

ORIGINAL ARTICLE

Differentiation from human pluripotent stem cells of cortical neurons of the superficial layers amenable to psychiatric disease modeling and high-throughput drug screening

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Cortical neurons of the superficial layers (II-IV) represent a pivotal neuronal population involved in the higher cognitive functions of the human and are particularly affected by psychiatric diseases with developmental manifestations such as schizophrenia and autism. Differentiation protocols of human pluripotent stem cells (PSC) into cortical neurons have been achieved, opening the way to *in vitro* modeling of neuropsychiatric diseases. However, these protocols commonly result in the asynchronous production of neurons typical for the different layers of the cortex within an extended period of culture, thus precluding the analysis of specific subtypes of neurons in a standardized manner. Addressing this issue, we have successfully captured a stable population of self-renewing late cortical progenitors (LCPs) that synchronously and massively differentiate into glutamatergic cortical neurons of the upper layers. The short time course of differentiation into neurons of these progenitors has made them amenable to high-throughput assays. This has allowed us to analyze the capability of LCPs at differentiating into post mitotic neurons as well as extending and branching neurites in response to a collection of selected bioactive molecules. LCPs and cortical neurons of the upper layers were successfully produced from patient-derived-induced PSC, indicating that this system enables functional studies of individual-specific cortical neurons *ex vivo* for disease modeling and therapeutic purposes.

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INTRODUCTION

Advances on the molecular understanding of psychiatric diseases have long been challenged by the limited access to live human neurons with a genetic background associated with the disease state. For obvious reasons, biopsies of neuronal tissues in affected patients are not feasible and do not allow the full *in vitro* recapitulation of key events involved in the initiation and progression of the disease. In addition, high-throughput screening of potential therapeutic compounds (HTS), which would greatly enhance the rate of drug discovery, is compromised by the fact that live human neurons are post mitotic cells that cannot be amplified to reach the amount of biological resources needed for such of large screening campaigns. Therefore, disease-affected neurons have to be produced on demand from validated banks of human neuronal progenitors. Human pluripotent stem cells (PSC) derived from mutation-bearing blastocysts (human embryonic stem cells, hESC) or directly reprogrammed from patient somatic cells (human-induced PSC, iPSC) open a new area of disease modeling as they can virtually be differentiated into any cell type, at a scale compatible with high-throughput cell-based studies, including neuronal progenitors and post mitotic neurons.^{1,2} However, it is critical to fully control the differentiation of these PSC into neuronal types relevant to psychiatric conditions in a robust, homogeneous and timely manner.

Cortical neurons are among the primary targets of psychiatric conditions of developmental origin such as autism spectrum disorder (ASD) and schizophrenia. Common neuronal phenotypes include altered neuronal differentiation and migration, potentially leading to cortical layers disorganization, reduced soma size, abnormal neurite arborization and impaired synaptogenesis.³ As a rule, these pathological phenotypes affect glutamatergic neurons of the superficial cortical layers (II-IV), a subtype of neurons predominantly involved in interconnectivity between cortical regions within a hemisphere.⁴⁻⁷ Cortical neurons of superficial layers are thought to underlie the enhanced cognitive abilities of humans⁸⁻¹⁰ and therefore represent an attractive neuronal subtype regarding modeling of psychiatric disorders and meaningful cellular targets for high-throughput drug screening. Differentiation of PSC toward cortical excitatory neurons has been successfully achieved in mouse and human cells by forming three-dimensional epithelial structures, named rosettes, containing early neuroepithelial cells (NEP) that progressively produce the neuronal types typical for the different cortical layers in a time-dependent manner.^{11,12} These studies have clearly demonstrated that, in contrast to mouse PSC, human PSC can form superficial layer neurons, respecting the key milestones of human cortical development. Specific neuronal subtypes are produced in a predictable temporal manner with the sequential generation of preplate neurons, deep layers neurons (V-VI) and, finally, more

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superficial layers (II–IV).^{12,13} However, the transposition of these protocols to high-throughput technologies, including drug screening, face limitations. The first one is the protracted time course of these protocols. Several weeks of differentiation are necessary to obtain late-born neurons because these protocols do not involve the capture of any stable, self-renewing population of neuronal precursors but rather a slowly evolving equilibrium between progenitors and neurons progressively generated through asymmetric divisions. Consequently, these protocols have led to a mixture of the different cortical layers sequentially generated and did not allow the homogenous production of specific neuron subtypes. Second, the involvement of compact three-dimensional multilayered structures, although representative of the complex cortical organization, excludes the possibility to use a systematic and automated image-based quantification of the different aspects of neurogenesis, an approach necessary to objectively compare disease-affected and control cells then to monitor appropriately the effects of potential therapeutic compounds.

Genetic manipulations in mice have shown that cortical development can be influenced to promote the production of superficial layers.^{14,15} Here we report that this strategy of preventing spontaneous neuronal differentiation cells while promoting neurogenic competence can be applied to human PSC-derived early NEP cells. This allows the production of homogeneous and stable population of late cortical progenitors (LCP) that mainly differentiate into glutamatergic neurons of the cortical superficial layers upon removal of the neuronal differentiation barrier. The rapid and on demand production of mature neurons from these progenitors has permitted the development of HTS assays based on the quantification of the efficiency of neuronal differentiation and of the neurite outgrowth and arborization capacities of the resulting neurons. A collection of 130 small molecules with known biological activities affecting signaling pathways potentially involved in those mechanisms was built and used to validate the scale-up of the assay.

MATERIALS AND METHODS

Human iPSC derivation

Induced PSC were derived from two autistic patients (ASD lines) following appropriate ethical rules. The two patients were diagnosed with non-syndromic autism at Robert Debré Hospital according to Diagnostic and Statistical Manual of Mental Disorders-IV for the classification of neuropsychiatric disorders. Both patients carried a *de novo* mutation on SHANK-3 gene (ASD1: E809X, ASD2 Q1243X,¹⁶). After patient's legal representatives approval and patient consent when appropriated, 8-mm skin punch biopsies were obtained (study approval no. C07-33). Fibroblasts were derived from the donated tissue using standardized in-house protocols and expanded in standard fibroblast culture medium. For control iPSC lines (GM4603 and GM1869), fibroblast were obtained from the Coriell Biorepository (Coriell Institute for Medical Research, Camden, NJ, USA). Reprogramming was performed using the four human genes *OCT4*, *SOX2*, *c-Myc* and *KLF4* cloned in Sendai viruses (Invitrogen, Cergy-Pontoise, France) and iPSC lines characterized according to.¹⁷

PSC culture

Next to the four iPSC lines, one hESC line (SA001, 46 XY, Cellartis, Goteborg, Sweden) was used in this study. All human PSC were maintained on a layer of mitotically inactivated murine embryonic STO fibroblasts in Dulbecco's modified Eagle's medium/F12 Glutamax supplemented with 20% knockout serum replacement, 1 mM nonessential amino acids, 1% penicillin/streptomycin, 0.55 mM 2-mercaptoethanol and 5 ng ml⁻¹ recombinant human fibroblast growth factor-2 (all from Invitrogen). Cultures were fed daily and passaged every 5–7 days. Manual dissection rather than enzymatic methods were routinely used to passage the cells.

