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Assembloid models of cell-cell interaction to study tissue and disease biology

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Abstract

Neurodevelopment involves the migration, projection, and integration of various cell types across different regions of the nervous system. Assembloids are self-organizing systems formed by the integration of multiple organoids or cell types. Here, we outline the generation and application of assembloids. We illustrate how assembloids recapitulate critical neurodevelopmental steps, like migration, axon projection, and circuit formation, and how they are starting to provide biological insights into neuropsychiatric disorders. Additionally, we review how assembloids can be used to study properties emerging from cell-cell interactions within non-neural tissues. Overall, assembloid platforms represent a powerful tool for discovering human biology and developing therapeutics

Introduction

Tissue physiology involves dynamic interactions between different cell lineages, which are particularly evident during the earlier stages of development. Cells typically originate from distinct stem cell niches, then migrate and interact with other cell types to develop specialized functions of individual tissues.[1] This coordinated effort is crucial in many biological systems, such as neural development. Diverse neuronal cell types are often born at distance from each other, coming together through migration and axonal projection to form functional neural networks.[2,3] For example, neural cells produced along the neural tube in response to spatial and temporal signals migrate and extend axons to assemble and connect with other cells, forming the complex arrangements of brain nuclei and regions in the human nervous system.[4] Interactions among neuronal and glial cells, both within and between different regions of the nervous system, are essential for initiating maturation programs and refining neural circuitry, ultimately influencing internal neural function and

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Declaration of interests

Stanford University holds patents for the generation of human cortical organoids, assembloids, and their transplantation.

external behavior.[5,6] Disruptions in these critical developmental processes, whether through genetic or environmental factors, can lead to neuropsychiatric diseases.

The generation of organoids– self-organizing three-dimensional (3D) cellular models derived from pluripotent stem cells or primary tissue[7,8]– is beginning to address some challenges associated with studying the developing human central nervous system directly[8–12]. However, since fate specification in regionalized neural organoids primarily relies on the addition of guidance molecules to the culture medium, these models do not generally produce multiple niches. Consequently, organoids often do not capture interactions across different distant brain regions or among different germ layer lineages. In this review, we will explore the derivation and application of assembloids as a platform to model emergent properties during development as well as disease states.

Assembloids

Assembloids are 3D preparations formed by the fusion and functional integration of different organoids with each other or with other specialized cell types. When designed with relevance to the nervous system, these multi-cellular models can mimic both inter-regional or intra-regional cell-cell interactions.[8,13,14] Below, we will discuss key interactions in the nervous system that can be modeled with assembloids. These include neural migration, axon guidance, circuit formation, and interactions with vascular and immune systems.[14] Given the large volume of literature on self-organizing human in vitro systems, we will limit the content of this review to only assembloid studies, excluding models such as mosaic organoids and “on-a-chip preparation”.

Migration of Cells in Neural Assembloids

The process of neural migration is crucial for the proper formation of neural circuits, requiring precise spatial and temporal coordination of neurons across different regions of the nervous system. Despite notable progress in the field, the detailed molecular and cellular mechanisms driving neural migration in primates, as well as the roles of genetic and environmental factors, remain poorly understood. This is further complicated by the difficulty in accessing the human brain during critical periods of development, particularly in the late prenatal and early postnatal stages when neural migration events are occurring.

Assembloids offer a modular approach to studying neural migration by combining multiple regionalized neural organoids. This allows for the reconstruction of specific neural pathways and the investigation of cell migration dynamics, such as to model cortical interneuron migration or the invasion of cancer cells (Figure 1).

During human cortical development, neural networks are formed by integrating circuits from glutamatergic neurons of the dorsal forebrain with GABAergic neurons originating from the specific subpallial domains such as the medial and caudal ganglionic eminences. These GABAergic neurons migrate to the dorsal forebrain, where they mature and synaptically integrate into cortical circuits.[2,15] By utilizing guided neural differentiation, pallial (dorsal forebrain) organoids containing primarily glutamatergic neurons can be generated in parallel with subpallial organoids containing mostly GABAergic neurons. These pallial and

subpallial organoids can be strategically placed in proximity to encourage morphological and functional integration to form forebrain assembloids[16–20]. This assembly facilitates the unidirectional saltatory migration of human interneurons from the ventral forebrain to the dorsal forebrain, culminating in their functional incorporation into microcircuits.[17] While these interneurons follow migration patterns similar to those observed in rodents, human subpallial-derived interneurons in forebrain assembloids exhibit larger processes with lower saltation frequency and speed, aligning with observations from mid-gestational human primary forebrain tissue explants.[17,21]

