig. 5C) and the average number of APs spikes fired increased
significantly from week 1 to week 5 (Fig. S4D). More detailed
analysis was made from ‘active’ neurons and, inherently consistent
with what would be expected of a population of developing
cortical neurons, these cells showed expected changes in their AP
firing threshold potential, AP amplitude, half-width and after-
hyperpolarisation (Fig. S4 E–I). Some cells could also maintain
their ability to repeatedly fire APs for more prolonged durations
(Fig. 5E). AP activity and properties are ultimately determined by
the co-ordinated activity of multiple voltage-gated ion channels
and therefore we assessed the maturation of current density
profiles of NaV1.6, Iκ, I1 (Fig. 5D), which showed good correlation
with regard to AP development (see also Text S1 and Fig. S4 J–L).
Collectively, these properties demonstrate the fidelity of the 3% O2
protocol to derive neurons with functionally consistent properties
that are comparable with those from native cortical excitatory
neurons and other human PSC-derived forebrain neurons [9,39].

3% O2 aNPC-derived Cortical Neurons form Functional
Excitatory Synapses

Whole-cell voltage-clamp recordings revealed the presence of
functional NMDA, AMPA, and GABA receptors in accordance
with native mammalian cortical neurons (Fig. 6A). The neuro-
transmitter subtype identity of these aNPC-derived cortical
neurons was assessed after 5 weeks of differentiation by
immunofluorescence and revealed that the majority of neurites
displayed extensive punctate staining for vesicular glutamate
transporter 1 (VGLUT1) (Fig. 6B) but only a small fraction of
neurons were positive for GABAergic interneuron marker
GAD65/67 (3.5±0.4%, n = 4) (Fig. S3B), consistent with a
predominant glutamatergic neurotransmitter profile. Such a
VGLUT1 profile was also recapitulated in hiPSCs lines (Fig.
S3E). The apposition of post-synaptic density protein (PSD-95)
and synaptophysin I (SYN) in processes confirmed synaptic
differentiation (Fig. 6C and D). The existence of functional
synapses was confirmed by the presence of AMPA receptor-
mediated miniature excitatory postsynaptic currents (mEPSCs;
Fig. 6E) in 40% of cells (19 from 47) albeit with variable mEPSC
event frequencies. This data indicate that synaptogenesis is clearly
present within the culture, and at levels in accordance with other
reports [9]. Collectively these data suggest that long-term
propagated aNPCs generate enriched glutamatergic neuronal
populations that form functional excitatory synapses. Neurons
differentiated from hiPSC-derived aNPCs exhibited AMPA,
NMDA and GABA-mediated currents in addition to mEPSCs
(data not shown).

Discussion

In the present study we have demonstrated that the removal of
EGF and use of physiological O2 levels permits maintenance of
NPCs with anterior identity. This enables long-term propagation
of aNPCs as a monolayer that can be cryo-preserved and
differentiated to highly enriched neuronal populations composed
of both deep- and upper-layer cortical excitatory neurons. In
addition, 3% O2-derived neurons display more uniform, predict-
able and accelerated functional development profile compared to
21% O2 counterparts.