PSC commitment to the dorsal telencephalic lineage

Commitment of PSC to the neural lineage was performed as described in.¹⁸ PSC were manually detached from the feeder layer, collected in N2B27

supplemented with human recombinant Noggin (500 ng ml⁻¹, Peprotech, London, UK), SB431542 (20 μM, Tocris Biosciences, Ellisville, MO, USA) and fibroblast growth factor-2 (4 ng ml⁻¹, Peprotech),. They were subsequently transferred to a low-attachment Petridish and incubated for at least 4 h in order to fully remove them from the influence of the feeders. Cells were then seeded on poly-ornithine and laminin-coated (Sigma, St Louis, MO, USA Petridishes. The Rock inhibitor Y27632 (Calbiochem, San Diego, CA, USA) was used at 10 μM at the time of plating to optimize cell survival and seeding. The differentiation medium was changed after 24 h, then every other day until day 10.

LCP derivation, amplification and banking

At day 10, neural rosettes containing NEP cells were manually collected under a binocular microscope under sterile conditions. They were plated *en bloc* in poly-ornithine/laminin-treated culture dishes in N2B27 medium containing epidermal growth factor (10 ng ml⁻¹), fibroblast growth factor-2 (10 ng ml⁻¹) and brain-derived growth factor (20 ng ml⁻¹). At confluence, the cells were passed using trypsin first as small multicellular clumps (passages 2–4) at a ratio of 1:3, then as a single-cell suspension with a density of 100 000 cells cm⁻². Mass amplification was performed until passages 8–10 and cells were frozen in a mixture containing 10% dimethyl sulfoxide and 90% fetal calf serum (Invitrogen). Cryotubes were stored in liquid nitrogen until used. The Rock inhibitor Y27632 was used at the time of thawing to enhance cell recovery.

Neuronal differentiation

Neuronal differentiation of LCP-like cells was induced by plating the cells at low density (50 000 cells cm⁻²) in poly-ornithine/laminin-treated multi-well culture plates, with or without round glass coverslips, in N2B27 without growth factors. The medium was changed every 4 days with systematic addition of 2 μg ml⁻¹ of laminin to avoid detachment and clumping of neurons.

Immunostaining

Cells were fixed in 4% paraformaldehyde and incubated for 10 min in blocking buffer (phosphate-buffered saline, 1% bovine serum albumin and 0.1% Triton X-100). Primary antibodies (Supplementary Table 3) were diluted in blocking buffer and applied overnight at 4 °C. After three washes in phosphate-buffered saline, secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Eugene, OR, USA) were diluted at 1:1000 in blocking buffer and applied for 2 h at room temperature. The cells were washed at least three times in phosphate-buffered saline and visualized on a Zeiss inverted fluorescence microscope (Axiovert, Carl Zeiss, LePecq, France). Image acquisition was performed using the Axiovision LE software (Carl Zeiss). For nuclear counterstaining, cells were incubated in 10 μg ml⁻¹ Hoechst 33258 (Sigma) for 10 min after immunostaining.

Flow cytometry (fluorescence-activated cell sorting)

Cells were trypsinized for 5 min and a solution of 10% fetal calf serum was added to stop the reaction. The cells were collected and washed by resuspension in N2B27 and centrifugation at 1500 g for 10 min. Cells were fixed in 200 μl of freshly prepared 2% paraformaldehyde solution for 10 min on ice, washed by centrifugation and permeabilized with a 0.1% solution of saponin. After centrifugation, cells were incubated 2 h at room temperature in a solution of 200 μl of primary antibody as described in Supplementary Table 3. Cells were washed and incubated 1 h at room temperature with Alexa 488-coupled anti-isotype antibodies. Fluorescence-activated cell sorting-based counting was performed on a MACSquant cell analyzer (Miltenyi, Paris, France). Proper gating, compensations and exclusion of auto-fluorescent cells were performed using several control conditions, which included cells only probed with primary antibodies or with isotype-related non-binding antibodies.

Quantitative reverse transcription-PCR (qPCR)

Total RNAs were isolated using the RNeasy Mini extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. An on-column DNase I digestion was performed to avoid genomic DNA amplification. RNA levels and quality were quantified using a Nanodrop spectrophotometer. A total of 500 ng of RNA was used for reverse transcription using the Superscript III reverse transcription kit (Invitrogen). Quantitative PCR assays were prepared by loading primer mixes (1 μM of each primer in the final reaction) in duplicate wells of 384-well plates

followed by the addition of SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) and 12.5 ng of complementary DNA. PCR reactions were run and quantified using an ABI 7900 light cycler (Applied Biosystems). The quantification of gene expression was based on the DeltaCt Method and normalized to 18S expression. Melting curve and electrophoresis analysis were performed to control PCR product specificities and exclude non-specific amplification. The list of primers is provided in Supplementary Table 4 excepting Sox5 primers that were purchased at Qiagen. In addition, extensive molecular profiling of cortical progenitors was performed using 'Neurogenesis' qPCR array (SA Biosciences, Qiagen)

Chemical collections

A list of 130 small molecules of interest was constituted from a commercially available collection of 80 inhibitors of kinases (Enzo Life Sciences, Villerbanne, France) and 50 compounds selected from the literature and previously shown to be active in models of neurogenesis¹⁹. A complete list of the molecules, as well as their reported efficient dose, is provided in (Supplementary Table 2).

Automated seeding and compound delivery in the 384-well plates assay format

LCP were dissociated and plated in N2B27 at a density of 3000 cells per well in poly-ornithine/laminin pretreated 384-well plates using the Bravo automated liquid handling platform equipped with a 384 pipette head (Agilent Technologies, Santa-Clara, CA, USA). Six hours after plating, compounds were added using the same platform. Half of the medium was removed on days 4, 7 and 11, and replaced with fresh compound containing N2B27 medium $\pm 2 \mu\text{g ml}^{-1}$ laminin.

Automated image-based high-content screening

For high-content screening assays, the fluorescent labeling was performed as described above in an automated manner using the Bravo liquid handling system. Image acquisition and analyses were performed using the Cellomics Arrayscan automated microscope (ThermoFisher, Hudson, NH, USA). The 'Colocalization' bioapplication was used to identify and count cells positive for HuCD (differentiation assay). Hoechst staining was used to identify objects to include in the analysis. A minimum of 1000 cells were analyzed per well in at least four wells at a $\times 10$ magnification to reach statistical robustness. To quantify neurite outgrowth and branching points, the 'neuronal profiling' bioapplication was used to track Tuj-1-positive neurites. A minimum of 200 neurons per well were analyzed in a minimum of four wells.

