

Multicellular Co-development Promotes Maturation of Human Stem Cell-Derived Neural Networks

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Abstract

Background

Human central nervous system development depends on the coordinated differentiation and reciprocal interactions of neurons and glia, whereas most stem cell-based in vitro models rely on lineage-directed differentiation. Whether allowing multiple neural lineages to differentiate concurrently improves the maturation and physiological relevance of human stem cell-derived neural cultures remains unclear. We developed lineage-unrestricted differentiation (LUD), enabling neurons, astrocytes and oligodendrocyte-lineage cells to emerge concurrently from human neural stem cells without enforced lineage specification.

Methods

LUD and lineage-directed cultures were compared using immunocytochemistry, synaptic analyses, transcriptomic profiling, calcium imaging, multi-electrode array recordings, axonal transport assays and glutamate-induced stress. Transcriptomes of LUD-derived neurons and astrocytes were compared with human fetal cortical reference cells. Statistical analyses used parametric or non-parametric tests with correction for multiple comparisons where appropriate.

Results

LUD generated multicellular neural cultures containing neuronal, astrocytic and oligodendrocyte-lineage populations. Compared with lineage-directed cultures, LUD cultures exhibited increased expression of neuronal maturation markers, larger and better-organised synaptic structures, and stronger and more sustained network activity. LUD-derived neurons shared 97.8% of expressed genes with human fetal cortical neurons, while LUD-derived astrocytes shared 75.5–85% of expressed genes with fetal astrocytes depending on culture conditions. LUD astrocytes exhibited reduced expression of CCL2, TGFB111, TGFB2 and VIM relative to primary human astrocytes, consistent with a comparatively non-reactive, developmentally oriented state. Multicellular cultures also displayed attenuated glutamate-evoked calcium responses and reduced glutamate-induced apoptosis, while astrocytes modulated neuronal axonal transport responses to glutamatergic stress.

Conclusions

Allowing neuronal and glial lineages to co-develop enhances structural, molecular and functional maturation of human stem cell-derived neural cultures. LUD provides a serum-free and experimentally accessible multicellular platform that combines the tractability of two-dimensional culture with developmentally relevant neuron–glia interactions. This approach may improve human stem cell-based

models for investigating neural development, neurological disease mechanisms, neurotoxicity and mechanisms relevant to neural repair and regeneration.

BACKGROUND

The human central nervous system develops through the coordinated emergence and maturation of multiple neural lineages. Neurons, astrocytes and oligodendrocyte-lineage cells do not mature in isolation; rather, their developmental trajectories are shaped by reciprocal signaling, cell-cell contact and activity-dependent interactions within an evolving multicellular environment [1, 2]. Reproducing these interactions *in vitro* is therefore important not only for understanding human neural development but also for generating physiologically relevant stem-cell-based models for studying neurological disease and mechanisms relevant to neural repair.

Stem cell-based differentiation protocols can efficiently generate individual CNS cell types using defined combinations of small molecules, transcription factors and growth factors. Such approaches reliably produce neurons [3, 4, 5, 6], astrocytes [7, 8, 9] and oligodendrocytes [10, 11, 12]. However, lineage-directed differentiation necessarily constrains progenitors to predefined fates and can separate cell types that normally develop together. This may restrict reciprocal interactions that contribute to synaptic maturation, network homeostasis and glial differentiation *in vivo* and may contribute to the incomplete maturation and molecular heterogeneity frequently observed in stem cell-derived CNS populations [13, 14].

Many differentiation protocols include an initial progenitor phase followed by lineage-specific maturation using neurotrophic factors, serum or restricted growth-factor combinations [3, 6, 11, 15] (Fig. 1). Although effective for generating defined cell populations, these approaches preclude the parallel emergence of neuronal and glial lineages that characterises CNS development. At the other end of the experimental spectrum, three-dimensional organoid systems permit multicellular self-organisation and reproduce important aspects of neural tissue development [16, 17], but their three-dimensional architecture can introduce diffusion-limited gradients and restrict high-resolution live imaging and experimental manipulation [18]. An experimentally accessible system that combines the tractability of two-dimensional culture with developmental advantages of multicellular differentiation could therefore complement existing stem cell-based neural models.

An important challenge in stem cell-based neuroscience is consequently not only the efficient generation of individual neural cell types, but also the establishment of culture conditions in which these cells acquire functionally relevant properties within an appropriate multicellular environment. Astrocytes regulate synapse formation and elimination, neuronal activity and metabolic homeostasis [19, 20], illustrating how separating neural lineages during differentiation may remove signals that normally contribute to neuronal maturation. We therefore hypothesised that lineage restriction itself may constrain maturation by preventing reciprocal neuronal-glial interactions that accompany neural development.

To test this hypothesis, we developed a lineage-unrestricted differentiation (LUD) paradigm that allows human neural stem cells to differentiate without enforced lineage specification. LUD omits a separate progenitor expansion phase and uses a minimal, serum-free medium supplemented with ciliary neurotrophic factor (CNTF), which supports neuronal and glial differentiation, survival and maturation [21, 22, 23]. This design enabled us to determine whether permitting multiple neural lineages to emerge together alters cellular composition, molecular identity, synaptic organization, network function and response to cellular stress.

Here, we show that LUD cultures generate neuronal, astrocytic and oligodendrocyte-lineage populations within a shared developmental environment and acquire structural, molecular and functional properties distinct from lineage-directed cultures. LUD cultures form larger and better-organised synaptic structures, develop stronger and more sustained network activity, show substantial transcriptomic correspondence with human fetal cortical neurons and astrocytes, and exhibit increased resilience to glutamatergic stress. These findings identify multicellular co-development as an important determinant of maturation in human stem cell-derived neural cultures and establish LUD as an experimentally accessible platform for studying human neural development, neuron-glia interactions and mechanisms relevant to neurological disease and regenerative neuroscience.

RESULTS

Lineage-unrestricted versus lineage-directed differentiation of human neural stem cells

To test whether removing lineage bias confers molecular, structural, or functional advantages, we compared three established lineage-directed differentiation protocols with the lineage-unrestricted differentiation (LUD) protocol (Fig. 1). Neuronal differentiation included a progenitor phase followed by serum-free maturation [6]. Astrocytic differentiation used serum to promote proliferation and maturation [15], and oligodendrocytic differentiation relied on lineage-directed growth factors [11]. In contrast, LUD omitted the progenitor expansion phase, used entirely serum-free conditions, and included CNTF supplementation.

Cell population balance, identity, and molecular signatures

Flow cytometry using canonical lineage markers [24, 25] (Fig. 2A) revealed comparable proportions of neurons, astrocytes and oligodendrocytes across protocols, with modest shifts between early and late astrocytic markers in LUD cultures (Fig. 2A; Supplementary Fig. S1; Supplementary Table S1A). qPCR analysis indicated higher expression of late neuronal markers in LUD cultures relative to neuron-directed differentiation, whereas astrocyte-directed cultures retained elevated early astrocytic markers (Fig. 2B; Supplementary Table S1B). No evidence of enhanced oligodendrocyte maturation was observed in LUD cultures, consistent with known *in vitro* limitations.

Neurogenesis-focused PCR arrays revealed enrichment of pathways related to neurogenesis, nervous system development, and neuronal differentiation in LUD cultures compared with lineage-directed

conditions (Fig. 2C-D, Supplementary Tables S1C-D). Network analysis identified 11 neuronal, 12 astrocytic and 12 oligodendrocytic key hub gene regulators enriched in synaptic, dendritic, and somatodendritic compartments and highlighted upregulation of glutamatergic signaling pathways (Fig. 2E; Supplementary Fig. S2A, Supplementary Table S1E). Moreover, key glutamatergic neuron markers and genes involved in glutamatergic signalling pathway were significantly upregulated in the lineage-unrestricted differentiation protocol (Fig. 2F), supporting the emergence of a glutamatergic phenotype in these cultures. Disease association analysis revealed enrichment of Alzheimer's disease-associated genes, including amyloid precursor protein (APP) and microtubule-associated protein tau (MAPT) (Supplementary Fig. S2B; Extended Data Table 1).