Forebrain assembloids have already become a valuable tool for elucidating disease mechanisms. For example, in Timothy syndrome– a neurodevelopmental disorder associated with autism spectrum disorder, intellectual disability and epilepsy–, cortical GABAergic interneurons display an abnormal migration pattern.[17] Studies using patient-derived forebrain assembloids revealed two distinct phenotypes and the underlying mechanisms contributing to these abnormalities: a decrease in saltation length, which is regulated by increased calcium influx through voltage-gated L-type calcium channels causing cytoskeletal disruptions during nucleokinesis, and an increase in saltation frequency, which is downstream of an upregulation of GABAergic receptors and GABA sensitivity.[21] These findings provide new insights into how L-type calcium channels regulate the development of human cortical interneurons in pathological contexts. Recently, we developed a therapeutic strategy that leverages antisense nucleotide-mediated switching from the exon carrying the Timothy syndrome mutation towards a counterpart unaffected exon. This approach has been successfully tested in assembloids, where it can restore migration defects. [22]

Forebrain assembloids can also be leveraged to conduct CRISPR screens and map hundreds of disease genes onto stages of human interneuron development. These pooled CRISPR screens, performed in ~1,000 assembloids, have pinpointed the endoplasmic reticulum related gene *LNPK* as a critical regulator of interneuron migration.[23] During interneuron migration process, the endoplasmic reticulum is displaced along the leading neuronal branch prior to nuclear translocation. Deletion of *LNPK* disrupts endoplasmic reticulum displacement, resulting in abnormal migration. This disruption likely contributes to imbalances in excitation-to-inhibition ratio in the cortex and the severe epileptic encephalopathy observed in patients with loss-of-function mutations. These findings underscore the potential of CRISPR-assembloid platforms to systematically identify the role of neurodevelopmental disorder genes in previously inaccessible stages of human development and to uncover disease mechanisms.

Furthermore, the assembloid platform offers significant promise for studying other complex migratory programs, such as the migration of neural crest cells. Neural crest migration is regulated by secreted molecules, extracellular matrix components, and various cell-cell interactions that guide the their direction, speed, and differentiation into terminal cell types.[24,25] The assembly of neural crest cells with various types of organoids, both neural and non-neural, could help identify some of the signals regulating neural crest cell fate decisions and the role of specific signaling molecules in migration. Recent advances in protocols for deriving neural crest cells from induced pluripotent stem (iPS) cells have enabled detailed studies on neural crest development and differentiation into

neural, mesenchymal, and melanocyte lineages.[26] Assembling hiPS cell-derived neural crest cells with neural organoids allows the study of migration patterns across various tissues, providing insights into how environmental contexts influence the fate of neural crest cells. This neural crest cell assembloid can be used to investigate disorders affecting the peripheral nervous system. For instance, it can be applied to identify the molecular pathways disrupted by genes associated with conditions such as Hirschsprung's disease, in which there is a failure of neural crest cells to migrate, proliferate, or differentiate properly resulting in the absence of enteric ganglion cells.[27] Moreover, human stem cell-derived cells hold promise for developing cell therapies.

Assembloids can also be used to investigate metastatic cancer events, such the infiltration and migration of tumor cells within the nervous system, by combining cancer cells with neural organoids.[28] For instance, the combination of tumor cells from primary tissue-derived glioblastoma organoids with hiPS-cell derived neural organoids enabled the infiltration of metastatic cells into neural organoids, These studies highlighted distinct patterns of tumor cell compartmentalization, migration and depth of invasion compared to noncancerous adult neural progenitors.[29] Additionally, this model has been effectively employed to assess different forms of immunotherapy-relevant interactions, including by co-culturing CAR-T cells with glioblastoma organoids.[30] Such applications underscore its potential for rapidly evaluating responses to antigen-specific CAR-T cell treatments, with implications for personalized medicine.

The assembloid approach holds great potential for modeling evolutionarily unique features of other cell migratory events. Comparative studies of adult primate and rodent telencephalon have revealed a primate-enriched population of striatal interneurons expressing the neuropeptide TAC3.[31] This discovery represents an interesting case where an evolutionarily novel class of neurons constitutes a significant proportion of the striatal interneuron population in primates. The regions behind the migration of these TAC3 positive interneurons to the striatum remain unknown. Notably, the human striatum is uniquely susceptible to dysfunction throughout neurodevelopment and is disrupted in various neuropsychiatric disorders, such as schizophrenia. Interestingly, the TAC3 pathway has been proposed to contribute to schizophrenia and has been a target for therapeutic intervention.[32] This underscores the importance of developing an organoid model containing TAC3 interneurons to elucidate the role of TAC3 pathway in both striatal development and disease. Assembling TAC3 interneuron organoids with striatal organoids could provide insights into the origin and development of TAC3 populations in the human striatum, potentially uncovering what makes it evolutionarily unique and susceptible to disease.