Statistical analyses

To assess the quality of the assay, the z' factor value was determined. This factor represents a dimensionless parameter, whose value can range between <0 and 1 . It is defined as $z' = 1 - ((3\sigma^+ - 3\sigma^-) / (\mu^+ \pm \mu^-))$, where σ and μ , are the s.d.'s and the means, respectively, of the positive (+) and negative (-) controls.²⁰

The raw data of the screening were subjected to analysis of variance following by a Dunnett's test to evaluate statistical differences between cells treated with tested compounds and dimethyl sulfoxide-treated controls.

RESULTS

Differentiation of hESC into cortical NEP cells

The first crucial step in the formation of specific cortical neuronal subtypes consists in efficiently inducing the differentiation of PSC into early NEP cells of the dorsal telencephalon (NEP). We induced early NEP differentiation in a monolayer system using the two SMAD inhibitors SB431542 and Noggin²¹ in combination with the defined medium N2B27.¹⁸ We have previously reported that the efficient neural commitment of hESC can be obtained using these conditions, avoiding the use of batch-dependent component like the knockout serum replacement and suppressing the need of using a medium preconditioned by feeder fibroblasts, thus removing a source of factors that may be instrumental in patterning the nervous system. After 10 days, more than 80% of

the cells organized in 'rosettes' expressed the protein SOX1, which is characteristically associated to early NEP (Figure 1a). They were identified as dorsal telencephalic cells by their immunopositivity for both the dorsal marker PAX6 (Figure 1b) and the canonical telencephalic marker BF1 (Figure 1c). Even more precisely, they were engaged in neocortical differentiation as shown by high levels of expression of EMX2 and OTX1 (Figure 1a), whereas they hardly expressed any of either the ventral forebrain markers DLX2 and GSH2 or the more caudal markers EN-1, PAX2, PAX5 and HOXB9. The cells organized as a polarized epithelium with a luminal side expressing the apical protein ZO-1 (Figures 1d and e). Belonging to the radial glia lineage was indicated by nestin immunoreactivity (Figure 1e). Consistent with previous descriptions, however, these progenitors only produced about 40% of differentiated HuC/D immunopositive neurons upon replating for 3 weeks after mitogens withdrawal (42.33 ± 9.71 ; Figure 2a). They primarily expressed Ctip2, a molecular marker of the deeper layers of the cortex (Figure 2b).

Derivation of a homogeneous self-renewing population of precursors generating glutamatergic neurons of the cortex superficial layers

We then reasoned that the late production of neurons of the superficial layers during development *in vivo* implied that cortical NEP performed a large number of divisions before producing them, and tested the hypothesis allowing them to do so *in vitro* before inducing differentiation would have a similar effect. Accordingly, rosettes were collected manually without cell dissociation and transferred into a pro-proliferative medium with N2B27, epidermal growth factor, basic fibroblast growth factor and brain-derived growth factor (EFB medium). Under these conditions, rosettes dissociated spontaneously over time, as cortical progenitors lost ZO-1 immunopositivity while maintaining nestin immunoreactivity (Figures 1f and g). After a second passage using trypsin, a population of morphologically homogenous cells growing as a monolayer was obtained and further amplified (Figure 1h). After eight passages in the EFB medium, the cells maintained the expression of the cortical radial glia markers nestin (Figure 1i), Pax6 (Figure 1j), Blbp (Figure 1k) and Sox2 (Figure 1l). In contrast, they did not express Tbr2, which is classically associated with intermediate cortical progenitors (Figure 1m). Treatment with the EFB medium successfully blocked spontaneous neuronal differentiation and promoted active cell cycling (Figures 1n and o). Molecular profiling of EFB amplified progenitors cells using qPCR arrays showed that they expressed high levels of polarized neural stem cells and radial glia markers, including Pax6, POU3F3 (Brn2), NRCAM and PARD3, in addition to genes involved in corticogenesis-like MEF2C, brain-derived growth factor, RTN4 (NOGO), ODZ1 (a target of EMX2), Filamin A and LIS1 (Figure 1p). These results were confirmed by additional qPCR analysis (Supplementary Figure 1). The same profile was maintained for at least 20 passages without either morphological changes or genomic instability (Supplementary Figure 2) and conserved upon freezing (Supplementary Figure 3). We named those cells 'LCP'.

LCPs were plated at low density, and mitogens removed in order to induce neuronal differentiation, and they quickly stopped cycling. Time course quantification of cells expressing the two neuronal markers Tuj-1 and HuC/D revealed an efficient differentiation along the neuronal lineage (Figures 2b, c and m). In parallel, quantitative reverse transcription analysis indicated the progressive appearance, over a period of 3 weeks, of Tau, MAP-2 and SNAP-25, associated to terminally differentiated neurons (Supplementary Figure 4) as well as the glutamatergic markers vGlut-1 and vGlut2 (Supplementary Figure 4). After 21 days of differentiation, more than 80% of the neurons were