Synaptic maturation and network activity

LUD cultures exhibited lower axonal and dendritic density but significantly increased volumes of pre- and postsynaptic structures, indicating altered synaptic organisation characterised by larger synaptic structures (Fig. 3A-D; Supplementary Tables S2B-D). Ultrastructural analysis confirmed enlarged synaptic profiles that nevertheless retained immature features (Fig. 3E). Multi-electrode array (MEA) recordings revealed delayed onset of activity followed by stronger and more sustained network function in LUD cultures, whereas neuron-only cultures showed earlier activity that subsequently declined (Fig. 3F; Supplementary Table S2F, Supplementary Fig. S3E). BDNF was detected across all protocols and was highest in neuron-only cultures, consistent with supplementation of the neuronal differentiation medium (Fig. 3G; Supplementary Table S2G). Total and phosphorylated tau levels were elevated in LUD and neuron-only cultures, with phosphorylated tau significantly increased in LUD cultures (Fig. 3H-I, Supplementary Tables S2H-I).

LUD-derived astrocytes showed reduced branching but increased territorial coverage (Supplementary Fig. S3A-B; Extended Data Table 2). These morphological features are consistent with a comparatively non-reactive, developmentally oriented astrocytic state rather than a strongly reactive phenotype. Oligodendrocyte morphology did not differ detectably across conditions (Supplementary Fig. S3C-D; Extended Data Table 2).

Transcriptomic fidelity to the human fetal cortex

RNA-sequencing of FACS-sorted neurons and astrocytes from LUD cultures demonstrated close correspondence with the respective populations from human fetal cortex (Fig. 4A-D; Supplementary Table S3B, S3D). LUD-derived neurons shared 97.8% of expressed genes with fetal neurons and showed a strong expression correlation (Spearman's $r = 0.88$, $p < 2.2 \times 10^{-16}$). LUD-derived astrocytes showed 75.5% overlap with primary human fetal astrocytes cultured under the standard comparison condition (Spearman's $r = 0.77$, $p < 2.2 \times 10^{-16}$; Fig. 4E). When compared with human fetal astrocytes cultured without serum, the proportion of shared expressed genes increased to 85% (Spearman's $r = 0.71$, $p < 2.2 \times 10^{-16}$), indicating that culture conditions substantially influence astrocytic state (Supplementary Fig. S4A-D,

Extended Data Table 3). Functional enrichment analysis showed that LUD-derived neurons were enriched for neurogenesis and synaptic pathways, whereas fetal neurons retained a stronger cortical-patterning signature. LUD-derived astrocytes were enriched for neurogenic and synapse-associated processes, while fetal astrocytes displayed more proliferative signatures (Fig. 4F-G; Supplementary Fig. S4E, Supplementary Table S3F-G; Extended Data Table 4). Cellular component analysis identified enrichment of extracellular matrix components in LUD-derived neurons (Fig. 4H; Supplementary Table S3H) and axonal and synaptic components, including presynaptic and glutamatergic synapse-associated terms, in LUD-derived astrocytes (Fig. 4I; Supplementary Table S3I).

CCL2, TGFB1I1, TGFB2, and VIM were significantly downregulated in LUD-derived astrocytes relative to primary human astrocytes. Together with the morphology and the greater overlap in expressed genes with serum-free fetal astrocytes, this coordinated expression pattern supports a comparatively non-reactive, developmentally oriented astrocytic state. Thus, unrestricted co-development generates neuronal and astrocytic populations with substantial molecular fidelity to the developing human cortex while preserving lineage-specific differences.

Glutamate-induced modulation of axonal transport, calcium dynamics, and viability

Assessment of glutamate effects on axonal transport, calcium dynamics and cell viability was performed using microfluidic chambers (Fig. 5A). Increasing glutamate concentrations reduced anterograde APP transport and increased the proportion of stationary vesicles in LUD cultures, an effect that was further amplified by astrocyte co-culture (Fig. 5B-D; Supplementary Table S4B-D). Thus, astrocytes modified the axonal transport response to glutamatergic stress. Live-cell calcium imaging demonstrated a distinct and attenuated glutamate-evoked calcium response in LUD cultures compared with neuron-only cultures, consistent with greater buffering of excitatory challenge in the multicellular condition (Fig. 5E; Supplementary Tab. S4E). LUD cultures also showed less glutamate-induced apoptosis, without a corresponding difference in necrosis, indicating greater resilience to the experimental challenge (Fig. 5F; Supplementary Tab. S4F). Collectively, these findings show that multicellular co-development alters several functional responses to glutamatergic stress, including axonal transport, calcium dynamics, and cell survival.

DISCUSSION

Our findings identify multicellular co-development as an important determinant of maturation in human stem cell-derived neural cultures. By allowing neuronal and glial lineages to emerge within a shared developmental environment, rather than imposing lineage-specific differentiation, LUD generates a multicellular neural system with structural, molecular and functional characteristics that differ substantially from conventional lineage-directed cultures. LUD cultures form larger and better-organised synaptic structures, develop stronger and more sustained electrophysiological activity, show substantial

transcriptomic correspondence with the developing human cortex, and display increased resilience to glutamatergic stress. These findings address an important limitation of stem cell-based neural models – the incomplete maturation that can result when individual neural populations are generated and studied in isolation – and suggest that developmental interactions between cell types can be exploited to enhance the physiological relevance of human neural cultures.

Stem cell models of human neural development span lineage-directed two-dimensional cultures and three-dimensional organoid systems. Lineage-directed cultures provide experimental control but typically separate cell types that normally mature together, whereas organoids permit multicellular self-organisation and reproduce aspects of neural tissue architecture [16, 17]. Their three-dimensional organisation, however, can introduce diffusion-limited gradients and restrict high-resolution live imaging and experimental manipulation [18]. LUD occupies a complementary experimental space: it retains the accessibility and optical transparency of the two-dimensional system while permitting multiple neural lineages to co-develop. This combination may be particularly useful for resolving how cell-cell interactions influence synaptic organisation, network dynamics and cellular responses to experimental perturbations in human stem cell-derived neural systems.

Despite the absence of enforced lineage instruction, LUD cultures produced cellular compositions comparable to lineage-directed protocols, while showing higher expression of late neuronal maturation markers. This is consistent with the established ability of astrocytes to promote neuronal and synaptic maturation [19, 20]. Network analysis further revealed spontaneous enrichment of glutamatergic signalling hub genes, including GRM2, NCAM1 and GRIA2, alongside disease-associated genes APP and MAPT. The emergence of this glutamatergic identity is compatible with the predominance of glutamatergic neurons in the developing human cortex and with astrocyte-derived signals that promote glutamatergic synaptogenesis [19]. Importantly, this phenotype emerged without imposing a dedicated glutamatergic lineage-specification programme, supporting the concept that the multicellular environment itself contributes to neuronal identity and maturation.