The positioning of dopaminergic neurons can be modeled using the assembloids platform. Midbrain dopaminergic neurons can be efficiently generated by specifying floor plate precursors, followed by neural differentiation and maturation.[33] By combining hiPS cell-derived floor plate cells with mesencephalon organoids, there is potential to model the origin, initial differentiation, and migration of midbrain dopaminergic neurons from the ventral floorplate to the ventral mesencephalon. Subsequent fusion with striatal or thalamic organoids could allow to incorporate neuronal inputs from medium spiny neurons and outputs to the diencephalon, respectively.

Assembloids to Study Human Axon Projection and Guidance

After neurons migrate and find their final position, they extend axons through the intricate extracellular and molecular environments of the nervous system.[34] The establishment of proper formation of neural circuits relies on precise communication between axon guidance and cell adhesion molecules.[35] This process involves detection of guidance molecules, which either attract or repel axons in the developing embryo.[36] In recent years, there has been increasing recognition of the role of axon guidance defects in human neurodevelopmental disorders.[37–39] This emphasizes the need for developing human models, particularly assembloids, which enable the investigation of cell-cell interactions, including the guidance of axonal projections (Figure 1).

Assembloids present a versatile framework for investigating axon projection and guidance by integrating multiple regionalized neural organoids. This modular approach enables the reconstruction of specific neural pathways, facilitating studies asking how axons navigate through complex environments to establish precise connections.

Cell-type specificity and directionality of axon projection in developing human brain have been captured and validated in several neural circuits within assembloids. For example, the unidirectional projection pattern observed in cortico-striatal circuits is preserved in cortico-striatal assembloids.[40,41] In addition, a combination of Cre recombinase-expressing rabies virus-mediated retrograde tracing with immunostaining of cell type specific markers demonstrated cell-type specificity of projections in cortico-spino-motor assembloids: $CTIP2^+$ and $MAP2^+$ deep layer cortical neurons preferentially connect with spinal motor neurons that preferentially connect with muscle fibers.[42] Furthermore, the integration of regionalized neural organoids resembling the thalamus and cortex led to the formation of reciprocal cortico-thalamic and thalamo-cortical projections, which resembling bidirectional projection pattern *in vivo* counterparts.[43–46] In a preprint paper, Rabies-mediated retrograde and AAV1 mediated anterograde viral tracing with Cre recombination confirmed the thalamocortical connectivity within the thalamo-cortical assembloids [44]. There have also been recent efforts to study interactions between ventral midbrain, striatal, and cortical organoids using assembloids[47].

In addition, assembloid approach could be a promising way to uncover fundamental mechanisms in human axon guidance. A prominent example is the crossing, in the developing spinal cord, of dorsal commissural axons in the ventral spinal commissure. Axons are initially guided towards the floorplate in a NTN/SHH-dependent manner, but later become sensitive to the chemorepellent Slit, resulting in repulsion across the midline.[48] While some factors driving midline crossing have been identified, other numerous signals likely regulate this process across various levels of the nervous system. In a recent preprint paper, human floor plate organoids have been established and assembled with spinal cord organoids to create midline assembloids.[49] Using this platform, classic developmental processes mediated by the floor plate such as Sonic hedgehog-dependent ventral patterning of human spinal progenitors and guidance of human commissural axons via Netrin have been modeled.[49] The versatility and scalability of the midline assembloid allowed for CRISPR-knockout screening of human enriched floor plate genes, implicating *GALNT2*,

a gene involved in O-linked glycosylation, in floor plate-mediated guidance of commissural axons.[49]

Additionally, the mechanism by which growth cones switch their responsivity from one intermediate target, such as the floorplate, to the next, remains elusive.[50] The generation of midline assembloids offer a promising avenue to model this process with human cells. Organizers in assembloids extend beyond the spinal cord midline to model various brain structures, including the corpus callosum in the human nervous system. Efforts to mimic corpus callosum formation often overlook the intricate signaling events governing axon guidance across the glial wedge. Additionally, organizer organoids for the roof plate and isthmic organizer illuminate dorsal and anterior-posterior patterning mechanisms. These models advance our understanding of human cell specification, axon guidance, and evolution, crucial for deciphering human neurodevelopment and neurodevelopmental disorders.

Circuit Formation in Neural Assembloids.

Assembly of neural circuits involves precise spatiotemporal integration of neurons across domains of the nervous system through cell migration and synaptogenesis.[51] Despite significant advancements, the molecular and cellular mechanisms of circuit assembly in primates, the influence of genetic and environmental cues on assembly, and how pathological conditions lead to malfunctioning and abnormal structure of neural circuits remain largely unknown.[52] This knowledge gap is primarily due to limited accessibility to the developing human brain, especially during late prenatal and early postnatal stages when circuits are assembled and refined.