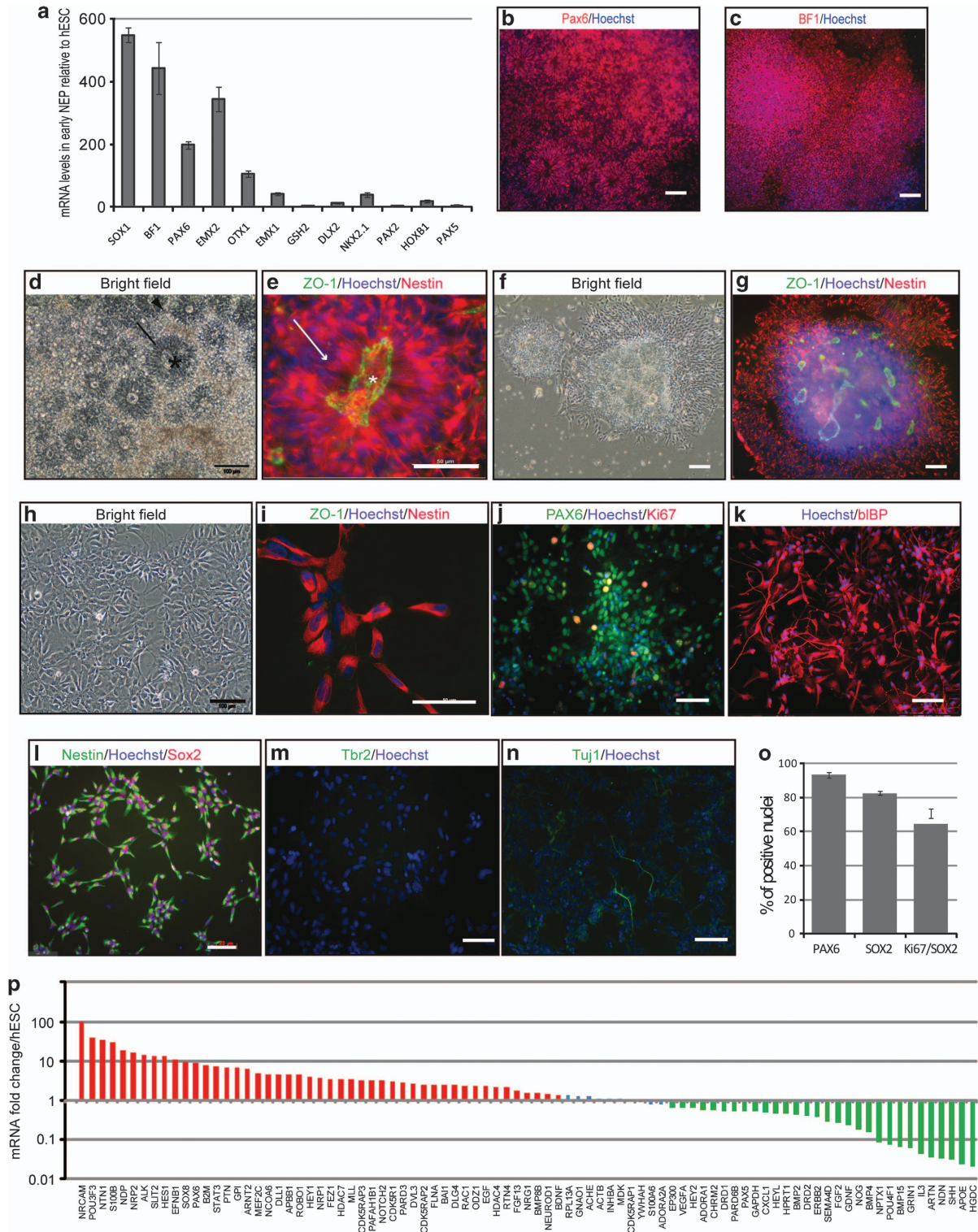


Figure 1. Differentiation of human embryonic stem cells (hESC) into late cortical progenitors (LCPs). **(a)** Regional profiling of neuroepithelial cells (NEPs) 10 days after induction of neural commitment. Results represent mean \pm s.d. of three independent biological replicates. **(b, c)** Representative photomicrographs illustrating PAX6 **(b)** and BF1 **(c)** expression in hESC-derived neural rosettes. NEPs organized into nestin immunopositive neuroepithelial structures (arrow) surrounding a lumen (star) with an apical side expressing ZO-1 **(d, e)**. Loss of expression of ZO-1 and maintenance of strong expression of nestin after the first passage **(g)**. After several passages homogeneous neural cell population **(h)** that does not express ZO-1 but maintains nestin expression **(i)** and express PAX6 **(j)**, Blbp **(k)** and Sox2 **(l)** but neither Tbr2 **(m)** nor Tuj-1 **(n)**. **(o)** Proportion of cells expressing radial glia markers, and of proliferating neural progenitors (Ki67/Sox2 double-immunopositive cells). **(p)** Expression profile of progenitors amplified in EFB medium. Green bars indicated genes downregulated in neural progenitors compared with the parental hESC, genes upregulated are shown in red. Results represent mean \pm s.d. of three independent experiments. Scale bar = 100 μ m.