LUD cultures formed fewer but significantly larger pre- and postsynaptic structures. Synapse size is closely related to synaptic strength [26], suggesting that the larger synaptic structures observed in LUD cultures may contribute to their enhanced functional properties. Ultrastructural profiles were enlarged but retained features of immature synapses, indicating that the cultures remain developmentally immature despite their enhanced structural organisation. These findings may therefore represent a developmental intermediate characterised by synaptic enlargement in the presence of incomplete ultrastructural maturation. Multi-electrode array recordings provided complementary functional evidence: LUD cultures developed activity more slowly but subsequently maintained stronger and more sustained network activity up to day 60, contrasting with the earlier activity followed by decline observed in neuron-only cultures. This protracted trajectory is compatible with the extended developmental timeline of the human fetal cortex [1] and supports an important role for the multicellular environment in sustaining developing neural networks. Firing rates were nonetheless lower than those reported for rapid-induction

protocols [27], which we interpret as a consequence of this slower developmental pace rather than as reduced network function.

Transcriptomic comparisons further demonstrated the biological relevance of the LUD system. FACS-sorted LUD neurons shared 97.8% of expressed genes with human fetal cortical neurons, whereas LUD-derived astrocytes shared 75.5–85% of expressed genes with human fetal astrocytes depending on the comparison culture condition. The greater overlap with serum-free fetal astrocytes is consistent with the well-established influence of serum exposure on astrocytic phenotype [9] and highlights the importance of culture conditions when evaluating stem cell-derived glia against primary reference populations.

In parallel, CCL2, TGFB1I1, TGFB2, and VIM were coordinately downregulated in LUD-derived astrocytes relative to primary human astrocytes. Together with their morphology and the greater overlap in expressed genes with serum-free fetal astrocytes, this expression profile supports a comparatively non-reactive, developmentally oriented astrocytic state. We do not interpret these findings as demonstrating complete absence of astrocytic activation. Rather, they indicate that LUD-derived astrocytes differ substantially from strongly reactive serum-exposed astrocytic states. This distinction is potentially important for stem cell-based neural models because astrocytic states can profoundly influence neuronal physiology and experimental responses.

Astrocytes also emerged as important functional modulators within the co-developing cultures. Their presence was associated with attenuation of glutamate-evoked calcium responses, reduced excitotoxic apoptosis and altered axonal APP transport towards stationary arrest, indicating that neuronal responses to glutamatergic stress are strongly influenced by the surrounding glial environment. These observations are consistent with established astrocytic roles in neurotransmitter regulation, metabolic support and calcium homeostasis [28, 29, 30] and illustrate how multicellular context can modify neuronal responses to perturbation. The greater resilience of LUD cultures to glutamatergic challenge further suggests that multicellular stem cell-derived systems may provide a physiologically more informative context for investigating neurotoxicity and disease-associated cellular stress than isolated neuronal cultures.

LUD cultures also showed increased phosphorylation of tau at Thr181. Although this epitope is widely used as a marker of Alzheimer's disease-associated tau pathology [31], tau phosphorylation is a normal feature of fetal brain development and contributes to regulation of microtubule dynamics [32, 33]. In the context of the strong transcriptomic correspondence between LUD cultures and fetal cortical cells, the increased phospho-tau observed here is therefore compatible with a developmental state and should not, by itself, be interpreted as evidence of pathological tau biology. Nevertheless, the concurrent expression of APP and tau and the ability to interrogate APP axonal transport illustrate the potential utility of this multicellular system for mechanistic studies of pathways relevant to neurodegenerative disease.

Several limitations should be considered. Although LUD cultures contain oligodendrocyte-lineage cells, their maturation remained limited, consistent with known constraints of *in vitro* oligodendrocyte

differentiation and myelination [34]. The principal functional effects demonstrated here therefore predominantly reflect neuron-astrocyte interactions. LUD also remains a two-dimensional culture system and does not reproduce the cytoarchitecture, vascular components, immune populations or complete cellular diversity of the human CNS. Moreover, the high transcriptomic correspondence with fetal neural populations indicates that the cultures primarily represent a developmental rather than fully adult neural state. These features should be considered when selecting the system for particular biological or disease-modelling applications. Future studies should determine whether prolonged culture, additional lineage-supporting factors, incorporation of other CNS cell types or application of the LUD paradigm to induced pluripotent stem cell-derived neural progenitors can further increase maturation and disease relevance.

From a stem cell and regenerative medicine perspective, these findings highlight a broader principle: optimisation of neural differentiation may require consideration not only of the efficiency with which individual lineages are generated, but also of the cellular environment in which those lineages develop. Many regenerative strategies ultimately depend on generating neural cells with stable molecular identities, appropriate functional properties and the capacity to interact with surrounding neural populations. Although the present study does not test transplantation or therapeutic efficacy, LUD provides an experimentally accessible system in which these multicellular interactions can be examined directly and may therefore complement more complex three-dimensional and *in vivo* approaches used in regenerative neuroscience.

In conclusion, LUD provides a simple, serum-free strategy for generating multicellular human neural cultures in which neuronal and glial lineages develop together. The resulting cultures demonstrate enhanced synaptic and network maturation, substantial molecular correspondence with human fetal cortical cells and altered responses to excitotoxic stress. By combining the accessibility of two-dimensional culture with developmentally relevant multicellular interactions, LUD provides a potentially useful platform for human stem cell research, neurological disease modelling, neurotoxicity studies and investigation of mechanisms relevant to neural repair and regenerative medicine. More broadly, our findings indicate that developmental potential is determined not only by lineage-intrinsic programmes but also by the cellular context in which differentiation and maturation occur.

CONCLUSION

This study demonstrates that permitting neural lineages to co-develop, rather than differentiating them separately, is itself an important determinant of human neural maturation. Lineage-unrestricted differentiation (LUD) generates neurons and astrocytes with substantial molecular fidelity to the developing human cortex, alongside larger synaptic assemblies, more sustained network activity, and greater resilience to glutamate-induced stress compared with conventional lineage-directed approaches. These findings indicate that developmental potential in human neural cultures is shaped not only by lineage-intrinsic differentiation programmes but also by the opportunity for neural and glial lineages to mature together within a shared multicellular environment.

By combining the scalability and experimental accessibility of two-dimensional culture with a multicellular composition more representative of the developing brain, LUD offers a practical platform for studying the cell-cell interactions that underlie human CNS development. This system is compatible with electrophysiology, live-cell imaging, microfluidics, and molecular profiling, and may provide a useful foundation for future applications in disease modelling, neurotoxicity screening, and the preclinical evaluation of cell-based therapeutic strategies.

METHODS

Human neural stem cell culture

Human neural stem cells (hNSCs) derived from the NIH approved H9 (WA09) human embryonic stem cell line were purchased from Gibco (Thermo Fisher Scientific). They retain their normal female human karyotype and the potential to differentiate into neurons and glial cell populations following multiple passages. Cells were maintained on Matrigel-coated 10-cm culture dishes. Plates were coated with Matrigel (Corning), diluted 1:100 in DMEM/F12, for 1 h at 37°C. Excess Matrigel was removed prior to seeding.

hNSCs were maintained and amplified up to passage 4 using neural stem cell medium (NSCM) consisting of KO/DMEM-F12 (Gibco), 2% StemPro Neural Supplement (Gibco), 2 mM GlutaMAX-I (Gibco), 20 ng/mL basic fibroblast growth factor (bFGF; Gibco), and 20 ng/mL epidermal growth factor (EGF; Gibco). Media were fully replaced every other day until cultures reached confluency (2–7 days). Cells were then washed with phosphate-buffered saline (PBS), detached using Accutase (Life Technologies), and reseeded for differentiation.

Neuronal differentiation

Neuronal differentiation was achieved using a modified published protocol [6]. Confluent hNSCs were first induced into neuronal progenitors using neural progenitor medium (NPM) composed of DMEM/F12, 0.5% B27 Supplement (50x; Gibco), 0.5% N2 Supplement (100x; Gibco), 2 mM GlutaMAX-I (Gibco), and 1% penicillin/streptomycin (VWR). Cultures were maintained for 6–9 days with daily replacement of half of the medium volume.