The modularity of assembloids enables the creation of circuits of interest through the integration of two or more regionalized neural organoids. To date, functional assembloids models of cortical microcircuits,[17] cortical-spinal-muscle,[42] cortico-striatal,[40] and thalamo-cortical[44] pathways have been successfully constructed (Figure 1).

Cortical microcircuits comprise both glutamatergic neurons and GABAergic interneurons. As previously described, microcircuits form in forebrain assembloids upon organoid integration. Studies with forebrain assembloids derived from individuals with Timothy syndrome has unveiled the intricate role of L-type calcium channel function in human interneuron migration and the consequences of abnormal migration on early circuit assembly.[17,21]

Cortico-striatal projections, which are critical components of motivation circuits, can be modeled in cortico-striatal assembloids in which axon projections from cortical neurons connect to striatal medium spiny neurons. These cortico-striatal connections can be monitored and manipulated using live calcium imaging and patch-clamp recording in combination with optogenetics.[41] Interestingly, in cortico-striatal assembloids, but not in individual, un-assembled, striatal organoids derived from patients with the *SHANK3*-related Phelan McDermid syndrome, striatal neurons display abnormal activity, suggesting that some disease phenotypes emerge through interactions between cells type across brain regions.[40]

Regionalized organoids resembling the diencephalon and containing excitatory thalamic neurons can be fused with cortical organoids to investigate thalamocortical projections.[43–46] We probed neuronal activity by extracellular recordings or live calcium imaging of thalamic neurons, coupled with optogenetic stimulation of cortical neurons, to demonstrate the functional integration of thalamocortical projections in a preprint paper [44]. The α1G subunit of the T-type voltage-gated calcium channel $Ca_v3.1$, which is encoded by the *CACNA1G* gene, is prominently expressed in thalamic neurons. The physiological role of $Ca_v3.1$ in the thalamocortical pathway has been extensively studied in adult rodent models.[53,54] However, the impact on developing human thalamic neurons have not yet been examined. Rare loss-of-function mutations of *CACNA1G* have been associated with schizophrenia while gain-of-function mutations are thought to related to absence seizures[55–58]. Knockout of *CACNA1G* in thalamo-cortical assembloids results in aberrantly increased thalamocortical projections along with hyperactivity in this pathway, while CRISPR-mediated gain-of-function mutations lead to increased residual T-type currents by delayed decay, as well as hyperactivity in this pathway.[44] Moreover, another group explored thalamocortical projection in the context of 22q11 Deletion Syndrome (22q11DS).[46] These studies illustrate how the cellular- and circuit-level consequences of pathogenic variants associated with neuropsychiatric disorders can be probed *in vitro* human models of early circuitry.

Moreover, functional neural circuits can be modeled in three–component assembloids, for instance, to create the cortico-spinal-muscle pathway[42]. Calcium activity imaging and patch-clamp recording, in conjunction with optogenetic stimulation, can be used to probe the functionality of the cortico-spinal circuit within these assembloids. More specifically, these experiments indicated that muscle fibers contract spontaneously and following stimulation in three-part assembloids indicative of the ability of motor neurons to regulate muscle activity via neuromuscular junctions.[42] This work holds potential for advancing our understanding of the underlying mechanisms associated with neuromuscular system dysfunction. Muscle contraction in human cortico-motor circuitry could serve as the powerful functional readout in studying disorders such as amyotrophic lateral sclerosis, spinal muscular atrophy as well as cell vulnerability following infection with poliovirus and non-polio enteroviruses.

Additionally, a four-part assembloid also has been developed to encompass key components of the entire sensory spinothalamic ascending pathway in a preprint paper[59]. Live calcium imaging following somatosensory stimuli, such as pain-related chemicals, captured the transmission of responses throughout the sensory pathway from sensory neurons to cortical neurons. Emergent synchronized activity across the pathway also has been observed which indicates the functional integration of the pathway. Abnormalities in the synchronized activity resulting from SCN9A gene knockout highlight its utility for studying circuit-level consequences of pathogenic gene variants associated with somatosensory function. This ascending somatosensory assembloid potentially provides a versatile platform for examining the intricate dynamics of neural networks in response to somatosensory stimuli, including pain, touch, and itch [59].

Looking ahead, there is potential to model more complex circuitries in assembloids and advance our understanding of primate brain circuit development and function. Specifically, the recapitulation of the neuro-modulatory systems within assembloids, by integrating

serotonergic or noradrenergic inputs from brainstem organoids into cortical organoids, could shed light on their role during brain development and unveil pathophysiological changes in disease. Circuits in assembloids could be arranged non-linearly to model loop circuits, such as the cortico-striato-midbrain-thalamo-cortical loop, which is critical for Parkinson's disease and other related disorders.