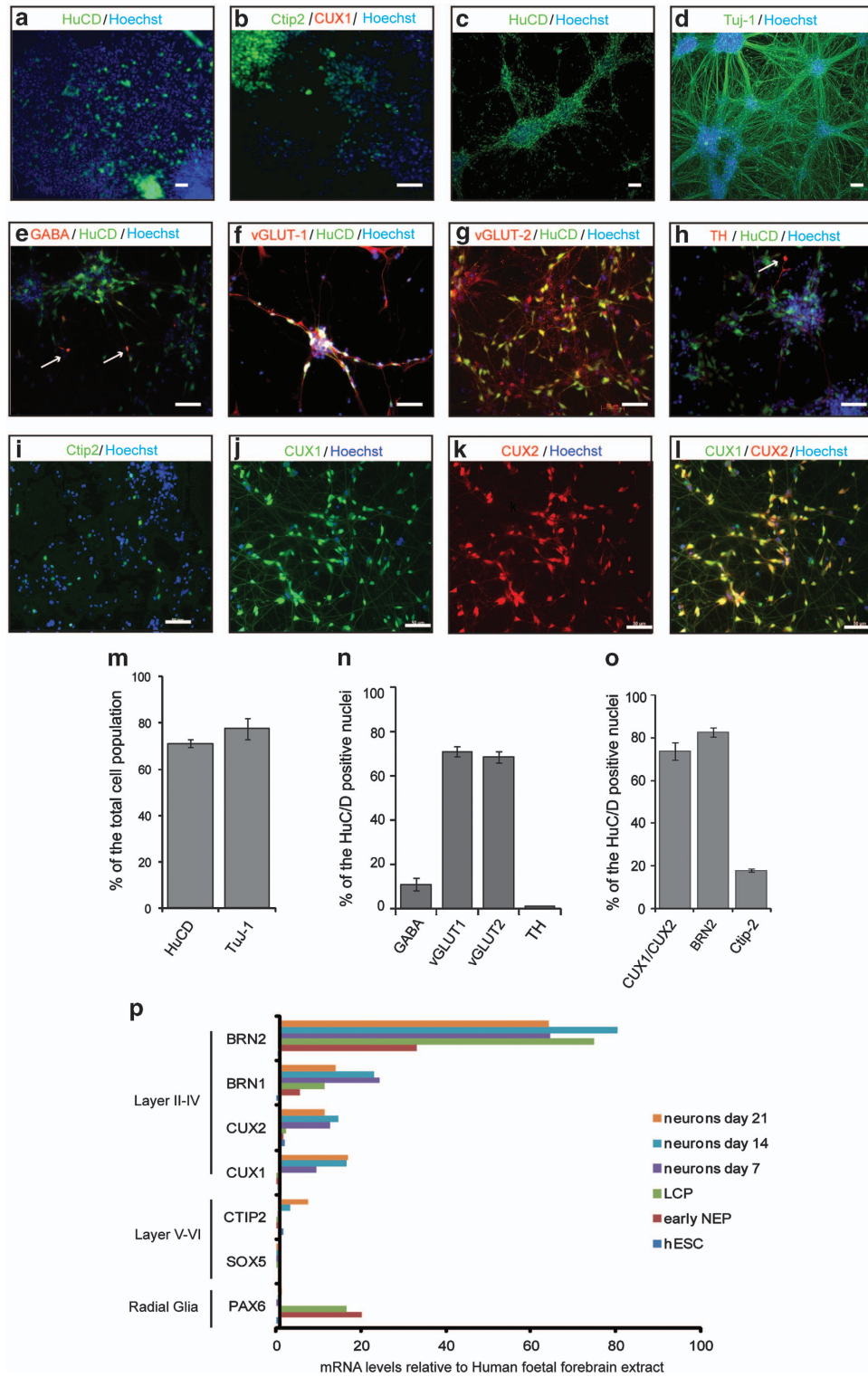


Figure 2. Production of superficial-layer neurons from late cortical progenitors (LCPs). **(a, b)** Cortical neurons produced by NEP differentiation 21 days after mitogen withdrawal. **(c)** HuC/D and **(d)** Tuj-1 immunopositive neurons produced by LCPs 21 days after mitogen withdrawal. **(e–i)** Immunocytochemical characterization of neurons produced by LCPs. **(m)** Automated quantification of the proportion of HuC/D and Tuj-1-positive neurons produced by LCPs after 21 days of differentiation. **(n)** Automated quantification of HuC/D-positive neurons expressing markers of a neuronal subtype or **(o)** of a cortical molecular layer. **(p)** Molecular profiling by RT-qPCR of hESC-derived early neuroepithelial cells (NEP), LCPs and differentiated neurons at different time points of differentiation. Results are expressed as mean \pm s.d. of three independent experiments. Scale bar = 50 μ m. TH, tyrosine hydroxylase.

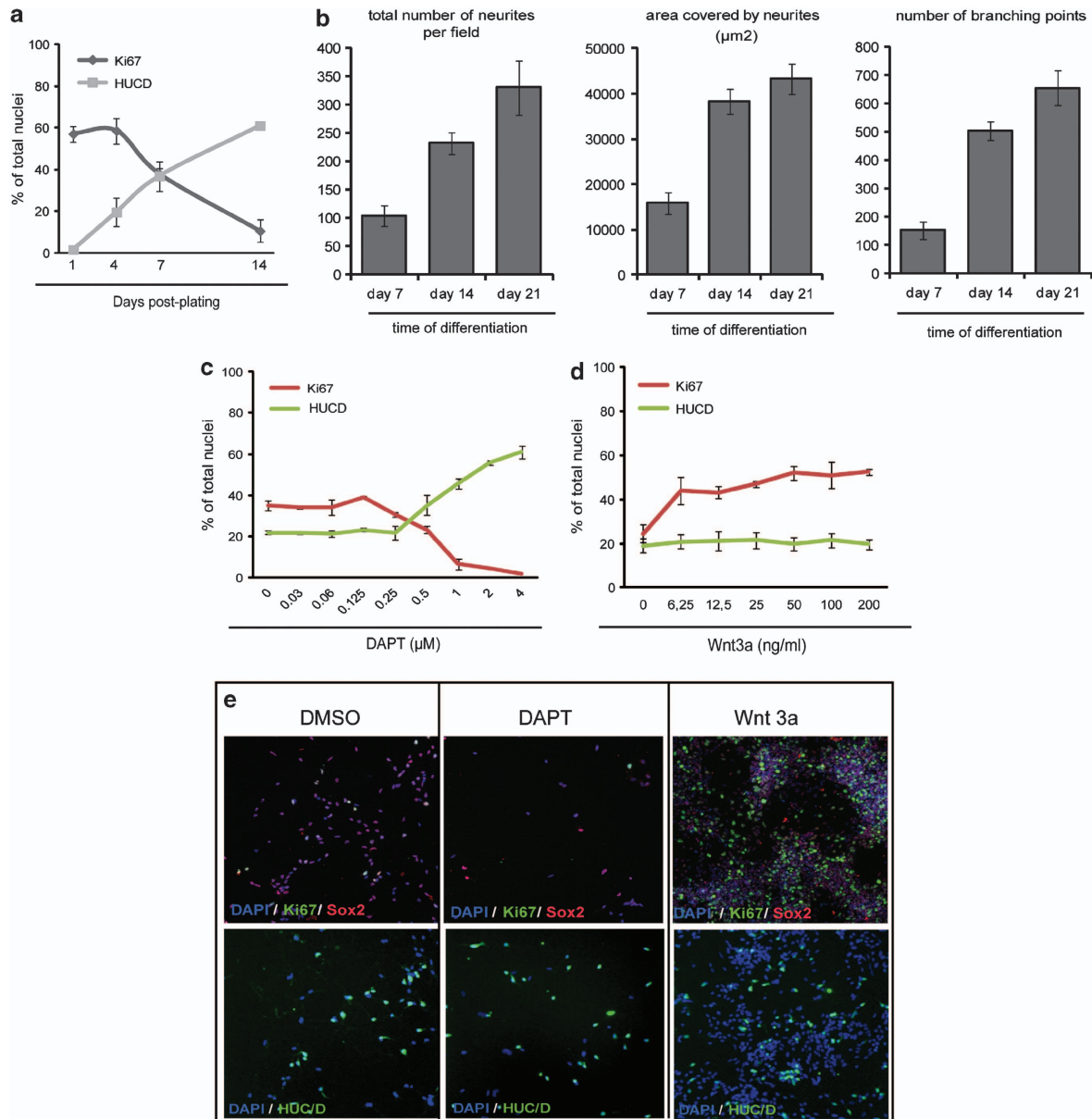


Figure 3. Amenability of late cortical progenitors (LCP) terminal differentiation into a high-throughput screening format. **(a)** Automated quantification showing the kinetics of neural proliferation (Ki-67) and neuronal differentiation (HuC/D) of LCPs in 384-well plates. **(b)** Automated quantification of the kinetic of neuritic outgrowth (Tuj-1-positive neurites). Results represent mean \pm s.d. of each parameter per field of 109 000 μm^2 in at least 16 independent wells (representative of one of three independent experiments). **(c, d)** Response of LCPs to increasing doses of DAPT **(c)** or Wnt3a **(d)**. Results are expressed as mean \pm s.d. **(e)** Representative photographs captured by the Arrayscan microscope.

immunopositive for vGlut-1 and v-Glut-2, whereas 10% expressed gamma-aminobutyric acid and less than 2% the catecholaminergic enzymatic marker tyrosine hydroxylase (Figures 2e–h and n). Neurons formed complex and branched neuritic networks as well as PSD-95 immunopositive glutamatergic synapses (Supplementary Figure 4). Over 70% of them expressed Cux1, Cux2 and Brn2, which are specifically expressed in upper layers II–IV (²² Figures 2j–l and o); thus validating our working hypothesis that an extended period of proliferation of cortical progenitors would strongly bias differentiation toward late-appearing neurons of the superficial cortical layers. The remaining neurons expressed CTIP-2, a marker of lower-layer cortical neurons (Figures 2i–o). This was confirmed by analyzing additional layer markers by qPCR (Figure 2p).