Neuronal progenitors were then plated onto poly-L-ornithine/laminin-coated 24-well plates. Plates were coated overnight at 37°C with poly-L-ornithine (0.1 mg/mL in PBS; Sigma), washed twice with distilled water, and incubated with laminin (10 µg/mL in PBS; Invitrogen) for 3 h at 37°C. Cells were detached using Accutase (2–3 min, at 37°C), pelleted (250 x g, 5 min), counted, and resuspended in neuronal differentiation medium (NDM). NDM consisted of Neurobasal medium (Gibco), 1% B27 Supplement (Gibco), 1% N2 Supplement (Gibco), 1% penicillin/streptomycin (VWR), 10 ng/mL brain-derived neurotrophic factor (BDNF; Gibco), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF; Gibco), 10 ng/mL insulin-like growth factor 1 (IGF-1; Gibco), 1 µg/mL laminin (Invitrogen), 1 µM cAMP (Sigma), and

200 ng/mL L-ascorbic acid (Sigma). Cells were seeded at 100,000 cells per well and maintained for 21–25 days, with medium changes once or twice weekly.

Astrocytic differentiation

Astrocytic differentiation was performed as previously described [15]. hNSCs were seeded at 60,000 cells per well on Matrigel-coated 24-well plates and cultured in astrocyte differentiation medium (ADM) consisting of high-glucose DMEM (Biotech), 1 mM sodium pyruvate (Gibco), 1% N2 Supplement (Gibco), 2 mM GlutaMAX-I, 1% fetal bovine serum (FBS; Sigma), and 1% penicillin-streptomycin. Cultures were maintained for 30 days with medium changes 2–3 times per week.

Oligodendrocytic differentiation

Oligodendrocytic differentiation was carried out using modified published protocols [11, 35]. hNSCs were seeded at 60,000 cells per well on Matrigel-coated 24-well plates and incubated overnight.

Differentiation was induced using oligodendrocyte differentiation medium (ODM) composed of DMEM-F12 (Gibco), 1% non-essential amino acids (Gibco), 1% N2 Supplement (Gibco), 1% B27 Supplement (Gibco), 60 ng/mL triiodothyronine (T3; Sigma), 100 ng/mL biotin (Sigma), 1 μ M cAMP (Sigma), 10 ng/mL platelet-derived growth factor receptor- α (PDGFR α ; Peprotech), 10 ng/mL neurotrophin-3 (NT-3; Peprotech), 10 ng/mL IGF-1 (Peprotech), and 1% penicillin-streptomycin. Cultures were maintained for 30 days with medium changes every 2–3 days.

Lineage-unrestricted differentiation

For lineage-unrestricted differentiation, confluent hNSCs cultured on Matrigel-coated plates were directly differentiated without a progenitor expansion phase. Cells were cultured in high-glucose DMEM supplemented with 1 mM sodium pyruvate (Gibco), 2 mM GlutaMAX-I (Gibco), 1% N2 Supplement (Gibco), 0.5% penicillin/streptomycin, 30 ng/mL T3 (Sigma), and 10 ng/mL ciliary neurotrophic factor (CNTF, Peprotech). Cultures were maintained for 30 days, with complete medium replacement every 2–3 days during the first two weeks and half-medium replacement every 3 days during the final two weeks.

Primary human fetal cortical cell cultures

Primary human neurons (Neuron^{human}, ScienCell), isolated from cerebral cortices of 20-week-old human fetal brains, were cultured for 30 days in neuronal medium according to the manufacturer's instructions (ScienCell) [36]. Primary human astrocytes (Astrocyte^{human}, ScienCell), also derived from fetal cerebral cortices, were cultured and passaged in human astrocyte medium for 6 days, with or without serum, following the manufacturer's instructions.

Flow cytometry

Cells were detached using 0.5 mM EDTA in PBS for 15 min at 37 °C and 5% CO₂, pelleted, and washed in FACS buffer (PBS without Mg²⁺/Ca²⁺, 0.5 mM EDTA, 0.5% BSA 1% penicillin/streptomycin). Cells were filtered through 70 μ m strainers and resuspended at 1 x 10⁶ cells/mL. All staining steps were performed at 4 °C.

Cells were incubated with fluorophore-conjugated primary antibodies for 30 min, washed, and incubated with secondary antibodies when required. Propidium iodide was used for nuclear staining. Data were acquired using a BD FACS Canto II cytometer (BD Biosciences) and analyzed using FlowJo software (v8.7.3). Gating was defined relative to secondary-only controls. At least three biological replicates were analyzed per condition.

Gene expression analyses

Total RNA was extracted using the RNeasy Mini Kit and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). cDNA was synthesized from 250 ng RNA. Quantitative PCR was performed with Power SYBR Green PCR Master Mix on a Bio-Rad real-time PCR system. Primer sequences are listed in the corresponding table. Gene expression was analysed using the $2^{-\Delta CT}$ method and normalized to RPL13, RPL27, and MB2. Each sample was analyzed using three biological and three technical replicates.

Neurogenesis RT² Profiler PCR Arrays were performed using 384-well plates containing primers for 84 neurogenesis-related genes. RNA (1 µg) was reverse-transcribed and analysed using the $2^{-\Delta CT}$ method. Three biological and three technical replicates were analyzed per condition.

Immunocytochemistry

Cells (28–30 days *in vitro*) were fixed with 4% paraformaldehyde and 4% sucrose for 30 min, permeabilized with PBS containing 0.01% Triton X-100, and blocked in 10% goat serum for 1 h at room temperature. Cultures were incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 2 h at room temperature. Nuclei were stained with DAPI. Coverslips were mounted with Mowiol and imaged using Zeiss LSM 780 confocal laser scanning microscope (Zeiss, Germany) equipped with a Plan-Apochromat 40x/1.3 NA oil objective. Quantification was performed with Imaris (v9.2). Three biological and three technical replicates were analyzed per condition.

Electron microscopy

Cells grown on glass coverslips were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1h, post-fixed with 1% osmium tetroxide and contrasted with 1% uranyl acetate. Samples were dehydrated through graded ethanol, embedded in Epon/Araldite resin, sectioned (70 nm), and mounted on formvar-coated copper grids for imaging.

Electrophysiology

hNSCs were seeded onto Matrigel-coated 96-well multi-electrode array (MEA) plates (Axion, M768-tMEA-96W) at 5,000 cells per well and differentiated as described. For recordings, DMEM-based media were replaced with BrainPhys medium (Stemcell Technologies). Recordings were performed up to day 60 using the Axion Maestro system. Data were analyzed using AxIS software (v2.4). Electrodes were classified as active using a threshold of 1 spike/min, lower than the manufacturer's default of 5 spikes/min, to preserve sensitivity to emerging, low-frequency activity during early network formation

and capture the developmental trajectory of network activity across the full culture period. Bursts were defined as > 5 spikes with inter-spike intervals < 100 ms. Each condition included three biological replicates and 32 technical replicates.

ELISA

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. Lysates were clarified by centrifugation (12,000 x g; 20 min; 4 °C), and protein concentration was determined using a BCA assay. Phosphorylated tau (pTau-181), total tau, and BDNF levels were quantified using commercial ELISA kits (Fujirebio, Gent, Belgium; R&D Systems, USA) according to manufacturers' instructions.

FACS sorting

Neuronal populations (CD24+/CD56+/CD44-) were identified by surface marker expression and sorted live. Astrocytic populations (GFAP+/NeuN-) were identified by intracellular/nuclear marker expression; cells were fixed in 50% ethanol (10 min, 4°C) prior to immunolabeling and sorting, consistent with established FACS protocols for intracellular marker labeling in neural tissue [37]. Sorting was performed on a MoFlo Astrios cell sorter, with unstained controls used to assess autofluorescence. Three biological replicates were analyzed per condition.