Assembloids to Model Interactions between Neural and Non-neuronal Cells.

Interactions between cells are crucial for supporting the development and maturation of neurons throughout their lifespan, which is essential for proper nervous system development.[60] For example, during brain development, astrocytes offer supports to neurons and help regulate synaptic connections, contributing to the formation of functional neural circuits.[61,62] However, many of these cell-cell interactions involve cells that are not of neuroectodermal origin. Differentiation conditions in neural organoids, especially regionalized organoids, favor a neuroectoderm fate. As a result, mesodermal lineages, such as vascular tissues and immune cells, are generally absent. To model interactions between these different lineages, assembloid can be used to separately derive organoids of different germ layer identifies or specialized cell types and then assembling them in three-dimensional cultures (Figure 2).[63]

Vascularization of the nervous system is critical for its development because it provides a network of blood vessels that supply oxygen, growth factors, hormones, and nutrients. Additionally, the vasculature plays a role in removing waste products and regulating the microenvironment of the brain. Blood vessels vascularize the central nervous system through angiogenic invasion of the perineural vascular plexus, subsequently sprouting the networks to cover the brain and spinal cord.[64] Attempts to recapitulate the vascularization of neural organoids have been carried out both *in vitro* and through *in vivo* transplantation.[65–68]

The integration of vascular cells into human neural organoids offers an opportunity to model infections of the nervous system. Pericyte-containing cortical assembloids are created by seeding a suspension of pericyte-like cells into a well containing a cortical organoid. This preparation allows the pericyte-like cells to interact with the outer surface of the neural organoid.[69] This assembly enables the study of SARS-CoV-2 infection, suggesting that pericytes are replication hubs that facilitate the spread of the virus to astrocytes, triggering inflammatory type I interferon transcriptional responses.[69] This effect is not observed in unassembled cortical organoids. Another application for integrating vascular cells with neural organoids is to study blood-brain barrier interactions. These 'blood brain barrier' assembloids[70] can be used to model aspects of cerebral cavernous malformations. Moving forward, the development of vascularized organoids or assembloids that contains physiologically relevant perfusion will further enhance the applications of these models.

Another method for introducing functional, blood circulating vasculature is through transplantation of organoids into animals. Human neural organoids, when grafted into the rat brain, especially at early stages of development, become vascularized as rat endothelial and pericyte cells come in contact with human astrocytes.[65,67] If these transplant *in vivo* models ultimately recapitulate the blood-brain barrier, particularly if transplantation includes human

pericytes and endothelial cells, they will allow for more physiological modeling of barrier crossing, infection, and the identification of viral serotypes for gene therapies.

Enhancing the physiological relevance of *in vitro* models involves integrating glial and immune cells to improve developmental processes and homeostasis. Since microglia are of mesodermal origin, they are not typically generated in neural organoid protocols, but recent evidence suggests that microglia requires a neural environment for specification.[71,72] Therefore, combining human-derived primitive macrophage progenitors with neural organoids could overcome these limitations. Assembloids incorporating human microglia can elicit a cytokine response when exposed to lipopolysaccharide.[73] These microglia-containing assembloids show increased secretion of IL6, IL8, IL10, and TNFα, providing a model for studying aspects of neuroinflammation.[73] Furthermore, viral infection with ZIKA, Dengue, and HIV-1 virus increase the expression of cytokine/chemokine genes in assembled macrophages upon.[74–77] These findings highlight the potential of microglia-containing assembloids to model neuroinflammatory responses and study viral immune responses and replication in the developing CNS.

Assembloids to Model Non-neural Tissue Cell-cell Interactions.

Beyond their application in neurodevelopment, assembloids have proven to be versatile tools for modeling emergent cell-cell properties in other tissues. These include the maturation of specific cell types, deciphering molecular signals following cell-cell interactions or studying the coordination of cell movement during development (Figure 2).

Post-implantation embryogenesis involves complex interactions embryonic and extraembryonic cell lineages, which are critical for gastrulation, proper body axis organization and further organogenesis and can now be modeled with human embryoid-like models.[78–85] For aggregated epiblast, hypoblast and/or trophectoderm like cells can be integrated to form embryo-like assembloids to study the role of WNT, BMP, and Nodal signaling pathways in peri-implantation lineage development.[81] In addition, the generation of key cells of the gastrointestinal tract, such as hepatocytes, cholangiocytes and pancreatic cells can be used to create hepato–biliary–pancreatic assembloids.[86,87] These are generated by fusion of separately differentiated anterior and posterior gut organoids.[87] The ability of models to replicate abnormal organogenesis, such as the segregation of the pancreas and bile ducts in HES1 knock-out conditions, underscores their value in elucidating the intricacies of developmental processes. Additionally, assembloids that combine retinal and retinal pigment epithelium tissues have been shown to accelerate the differentiation of photoreceptor cells compared to single retinal organoids, emphasizing the beneficial interactions between retinal pigment epithelial cells and retinal photoreceptors.[88] Moreover, tumor–lymphocyte assembloids effectively mimic T cell responses to epithelial cancers.[89] They facilitate the activation of tumor-reactive T cells using autologous cells, enhancing the selection of T cells that are responsive to the tumor.[89] These activated T cells are particularly adept at identifying and destroying cancer cells within organoids, illustrating the potential for T cell-mediated immunity to target tumor cells effectively at an individual level.