We identified two factors to be critical for the maintenance of
aNPCs derived from hiPSCs: physiological O2 levels for long-term
propagation and the omission of EGF to maintain anterior
identity. Oxygen levels have been shown to regulate survival,
proliferation and neuronal fate of both rodent and human NSCs
as well as limiting their precocious differentiation [19,21,26,41].
Clonal analysis of mouse cortical NSCs have revealed that culture
at 21% O2 leads to rapid depletion of multipotential NSCs
whereas expansion at 5% O2 permits long-term maintenance of
distinct NSC populations [20]. The classical view of reactive
oxygen species only in the context of cellular toxicity has recently
been challenged by a number of studies demonstrating the
regulation of processes such as DNA repair and NSC self-renewal
by redox signaling [22,42]. Therefore, the culture conditions for
NPCs should also take into account the concept of ‘oxidative optimun’,
where redox signalling present at physiological O2 levels is
actively integrated with other intracellular signaling cascades
such as PI3K/Akt in the maintenance of self-renewing NPCs [22,42]. The derivation of self-renewing NPCs with a stable identity from hPSCs is further complicated by the heterogenous nature of NPCs that emerge from neural conversion and subsequent expansion with mitogens. EGF and FGF2 are the most frequently used growth factors for the propagation of embryonic forebrain NPCs in vitro [5,15,40,43,44]. The expression patterns of FGFR1 and EGFR, the key receptors for FGF and EGF signaling, in the developing rodent and human CNS germinal zones is conserved with FGFR1 being expressed earlier than EGFR in the developing telencephalon [18,45,46]. Several lines of evidence suggest that more than one type of neural stem cell exist in the developing cortex and these cells display differential response to FGF2 and EGF mediated signaling in a dose- and context-dependent manner. Specifically, EGF signaling can alter NSC identity and differentiation potential. For instance, over-activation of EGF signaling in vivo biases cortical progenitors to astrocyte lineage and reduces NSC self-renewal in the adult SVZ whereas in vitro propagation of NSCs with EGF yields more glia than FGF [18,47–52]. Accumulating evidence suggests that the homeobox gene Otx2 lies at the intersection of pluripotent stem cell maintenance and anterior neuroectoderm commitment and differentiation. Otx2 is proposed to be an intrinsic determinant of embryonic stem cell state, is expressed in the epiblast as well as ESCs and is also required in the anterior neuroectoderm to induce telencephalic gene expression for forebrain specification [30,53]. Independent of the method of derivation, PSC-derived NPCs propagated in EGF and FGF2 lose this inherent OTX2 expression in culture over time, consistent with the loss of anterior identity [16,17,54]. We have shown that NPCs derived from hPSCs express EGFR as early as neural-rosette stage and expansion in EGF leads to
downregulation of OTX2 expression over successive passages. The ability to propagate aNPCs with FGF2 only as described here should facilitate future studies to delineate the effects of different signaling pathways on neural progenitor identity and potentially enable the isolation of NPC populations with different differentiation potentials.

Anterior NPCs maintained at physiological O2 levels with FGF2 alone assume dorsal telencephalic identity by default upon differentiation, generating VGLUT1+ excitatory cortical neurons, including CTIP2+ layer 6–5 subcortical projection neurons [33], BRN2+ layer 2–4 neurons [34] and SATB2+ layer 2–4 callosal projection and upper-layer neurons [55,56], in contrast to other propagated NPCs that show deregulation of positional identity and assume a GABAergic fate [15,16,36]. The differentiation output of 3% O2 propagated aNPCs is also stable through serial passaging and cryopreservation as demonstrated by comparable numbers of CTIP2+ neurons generated from passage 5 aNPCs and late passage (20–30) aNPCs recovered from cryopreservation (30% vs 32%, respectively). The system described here not only achieves the long-term maintenance of bankable anterior neuronal precursors from hPSCs including iPSCs but also presents a significant functional improvement over conventional methods, yielding uniformly active cortical neuronal populations that display activity-dependent gene regulation, basic neuronal physiology consistent with native cortical neurons and spontaneous synaptic activity by 5 weeks of differentiation.

The culture of neurons at ambient O2 levels is a significant departure from that of the brain, which range from 1–5% O2 [57]. Physiological O2 levels is shown to improve neuronal survival [24,58–60] and modulate neuronal metabolism [61,62]. Neurons are particularly vulnerable to oxidative stress due to their high metabolic rate, relatively low levels of antioxidant enzymes and being post-mitotic cells more vulnerable to accumulation of reactive oxygen species [63]. This is of particular importance as redox signalling and oxidative stress are thought to play an important part in neuronal injury in a range of developmental and disease conditions.