RNA sequencing and analysis

RNA was extracted using the Direct-zol RNA MicroPrep Kit (Zymo Research), with on-column DNase I treatment. RNA integrity was assessed by Agilent 2100 Bioanalyzer. Sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA protocol with poly(A) selection and sequenced on an Illumina NovaSeq 6000 platform (paired-end, 150 bp). Reads were adapter- and quality-trimmed using Cutadapt and assessed with FastQC, then aligned to the human reference genome (GRCh38, Ensembl release 96) using HISAT2 [38]. Transcript assembly and quantification were performed with StringTie [39], and differential expression was analyzed using DESeq2, with genes considered significantly changed at $FDR \leq 0.05$.

Microfluidic chambers

Microfluidic chambers (Xona Microfluidics) were bonded to poly-ornithine- and Matrigel-coated coverslips. NSCs or NPCs were seeded into the neuronal compartment, and astrocytes were added to the glial compartment at DIV30. Axonal crossing was observed by DIV40 [40].

Transfection, live-cell imaging, and functional assays

Transfections were performed using Lipofectamine 2000 and pcDNA APP-GFP. Calcium imaging was conducted using Fluo-4AM, and glutamate responses were quantified by $\Delta F/F$ analysis. Apoptosis and necrosis were assessed using TUNEL and propidium iodide staining, respectively. Axonal transport was analyzed using time-lapse imaging with a Zeiss Confocal LSM780 confocal microscope.

Gene ontology

Gene ontology enrichment analysis was carried out using WebGestalt [41]. Differentially expressed genes were filtered using a minimum threshold of 10 genes per ontology term, and statistical significance was assessed using Benjamini-Hochberg false discovery rate (FDR) correction. Disease association network analysis was conducted using the Disease Alliance database.

Statistical analysis

Statistical analyses were selected according to the experimental design and are specified for each analysis and in the corresponding figure legends. For simple two-group comparisons, unpaired two-sided Student's t-tests were used where appropriate. Analyses involving multiple factors or conditions used analysis of variance (ANOVA) with the indicated post hoc comparisons. Statistical significance was defined as $p < 0.05$ unless otherwise specified. Biological replicates represent independent cultures/experiments; technical replicates represent repeated measurements within a biological replicate and were not interpreted as independent biological samples.

Longitudinal MEA data were analyzed using growth models incorporating linear and quadratic time terms to compare temporal trajectories between conditions. Fourth-degree polynomial curves were used for visualization only and were not used for inferential testing.

Axonal transport was classified by direction and velocity using the prespecified thresholds applied in the original analysis. Velocity comparisons and particle-distribution analyses were performed as specified for the corresponding experiments.

Calcium-response data were analyzed using mixed linear regression across baseline, reaction, and post-reaction phases, accounting for repeated measurements across phases.

Differential expression and PCA analyses were performed using DESeq2 with an FDR threshold of < 0.05 . Hub gene identification was achieved using Cytoscape (StringApp, v.1.7.1) and CytoHubba (v.0.1), with consensus hubs identified using UpSetR. Analyses were conducted using RStudio, Cytoscape and GraphPad Prism 9.

Ethics statement

All methods were performed in accordance with the relevant guidelines and regulations. Ethical approval was not required for the studies on humans in accordance with local legislation and institutional requirements, because only commercially available, established human cell lines were used (H9/WA09 human embryonic stem cells, Thermo Fisher Scientific; primary human fetal cortical neurons and astrocytes, ScienCell Research Laboratories). However, informed consent was obtained from all original subjects and/or their legal guardians by the deriving institutions and commercial vendors prior to the derivation and commercial distribution of these cell lines, in accordance with applicable ethical standards.

Abbreviations

ADM
astrocyte differentiation medium

APP
amyloid precursor protein

BDNF
brain derived neurotrophic factor

CNS
central nervous system

CNTF
ciliary neurotrophic factor

DEGs
differentially expressed genes

DMEM
Dulbecco's Modified Eagle Medium

ELISA
enzyme-linked immunosorbent assay

FACS
fluorescence-activated cell sorting

FDR
false discovery rate

GFP
green fluorescent protein

GO
Gene Ontology

hNSCs
human neural stem cells

LUD
lineage-unrestricted differentiation

MAPT
Microtubule-Associated Protein Tau

MEA
multi-electrode array

NDM
neuronal differentiation medium

ODM
oligodendrocyte differentiation medium

PBS
phosphate-buffered saline

PCA
principal component analysis
RIPA
radioimmunoprecipitation assay (buffer)
SEM
standard error of the mean
TEM
transmission electron microscopy
TUNEL
terminal deoxynucleotidyl transferase dUTP nick end labeling

Declarations

Ethics statement

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Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

M.Č. and G.B.S. conceived this study. M.Č. performed most experiments and prepared the figures. M.F, J.B, and T.L. contributed to cell sorting. K.K. and G.V.J. performed axonal transport assays. V.M.P.D. conducted electrophysiology experiments. D.M.J. and I.G.O. performed ELISA assays. N.D., L.S., and T.S. analyzed immunocytochemistry. P.A. performed RNA-sequencing experiment. M. Č. and J.S.N. analyzed source data. M.Č. and G.B.S. wrote the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its supplementary information files. Additional data not included in the supplementary materials are available from the corresponding author upon reasonable request.