Understanding molecular signaling between different cell types is essential for deciphering the complex mechanisms involved in organ development. The bladder assembloid is particularly instructive as it investigates cell-cell interactions across compartments– inner epithelium, connective stroma, and outer muscle layers, and overall exemplifies self-assembly of multilayered structures.[90] Genetically modified bladder assembloids revealed that the interaction between epithelial and stromal cells, mediated by the FOXA1-BMP-hedgehog signaling axis, acts as a determinant of sub-types of urothelial carcinoma.[90] Similarly, gastrointestinal assembloids have demonstrated self-organization of crypts driven by interactions between epithelial and stromal cells.[91] These studies highlight the importance of BMP receptors on either side as a key molecular signaling pathway for the formation of crypt structures. Additionally, mesenchymal-epithelial assembloids revealed that mesenchymal cells play a pivotal role in triggering ameloblast differentiation through TGFβ signaling, further underscoring the importance of intercellular communication in organogenesis.

Several assembloids systems have been developed to investigate cellular migration– a crucial aspect of organogenesis. Endometrial assembloids, comprising gland-like organoids and stromal cells, recapitulate cellular migration toward the embryo during implantation and the accompanying transcriptomic changes in endometrial cells.[92,93] Using pharmacological interventions, this model has pinpointed that tyrosine kinase-dependent stress responses influence the fate of decidual cells in the glandular epithelium during embryo transplantation.[94] In another example, assembloids combining human Wharton's jelly stromal cells with neonatal epidermal keratinocyte progenitor cell and comprising mesenchymal and epithelial cells, replicate the palatal fusion processes.[95] Toxicology screenings conducted with this platform highlighted the importance of various growth factors, such as EGF, IGF, and FGF in palatogenesis.[95] Furthermore, neuro-mesodermal assembloids model the migration of neural crest cells and the formation of sympathetic ganglia, including the interactions between peripheral ganglia and a co-developing vascular plexus, providing insights into complex developmental processes.[26]

Concluding Remarks

The intricate processes of neurodevelopment rely not only on generating specific cell types but also on the precise coordination of timing and integration of these cell types across various domains. Organoids have already begun unraveling cell diversification in the human nervous system. Now, assembloid platforms are providing insights into post-specification processes, including migration, axon guidance, and the establishment of connectivity. This approach is particularly promising for modeling neuropsychiatric disorders and developing and testing therapeutics. Moreover, transplantation of organoids– and assembloids in the future, will facilitate the study of disease phenotypes within circuits and in behaviorally relevant contexts, and ultimately enhancing our understanding of complex brain functions and disorders.

Despite the potential of these 3D human cellular models in studying neuropsychiatric disorders, several challenges remain in fully harnessing their capabilities. One major hurdle is deriving more (if not all) of the diversity of cell types in developing human nervous

system, which may require improvements in differentiation timing or the development of novel reagents to generate specific critical cell populations. There are approaches to study cell-cell interactions in self-organizing cultures, such as by generating broader cell diversity in organoids that may enable, for instance, neuromuscular interactions[96], or by engineering morphogen gradients with microfluidic devices.[97,98]

Another challenge is the difficulty in modeling morphogenic gradients and spatial tissue patterning that are observed *in vivo*. Addressing this gap could involve experiments that mimic organizer regions and their interactions in assembloids. For example, it is possible to concurrently generate multiple brain regions and domains by assembling neural organoids with an organoid that simulates the floorplate, creating a sonic hedgehog gradient to simultaneously generate ventral and dorsal spinal domains. Additionally, organoids that mimic the isthmic organizer can be developed to create domains along the rostro-caudal axis, specifying the midbrain and hindbrain regions using an FGF8 gradient. These approaches can lead to more accurate representations of brain development and function.

Expanding our understanding of neural circuits relevant to diseases by focusing on those that have not yet been adequately modeled is crucial. For instance, deep circuits in the brain, including the mesolimbic and mesocortical pathways, are essential for regulating motivation and reward. Dysfunctions of these circuits are linked to mood disorders, addiction, and schizophrenia. Additionally, the mesocortical and cortico-cerebellar circuits are integral for cognitive functions, motor coordination, and learning. These circuits represent key areas where further research and modeling could yield substantial breakthroughs in understanding complex diseases.