Responses of LCPs to a collection of 130 compounds in a HTS plate format

The amenability of LCP-differentiated neurons to drug screening was then analyzed. LCPs were successfully adapted to a miniaturized culture format in 384-well plates and differentiated into post mitotic neurons with a time course similar to that observed in larger culture dishes. Over 60% of HuC/D immunopositive neurons were detected after 14 days of differentiation, whereas less than 15% of Sox2 cycling cells were still present (Figure 3a). Neurons progressively formed a complex neuritic network starting at day 14 (Figure 3b). Control molecules with known opposing impact on neural differentiation were first assayed, with Wnt3a as a pro-proliferative agent²³ and Notch signaling inhibition by the gamma-secretase inhibitor DAPT as a pro-differentiating agent,²⁰

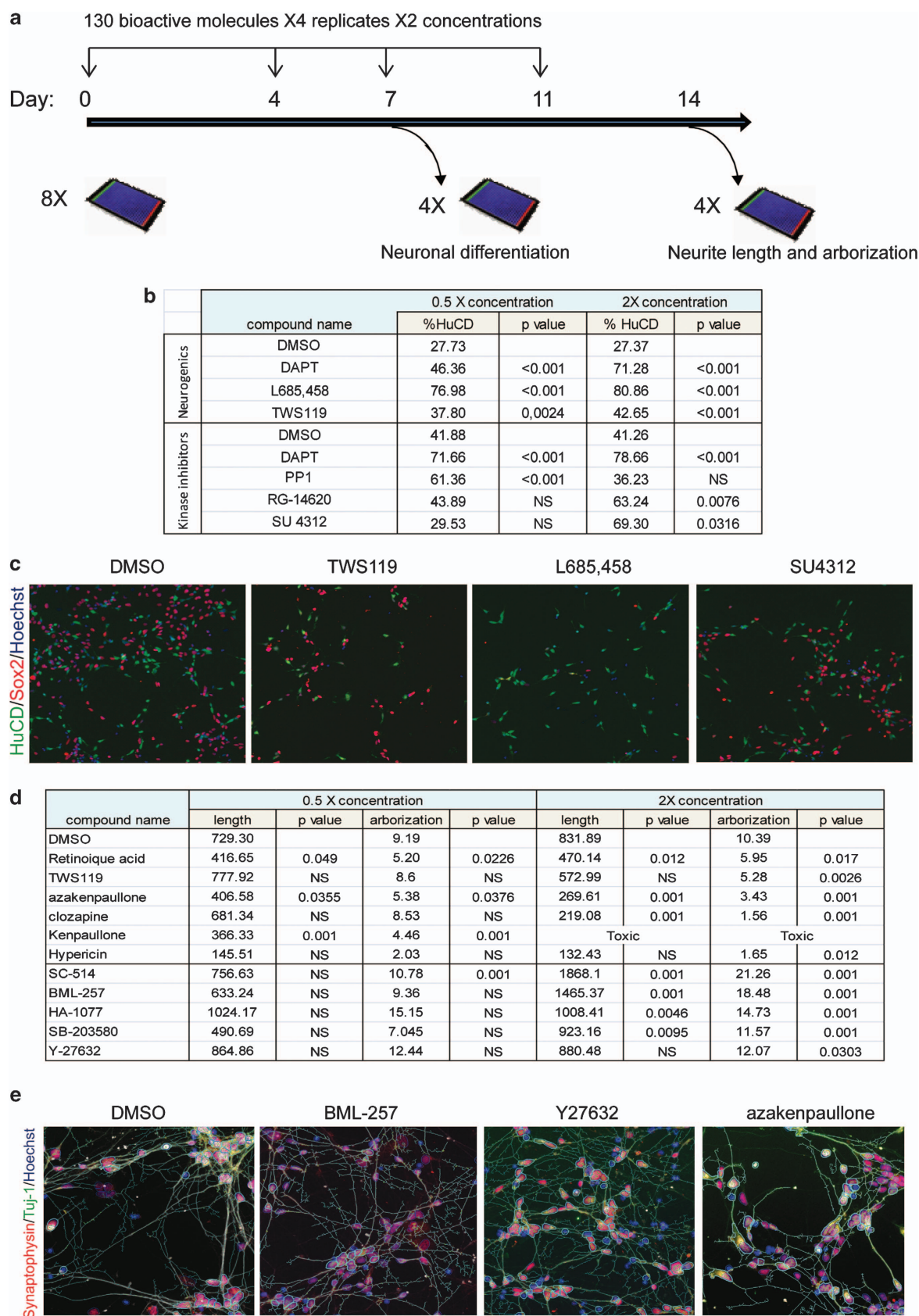


Figure 4. High-content screening of a library of 130 bioactive chemical compounds. **(a)** Screening flowchart. **(b)** List of compounds that increased the proportion of HuCD-positive neurons in the culture by more than 15%. **(c)** Representative results captured by the Arrayscan microscope. **(d)** List of compounds that modified significantly neurite length and/or arborization. **(e)** Representative results captured by the Arrayscan microscope, including the tracking of neurites. *P*-values were calculated using the Dunnett's test after an analysis of variance, NS; non significant.