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Figures

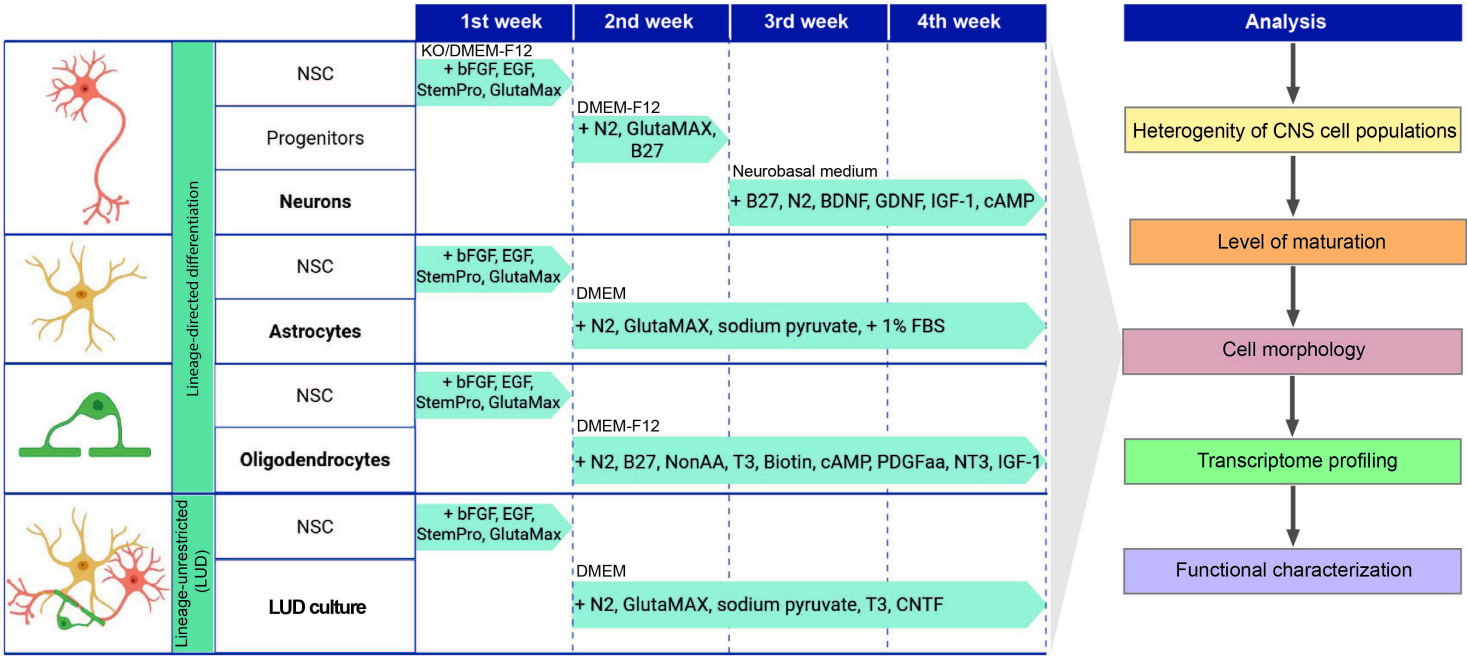


Figure 1

Differentiation overview and experimental design

Schematic representation of the differentiation protocols used, including neuron-, astrocyte-, oligodendrocyte-directed conditions, as well as the LUD system. Media formulations (DMEM, DMEM/F12, KO/DMEM-F12, and Neurobasal) and supplements are indicated across the differentiation timeline. Analytical approaches including morphological assessment, transcriptome profiling, and functional characterization are summarized, illustrating the multi-level strategy used to evaluate CNS cell populations and maturation.

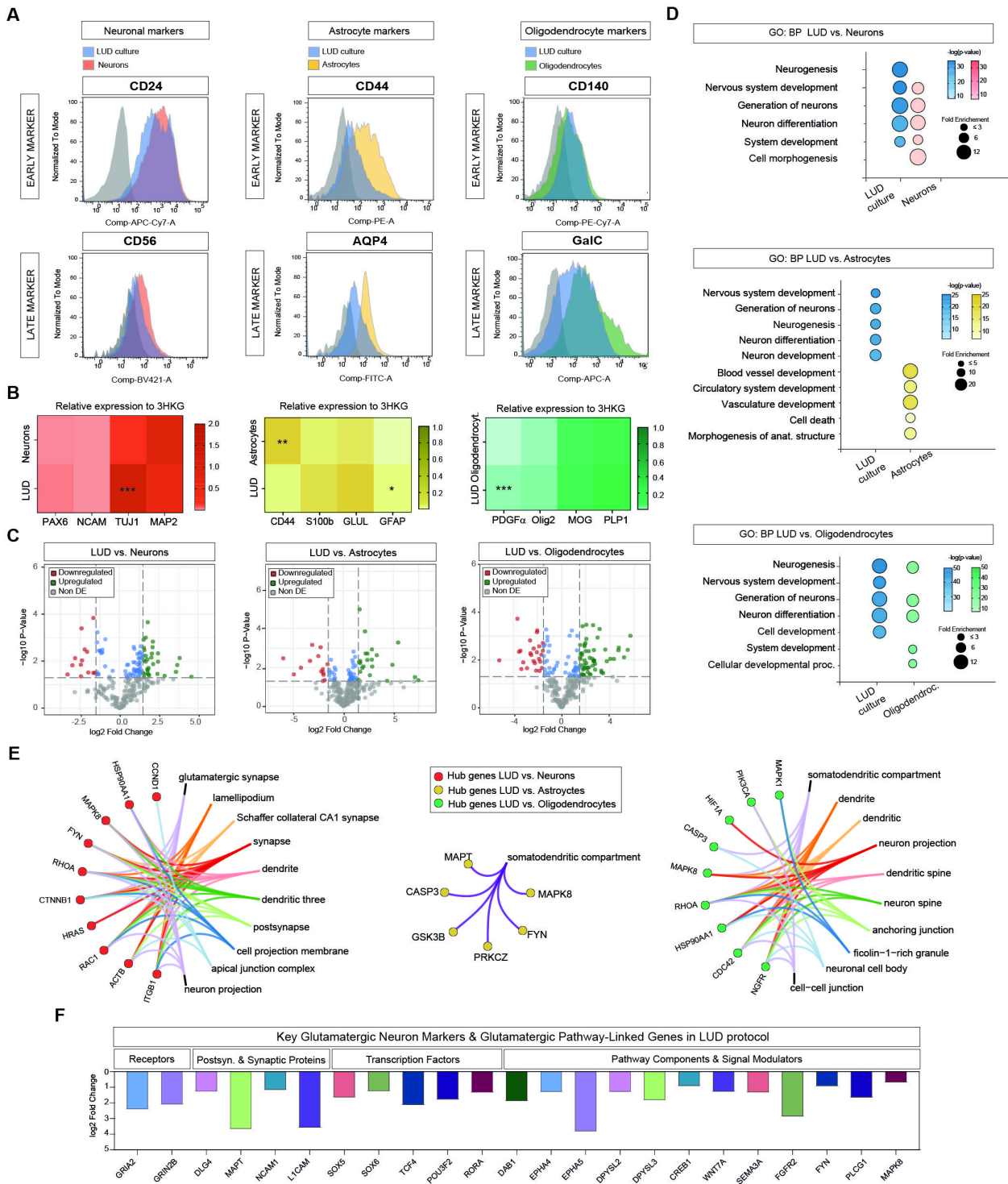


Figure 2

Cell population balance and identity

A) Representative flow cytometry histograms showing expression of neuronal (CD24, CD56), astrocytic (CD44, AQP4), and oligodendrocytic (CD140, GalC) markers in LUD and lineage-directed cultures.

B) Quantitative PCR analysis of lineage-specific markers demonstrating comparable proportions of neurons (PAX6, NCAM, TUJ1, MAP2), astrocytes (CD44, S100 β , GLUL, GFAP), and oligodendrocytes (PDGF α , Olig2, MOG, PLP1) across differentiation conditions. Data are mean $\pm$ SEM from three biological replates, each with three technical replicates. Statistical significance was assessed using unpaired t-test (* p < 0.05, ** p < 0.005, *** p < 0.0005).

C) Volcano plots depicting differentially expressed genes (DEGs) between LUD cultures and neuron-, astrocyte-, or oligodendrocyte-directed protocols (FDR-adjusted p -values).

D) Gene Ontology (GO) enrichment analysis of biological processes upregulated in LUD cultures, neurons, astrocytes, and oligodendrocytes.

E) Identification of hub genes and enriched cellular components in LUD cultures relative to lineage-directed conditions.

F) Selected glutamatergic neuron markers and genes associated with glutamatergic signaling significantly upregulated in LUD cultures, supporting a glutamatergic neuronal phenotype.