Non-neural and neural assembloids provide a promising approach to model interactions between different cell lineages that can be crucial for understanding disease mechanisms and developmental processes. By assembling organoids derived from various germ layers, these models study interactions between neural cells and non-neuronal cells, such as vascular and immune cells, enhancing the physiological relevance of in vitro systems. These assembloids facilitate the investigation of complex cellular interactions involved in organogenesis, immune and infection responses, and could offer insights into the disease molecular signals.

The maturation of cell types in neural organoids and assembloids requires further advancement to effectively study later-stage neurodevelopmental processes and neurodegenerative disorders. Transplanted organoid models achieve more advanced neural maturation *in vivo*. Identifying *in vivo* components currently absent from culture media or the specific role of activity could significantly enhance the maturation of organoids and assembloids *in vitro*. This feat may be accomplished by further experimentation on the effects of non-neural cellular interactions on neural maturation as many assembloids containing non-neural lineages appear to contribute to maturation of neural morphology and transcriptomic signatures.

Taken together, these recent developments in human cellular models, mark an exciting period in neuroscience, offering new opportunities to ask big questions in human biology.

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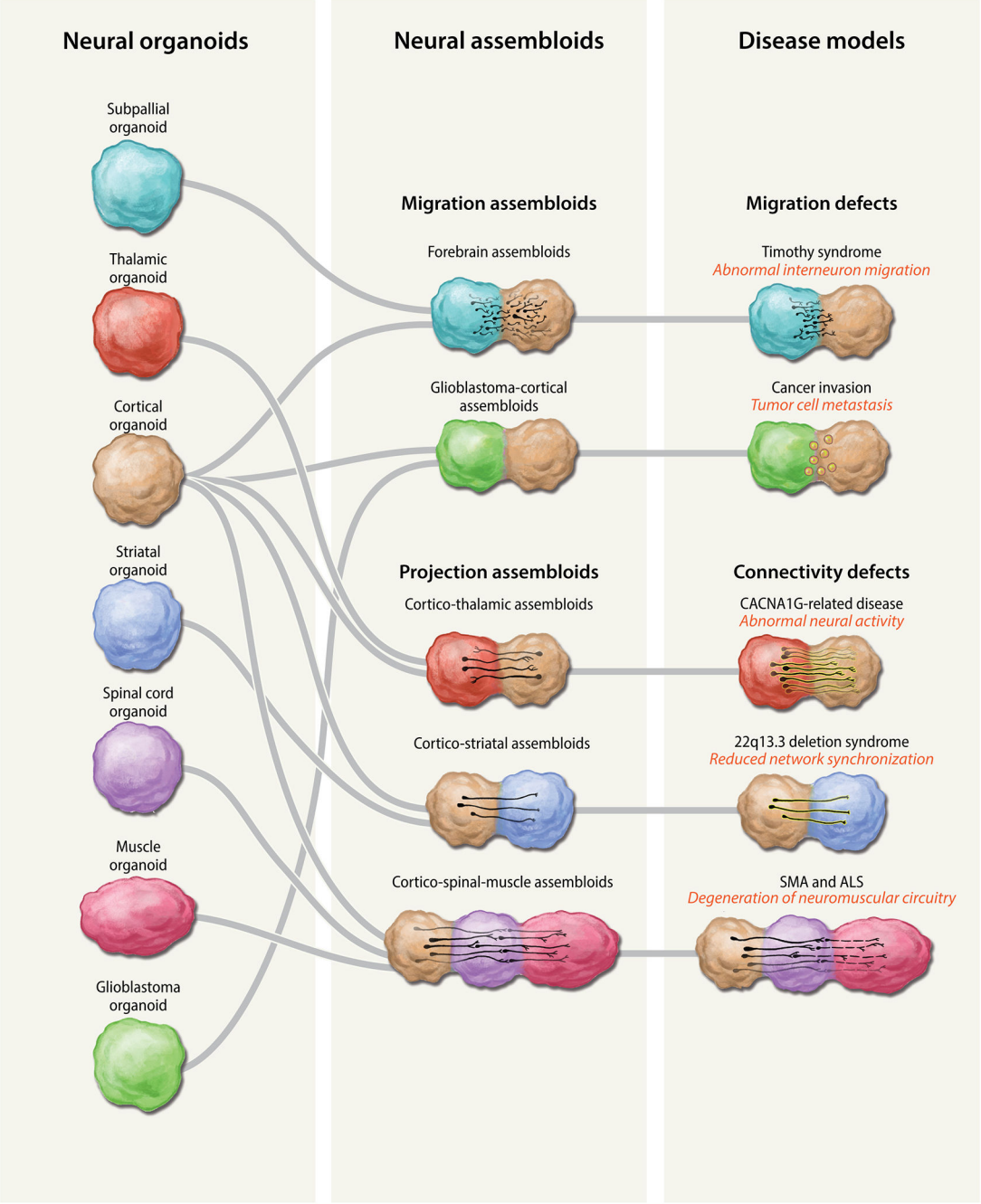

Figure 1: Generation of assembloids to model human neural development and disease

Neural organoids resembling domains of the nervous system (left) can be integrated to generate assembloids used for studying migration or connectivity (middle). These assembloids can then be used to model aspects of disease *in vitro* (right).