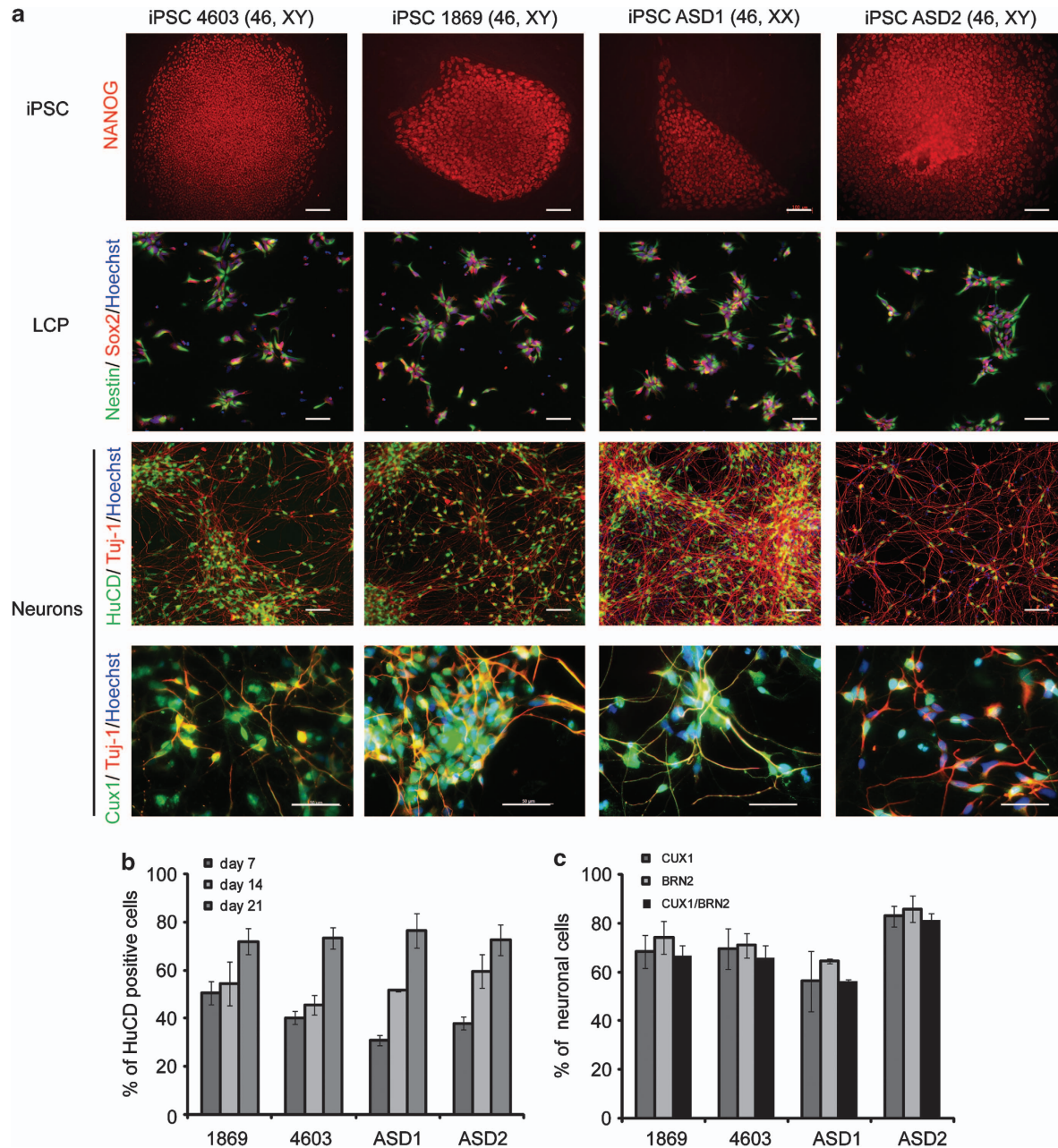


Figure 5. Differentiation of induced pluripotent stem cells (iPSC) into neurons of the superficial layers. **(a)** Representative immunocytochemistry of late cortical progenitors (LCP) and cortical neurons produced from four different iPSC lines. 4603 and 1869 iPSC lines were derived from control donors and autism spectrum disorder 1 (ASD1) and ASD2 from autistic children with SHANK-3 mutations. Scale bar = 50 μ m **(b)** Kinetic of differentiation of iPSC-derived cortical neurons. **(c)** Percentage of upper layers neurons produced after 17 days of differentiation of iPSC-derived LCP. For **(b, c)** results are expressed as mean \pm s.d. of three independent experiments.

respectively. Cells were exposed to increasing doses of either of the two molecules at the time of plating and at day 4, and the analysis was performed at day 7. As expected, Wnt-3a increased the proportion of Ki67/Sox2 immunopositive cycling LCPs while blocking the appearance of HuC/D-positive neurons, whereas DAPT strongly promoted LCPs differentiation as HuC/D immunopositive neurons (Figures 3c and d). Z' factor calculation validated the robustness of the screening assays according to (Z' factor of dimethyl sulfoxide vs controls >0.2 and Z' factor of control min vs control max >0.5 , Supplementary Figure 5,^{24,25}).

A primary screening was then performed in a search for regulators of those two functions based upon a selected chemical compounds library of 130 small molecules with known effects on

discrete signaling pathways. These included 80 kinase inhibitors and 50 additional compounds that had been reported as modifiers of neurogenesis in a variety of cellular and animal models¹⁹ (Supplementary Table 1). Changes in the production of post mitotic neurons from LCPs—revealing compounds promoting neural differentiation were analyzed after 7 days of treatment using HuC/D nuclear labeling. Modulation of neurite outgrowth and arborization was analyzed after 14 days—revealing compounds acting on neuronal maturation—using Tuj-1 cytoplasmic labeling (Figure 4a). Hit compounds were obtained in the two assays (Supplementary Table 2). Compounds stimulating differentiation of LCPs into post mitotic neurons included a gamma-secretase inhibitor different from DAPT (L695,458), a highly potent

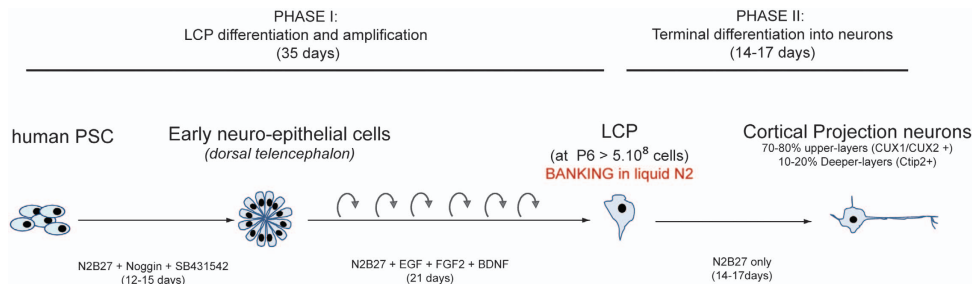


Figure 6. Overview of the timelines of the differentiation protocol. Phase I (days 0–35) corresponds to the steps of differentiation of human pluripotent stem cells (hPSC) into early cortical progenitors, the subsequent phenotypic transition to late cortical progenitors (LCP) and further amplification to create a large cell bank. After this phase, neuronal differentiation into cortical projection neurons is achieved in 14–17 days (phase II). As neuronal differentiation can be started directly from the stock of frozen LCP, the phase I does not need to be completed at each experiment. Liquid N2; liquid nitrogen.

and selective inhibitor of Src family tyrosine kinases (PP1), an epidermal growth factor receptor inhibitor (RG14620), a selective inhibitor of vascular endothelial growth factor receptor 2 (SU4312) and a glycogen synthase kinase-3 (GSK-3) β inhibitor (TWS 119) (Figures 4b and c). Among the compounds that did not modify the rate of neuronal differentiation were five molecules that, nevertheless, promoted neuronal maturation as revealed by increased neurite length and/or arborization (number of branching points): a mitogen-activated protein kinase/p38 pathway inhibitor (SB203580), two Rock inhibitors (HA-1077 and Y27632), an IKK-2 inhibitor (SC-514) and an Akt-1 inhibitor (BML-257). Six compounds decreased the rate of neuronal maturation without altering either cell survival or differentiation: three GSK-3 inhibitors (kenpaullone, azapallone and TWS 119), two antipsychotics or mood regulators (hypericin and clozapine), and retinoic acid (Figures 4d and e).