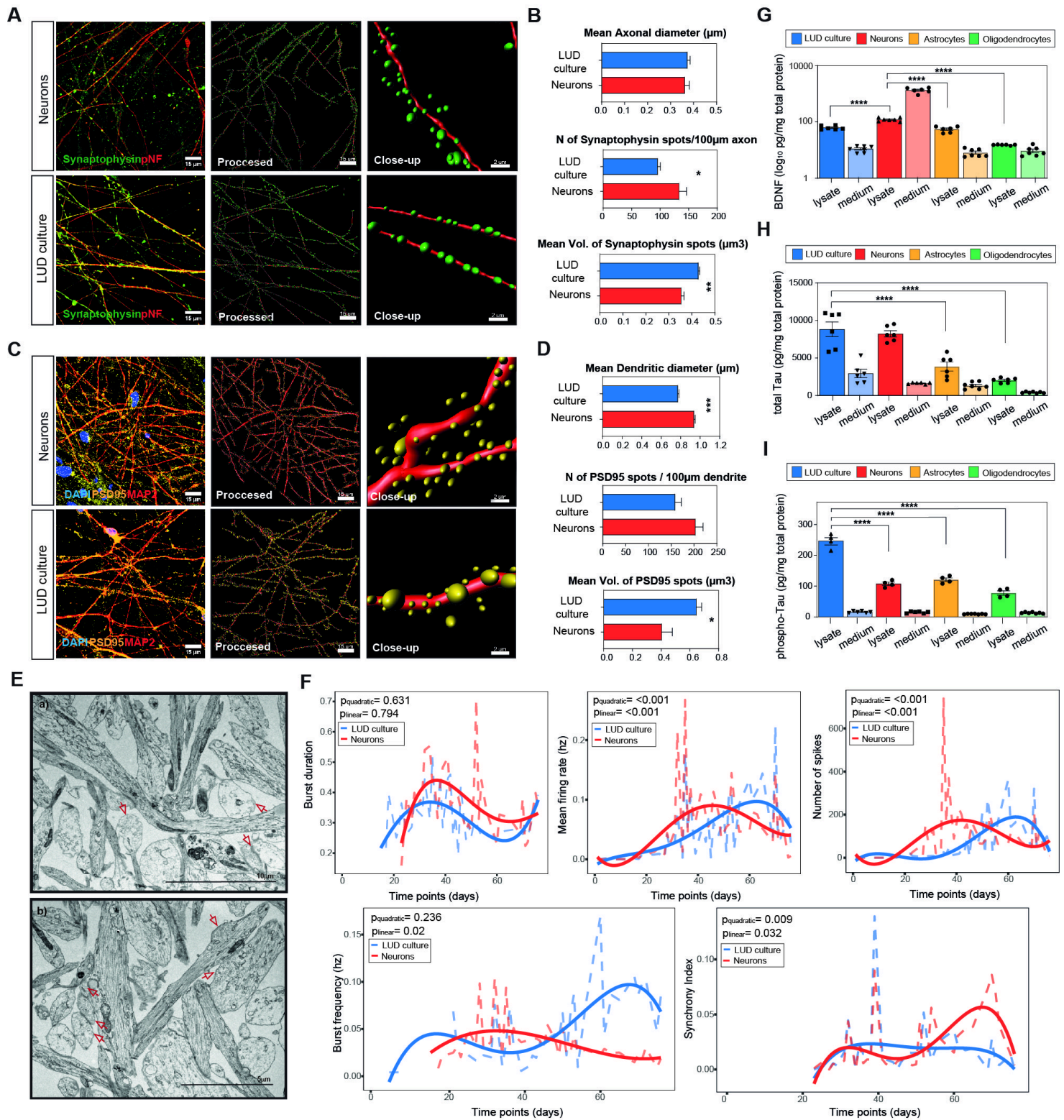


Figure 3

Synaptic maturation and electrophysiological activity

A) Representative confocal Z-stack images and Imaris reconstructions of LUD and neuron-only cultures stained for axons (phosphorylated neurofilament heavy chain, pNF), presynaptic puncta (synaptophysin), and nuclei (DAPI). Scale bars: 15 μm and 2 μm .

B) Quantification of presynaptic features: puncta diameter, number, and volume of synaptophysin-positive structures. Data are mean $\pm$ SEM from three biological replicates with three technical replicates each (* p < 0.05, ** p < 0.01; unpaired t-test).

C) Representative confocal Z-stack images and Imaris reconstructions stained for dendrites (MAP2), postsynaptic puncta (PSD95), and nuclei (DAPI). Scale bars: 15 μ m and 2 μ m.

D) Quantification of postsynaptic features: dendritic diameter and PSD95-positive puncta number and volume. Data are mean $\pm$ SEM from three biological replicates with three technical replicates each (* p < 0.05, *** p < 0.001; unpaired t-test).

E) Transmission electron microscopy (TEM) images showing synaptic ultrastructure in LUD cultures. Scale bars: 10 μ m and 5 μ m.

F) MEA recordings showing delayed onset but stronger, and more sustained network activity in LUD cultures compared with neuron-only cultures. Metrics include burst duration, mean firing rate, spike counts, burst frequency and synchrony index. Dashed lines indicate means from three biological replicates (32 technical replicates per time point); solid lines show fourth-degree polynomial regression fits. Used for visualization. Longitudinal differences were evaluated using statistical approach described in Methods.

G-I) ELISA quantification of BDNF, total tau, and phosphorylated tau levels in cell lysates and conditioned media from LUD, neuron-, astrocyte-, and oligodendrocyte-derived cultures. Data are mean $\pm$ SEM from three biological replicates with two technical replicates each (**** p < 0.0001; unpaired t-test).

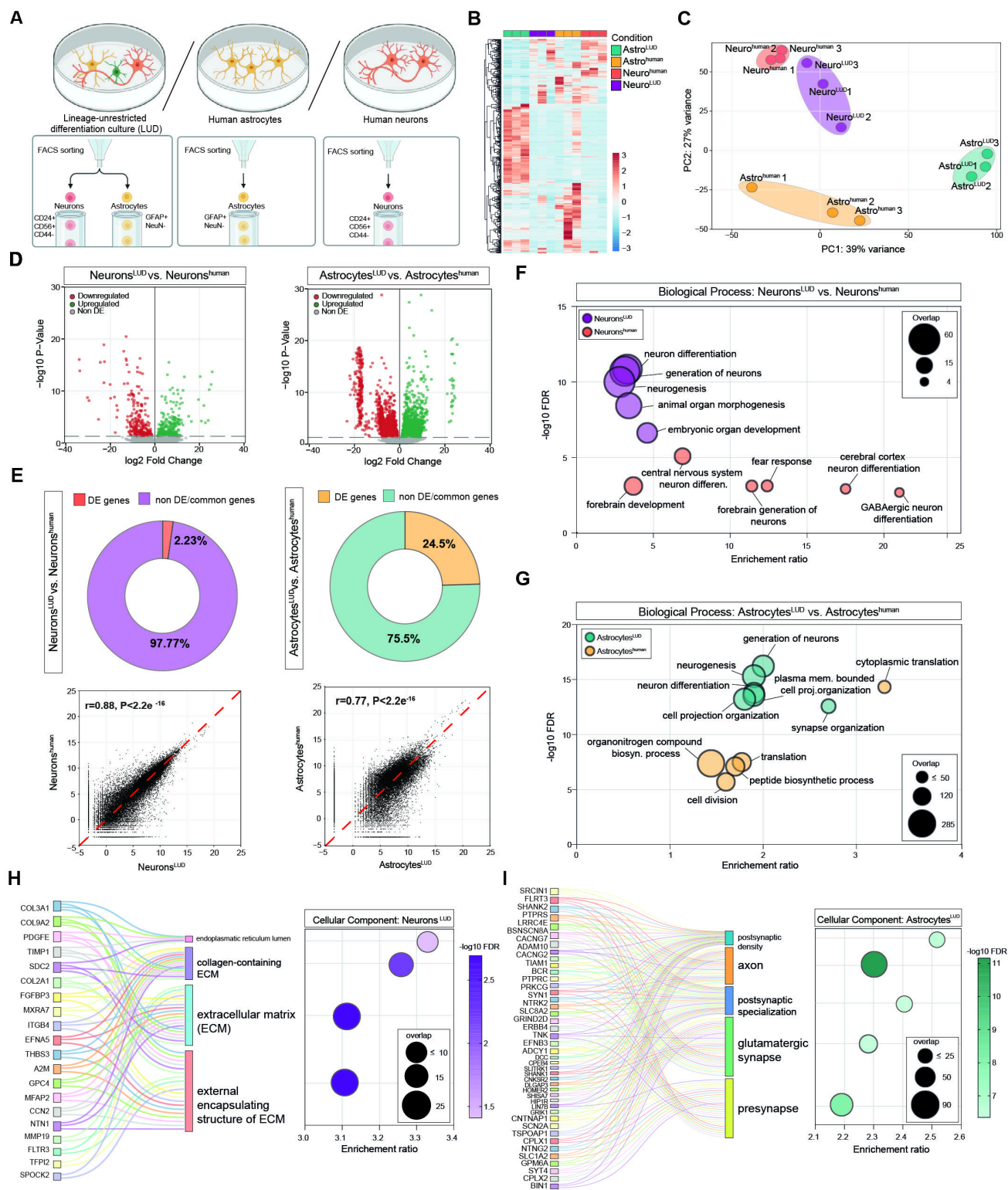


Figure 4

Transcriptomic fidelity of LUD cultures to human fetal brain

A) Schematic of FACS-based isolation of neurons (CD24⁺, CD56⁺, CD44⁻) and astrocytes (GFAP⁺, NeuN⁻) from LUD cultures and 24-week human fetal cortical tissue cultured for 30 days *in vitro*, followed by RNA sequencing.