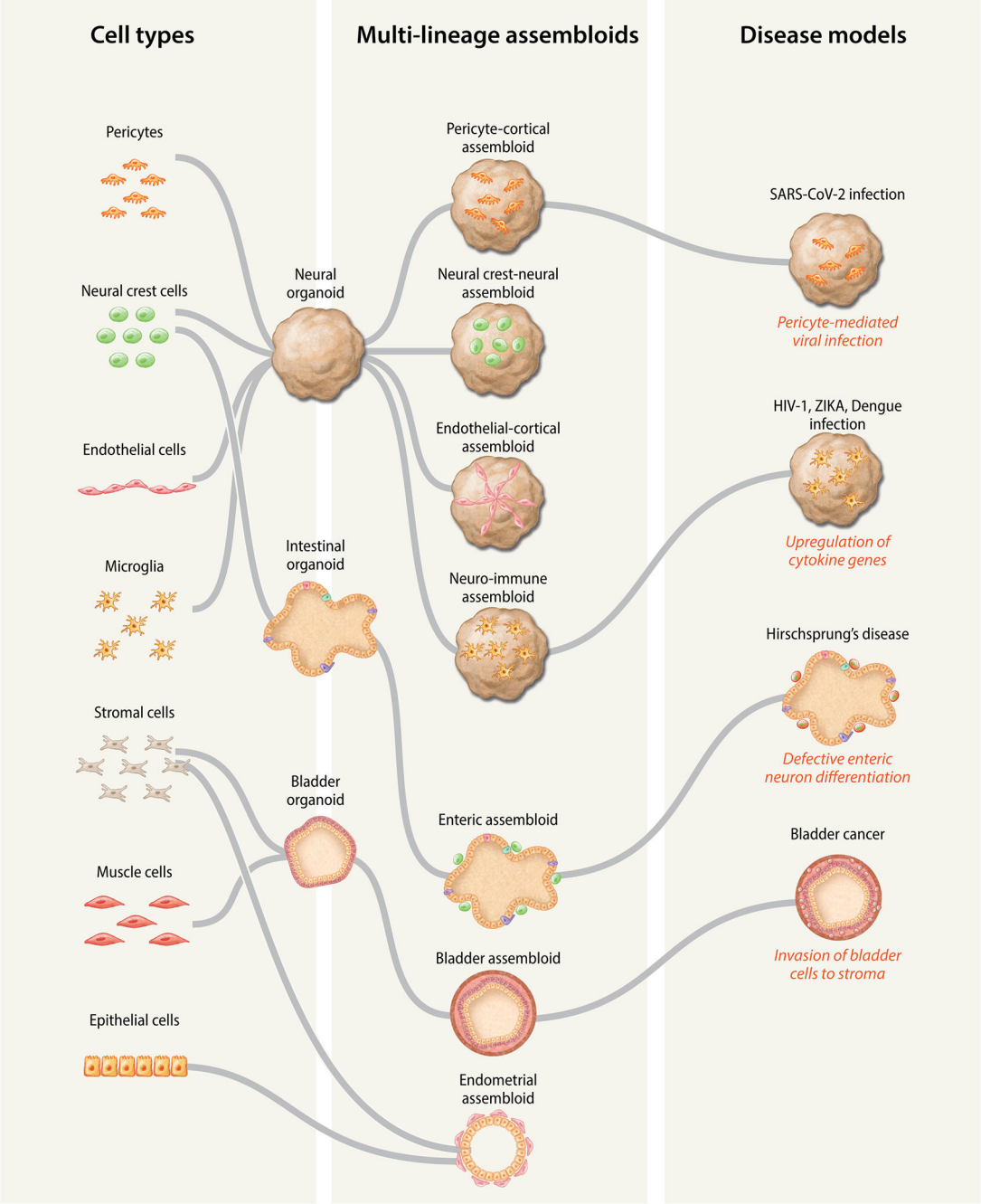

Figure 2: Modeling interactions with assembloids containing non-neural lineages.

Various cell types generated from human pluripotent stem cells (left) can be combined to generate multi-lineage assembloids and model genetic, cancer or infectious disorders.