Figure 5. Functional characterization of cortical neurons differentiated from 3% O2 FGF2-propagated aNPCs. (A): 3% O2 aNPC-derived neurons were subjected to Fluo-3 Ca2+ imaging before and during treatment with elevated K+ (50 mM final) in the presence of FPL 64176 (5 μM). For each cell, the fold-increase in cytoplasmic Ca2+ concentration was calculated (n = approximately 350 cells from n = 7 independent differentiations; passages 10–20). (B): FOS mRNA fold induction in 3% O2 aNPC-derived neurons in response to membrane depolarisation as determined by qRT-PCR. Expression is normalised to GAPDH, n = 7. (C): Development of induced AP activity in 3% O2 aNPC-derived neurons over 5 weeks (n = 61 from 5 de novo derivations). (D): Developmental increase in current density of voltage-gated ion channels (NaV, I<sub>K</sub>, I<sub>A</sub>) in 3% O2 aNPC-derived neurons. All current density values for each ion channel at week 3 are significantly higher (significance not indicated for clarity) than week 1 (P<0.001; Kruskal-Wallis test with post hoc Dunn’s test; n = 19–30 for each week, from 3 de novo derivations). (E): Current-clamp recording of a 3% O2-derived neuron that exhibited sustained repetitive firing at a holding potential of −45 mV. doi:10.1371/journal.pone.0085932.g005
along with the capacity to generate excitatory glutamatergic cortical neurons [16,17]. Radial-glia like NS cells were also previously established from mouse ES cells and adult and foetal forebrain in adherent culture conditions with FGF2/EGF [15,40]. It is important to note that derivation of NS cells require both EGF and FGF2; cultures established in FGF2 alone are prone to spontaneous differentiation, have heterogeneous morphology and display increased cell death [40]. In contrast, aNPCs propagated at 3% O₂ with FGF2 as described in this study display a stable anterior identity in culture and assume a dorsal transcriptional identity upon differentiation, giving rise to uniformly active, enriched cortical excitatory neuronal populations. Hence, aNPCs present a platform that not only reduces variation and experimental noise inherent to de novo differentiation runs from human PSCs but is also suitable for scalable functional, biochemical and imaging-based high-throughput studies.

**Conclusion**

Our report of a robust method to generate stable, scalable and cryopreservable aNPCs that reliably generates neurons with functional properties consistent with native cortical excitatory neurons establishes a platform for human neurological disease modelling.

**Supporting Information**

**Figure S1** Characterisation of 3% O₂ aNPCs. (A) Human PSCs were neuronalised at 21% O₂ in suspension in CDM and plated down for the mechanical isolation of neural rosettes. For each experiment several neural clusters were collected, dissociated into single cells and split into different conditions for pair-wise comparison (scale bar: 400 μm). (B) Radially-organised neuroepithelium express PAX6 as determined by immunofluorescence analysis of neurosphere cryosections before platedown (scale bar: 20 μm). (C) aNPCs differentiated for 5 weeks contain GFAP+ (red) and S100β (red) astrocytes. β-3 tubulin immunohistochemistry is shown in green and DNA is counter-stained with DAPI (blue) (scale bars are 20 μm).

**Figure S2** Generation of aNPCs from human iPSCs. (A) Neural rosettes derived from human iPSCs give rise to aNPCs that can maintain anterior marker expression OTX1, OTX2 and LHX2 in extended culture as determined by RT-PCR (ppassage). (B) Immunofluorescence analysis of OTX2 and RESTIN expression in proliferating iPSC-derived aNPCs. Immunohistochemical staining against CTIP2, SATB2, VGLUT1 and β-3 tubulin revealed that human iPSC-derived aNPCs can give rise to glutamatergic cortical neurons by default differentiation (C-E). (F) iPSC-derived aNPCs upregulate OLIG2 expression in response to patterning with RA and SHH agonist purmorphamine.

**Figure S3** Characterisation of 3% O₂ aNPCs. (A) Example experiment showing Fluo-3 Ca²⁺ imaging upon membrane depolarisation of a single 3% O₂ derived cortical neuronal culture. The mean ± s.e.m. of cytoplasmic Ca²⁺ concentration is shown, expressed as a multiple of the Kd ([F-Fmin]/[Fmax-F], n ≥ 50). (B) aNPC-derived cortical neuronal cultures are occasionally positive for GAD65/67+ (green) neurons. β-3 tubulin immunohistochemistry is shown in red and DNA is counter-stained with DAPI (blue).

**Figure S4** (A–C) Mean ± s.e.m. whole-cell capacitance (C_w), resting membrane potential (RMP) and input...
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