B) Heatmap showing expression profiles of top 500 most variable genes (log2 of r-transformed counts) found in LUD-derived neurons and astrocytes compared with primary human fetal cortical neurons and astrocytes.

C) Principal component analysis (PCA) showing clustering of LUD-derived neurons with fetal neurons and overlap of LUD astrocytes with fetal astrocytes.

D) Volcano plots showing DEGs between LUD-derived cells and fetal cortical neurons (left) or astrocytes (right).

E) DEG overlap and correlation analysis: LUD neurons showed a 97.8% overlap with fetal neurons ($r = 0.88, p < 2.2 \times 10^{-16}$), LUD astrocytes showed a 75.5% overlap with fetal astrocytes ($r = 0.77, p < 2.2 \times 10^{-16}$).

F-G) GO enrichment analysis of biological processes upregulated in LUD-derived neurons (F) and LUD-derived astrocytes (G) relative to fetal populations.

H-I) Sankey plots of top significantly enriched GO cellular components in LUD-derived neurons (H) and LUD-derived astrocytes (I). The plot displays enriched cellular components (right) and the corresponding genes (>3 genes per term) (left). Enrichment ratio and FDR values for each component are shown alongside to indicate significance.

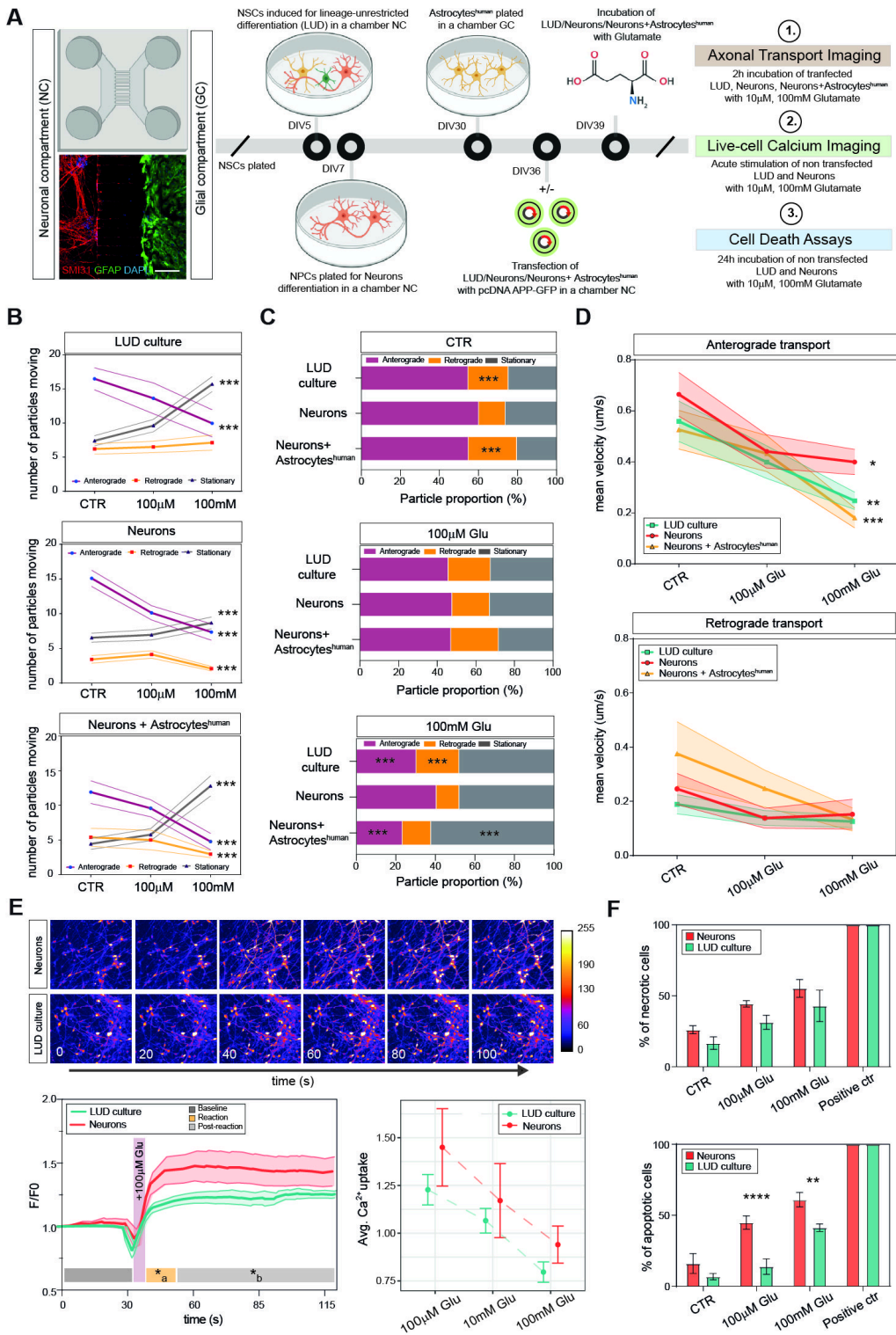


Figure 5

Glutamate-induced alterations in axonal transport, calcium dynamics, and neurotoxicity

A) Experimental timeline for neuronal-only cultures, neuron-astrocyte (ScienCell) co-cultures, and LUD cultures, followed by APP-GFP transfection and glutamate challenge.

B) Total anterograde, retrograde, and stationary APP-GFP particles per axon under control conditions and after 100 mM/100 mM glutamate across culture conditions. Data are mean $\pm$ SEM from three biological replicates (min 10 axons per condition). Statistical significance was assessed by one-way ANOVA with post-hoc tests ($***p < 0.001$).

C) Proportion of anterograde, retrograde, and stationary particles under control conditions and glutamate conditions. Statistical significance was assessed using the tests specified in Methods ($***p < 0.001$).

D) Mean velocity of anterograde and retrograde APP-GFP transport under control or glutamate-treatment conditions. Data are mean $\pm$ SEM from three biological replicates. Statistical significance was assessed as described in Methods ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

E) Live-cell calcium imaging showing glutamate-evoked Ca^{2+} responses in LUD and neuron-only cultures during baseline, reaction, and post-reaction phases. Line plots depict response dynamics; asterisks indicate significantly attenuated responses in LUD cultures during reaction (interaction, $*_a$) and sustained post-reaction elevation ($*_b$), determined by mixed linear regression. Error-bar plots summarize the post-reaction response. Statistical significance was assessed using mixed linear regression as described in Methods ($*p < 0.05$).

F) TUNEL and propidium iodide staining quantifying apoptotic and necrotic cells after 24 h glutamate exposure in LUD *versus* neuron-only cultures. Data are mean $\pm$ SEM from four biological replicates. Two-way ANOVA with multiple comparisons ($**p < 0.01$, $****p < 0.0001$).

Supplementary Files

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