LCPs and cortical neurons of the superficial layers can be produced from control and autistic patient-derived iPSCs

In order to evaluate whether our protocol was applicable in the context of psychiatric diseases, LCP and neurons of the cortical superficial layers were produced from iPSCs, both from controls and from two autistic patients bearing *de novo* mutations on *SHANK-3* (¹⁶). All four lines were efficiently converted into early cortical NEPs and stabilized as proliferating LCPs expressing Sox2 and nestin (Figure 5a). LCPs readily differentiated into HuCD/Tuj-1-positive neurons with the same efficiency than hESC (Figures 5a and b). The majority of neurons produced were also similarly positive for superficial layers marker in the four lines.

DISCUSSION

The goal of this study was to use human PSCs as a biological resource to robustly produce live glutamatergic neurons of the superficial layers of the cortex, a population of neurons particularly affected by psychiatric diseases, in order to study the impact of these diseases on their development and perform HTS campaigns for drug discovery. We successfully differentiated hPSCs into stable population of late cortical precursors that preferentially generated neurons of the superficial layers in a rapid and standardized manner. We showed that these cells could be used to conduct large throughput drug screening studies, identifying modifiers of human corticogenesis that may be involved in the etiology of psychiatric diseases, or conversely pointing to potential therapeutic strategies.

Superficial layer neurons represent a pivotal neuronal population particularly affected by psychiatric diseases.^{4–6,26,27} As expanded superficial layers of the cortex are among the most distinguishing features of the human brain, modeling the impact

of psychiatric diseases using these neurons may help revealing human-specific aspects. Superficial layers of the cortex are poorly developed in rodents as compared with humans, a situation replicated *in vitro* when corticogenesis is recapitulated from PSCs.^{11,12} Superficial layers neurons are late-born cells and, accordingly, their derivation from human PSCs only occurs after an extended period of differentiation. In their pioneer study, Shi *et al.*^{12,28} described a protocol recapitulating key features of corticogenesis starting from human PSC. In this three-stage process, PSC first differentiated to cortical progenitors cells resembling the cortical sub-ventricular radial glia cells that will spontaneously produce intermediate progenitors and the different types of projection neurons of the deep and superficial layers in stereotypical temporal order reminiscent of *in vivo* corticogenesis. Accordingly, superficial layer neurons appeared in small amount ($\sim 30\%$) after 80–90 days of differentiation, mixed with undifferentiated progenitors and earlier-born neurons of the deeper layers. Although very elegant, this approach, which was to our knowledge the only producing superficial layer neurons from hPSC, precluded a robust and a systematic study of the impact of a disease state on genesis and maturation of these neurons because of this protracted time of differentiation. Consequently, studies of neurons derived from neuropsychiatric patients iPSC were so far conducted using quicker protocols of differentiation leading to a mixture of GABAergic and glutamatergic neurons with not known regional identity.^{29,30} This temporal challenge is also particularly critical when addressing drug discovery using HTS as this powerful technique requires that the terminal differentiation and maturation steps of these neurons occurs in a rapid, reproducible and synchronous manner. The approach described here allowed us to differentiate and stabilize cortical precursors that could be largely amplified and preferentially produced glutamatergic neurons of the superficial layers. This was achieved by mimicking the chronobiology of the human corticogenesis, that is, by preventing the spontaneous neuronal differentiation of early NEPs patterned to adopt a dorsal telencephalic fate while promoting active cell divisions. Accordingly, the neurogenic potential of the PSC-derived cortical precursors changed from deep to superficial layers after the period of active division. Consequently, large amounts ($>70\%$) of superficial layers neurons can be produced in less than 50 days starting from PSC (instead of 80–90 days). Importantly, as the self-renewing LCP can be cultivated and amplified as single-cell culture, they can be stored frozen as very large banks. Terminal differentiation can therefore be restarted directly from the LCP at any time, and superficial layer neurons were produced in less than 17 days (Figure 6).

The results obtained by large throughput drug screening in the present study validated the biological pertinence of our model by highlighting signaling pathways that are known to be involved in

psychiatric diseases, namely the RhoA kinase and the GSK-3-dependant pathways. Inhibitors of the Rho kinase increased neurite outgrowth and arborization in our model. The role of Rho proteins in controlling neurite growth and morphology has been largely documented, in particular for RhoA. Activation of RhoA-dependant signaling cascades indeed inhibits neurite outgrowth in response to various stimuli,^{31,32} whereas neurite outgrowth induced by neurotrophin is dependent on Rho A inhibition.³³ Mutations in several genes involved in RhoA-dependant signaling pathways have been linked to genetic forms of mental retardation, autism and schizophrenia.^{20,34–37} Conversely, three GSK-3 inhibitors strongly blocked neurite growth and arborization while impairing neither cell survival nor neuronal differentiation. Impaired GSK-3-dependant pathways have been reported in several mood disorders and are the targets of many anti-psychotic agents,³⁸ including clozapine which also showed a blocking effect on neurite growth in our screening assay. Inhibition of GSK-3 represents a promising therapeutic option for Fragile X-syndrome as phenotypic improvements have been observed in mouse models of the disease after treatment with GSK-3 inhibitors, including a normalization of the pathological increase in neurite arborization and spine density.³⁹ This has been corroborated by the results of a pilot clinical study of lithium, which targets GSK-3.⁴⁰ Interestingly, thanks to our cell-based assay, we also identified pathways involved in the very early steps of neuronal differentiation, a developmental stage hardly accessible *in vivo* but particularly important in the context of psychiatric diseases of developmental origins, where impaired neuron number and abnormal cortical organization are commonly found.

To date, several PSC lines, from embryonic origin or induced from patient somatic cells, have been obtained for diseases involving cortical dysfunction. For some of these, evidence has been obtained that known cell-autonomous phenomena can be replicated *in vitro*, including the Fragile-X syndrome,^{41,42} Rett's syndrome,⁴³ Down's syndrome⁴⁴ and schizophrenia.³⁰ In this context, our results point to the possibility of specifically exploring the impact of a mutant gene deemed responsible for neuropsychiatric diseases on the differentiation and maturation of live neurons of the superficial layers of the cortex, which are one of the most relevant cell populations in such studies. High-throughput treatment of these cells with either pharmacological agents or small interfering RNA would greatly enhanced the identification of signaling pathways involved in the development of the neuronal phenotypes associated to such diseases and improve the rate of discovery of potential therapeutic molecules.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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