Psychiatry in a Dish
Stem Cells and Brain Organoids Modeling Autism Spectrum Disorders
Ilieva, Mirolyba; Fex Svenningsen, Åsa; Thorsen, Morten; Michel, Tanja Maria

Published in:
Biological Psychiatry

DOI:
10.1016/j.biopsych.2017.11.011

Publication date:
2018

Document version
Peer reviewed version

Document license
CC BY-NC-ND

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk
PSYCHIATRY IN A DISH: STEM CELLS AND BRAIN ORGANOIDS MODELING AUTISM SPECTRUM DISORDERS (ASD)

Short title: BRAIN ORGANOIDS MODELING AUTISM SPECTRUM DISORDERS

Mirolyba Ilieva\textsuperscript{1*}, Åsa Fex Svenningsen\textsuperscript{2}, Morten Thorsen\textsuperscript{1}, Tanja Maria Michel\textsuperscript{1,3}

\textsuperscript{1}Department of Psychiatry, University of Southern Denmark, Odense, Denmark

\textsuperscript{2}Department of Neuroscience research, University of Southern Denmark, Odense, Denmark

\textsuperscript{3}Odense Center for Applied Neuroscience BRIDGE, University of Southern Denmark, Psychiatry in the Region of Southern Denmark, Odense University Hospital

*Corresponding author: Sdr. Boulevard 29, Odense 5000, Denmark; +45 22 66 81 33; milieva@health.sdu.dk

Key words: autism spectrum disorders, induced pluripotent stem cells, brain organoids, neuronal stem cell niche, neurogenesis, cellular models in psychiatry

number of words in the abstract: 244

number of words in the text: 3982

number of tables: 2

number of figures: 3

number of supplementary material: 1
Abstract

Autism Spectrum Disorders (ASD) are a group of pervasive neurodevelopmental conditions with heterogeneous etiology, characterized by deficits in social cognition, communication, and behavioral flexibility. Despite an increasing scientific effort to find the pathophysiological explanations for the disease, the neurobiological links remain unclear. A large amount of evidence suggests that pathological processes, taking place in the early embryonic neurodevelopment, might be responsible for later manifestation of autistic symptoms. This dysfunctional development includes altered maturation/differentiation processes, disturbances in cell-cell communication, and unbalanced ratio between certain neuronal populations. All those processes are highly dependent on the interconnectivity and three-dimensional (3D) organizations of the brain. Moreover, in order to gain a deeper understanding of the complex neurobiology of ASD, valid disease models are pivotal. Induced pluripotent stem cells (iPSC) could potentially help to elucidate the complex mechanisms of the disease and lead to the development of more effective individualized treatment. The iPSC approach allows comparison between the development of various cellular phenotypes generated from cell lines of patients and healthy individuals. A newly advanced organoid technology makes it possible to create three dimensional (3D) in vitro models of the brain development and the structural interconnectivity, based on iPSC derived from the respective individuals. Since the biggest challenge for modeling psychiatric diseases in vitro is finding and establishing the link between cellular and molecular findings with the clinical symptoms, this review aims to give an overview over the feasibility and applicability of this new tissue engineering tool in psychiatry.
Introduction

Autism Spectrum Disorders (ASD) are characterized by deficits in social cognition and communication as well as behavioral inflexibility. These neurodevelopmental disorders are pervasive in nature with an early onset [1]. The etiology is heterogeneous and despite an increasing scientific effort, the neurobiological underpinnings remain uncertain [2]. A growing body of evidence suggests that the pathological mechanisms leading to the manifestation of autism spectrum disorders (ASD) might be a result of very early disruption, happening in the second trimester of fetal development [3, 4]. The severity of symptoms varies greatly between individuals and the prevalence of intellectual disability, epilepsy, attention deficit/hyperactivity disorder (ADHD), and obsessive-compulsive disorder is markedly higher in people with ASD than in unaffected individuals [5]. ASD has a strong and complex genetic basis, and hundreds of candidate genes have been identified, each with many different putatively disruptive variants [6].

The genetic links have been confirmed in twin studies with concordance rates up towards 90% in monozygotic twins, in contrast to 30% in dizygotic twins [7, 8, 9, 10]. Disruptive variants of genes involved in the glutamatergic pathway have been associated with autism development [11]. Synaptic proteins such as neurexin, neuroligin [12, 13], and SHANK1, 2, 3 [14, 15, 16, 17, 18], neurotrophic factors (BDNF) [19], oxidative stress (SOD 1, 2) [20, 21] and transcription factors (FOXG1) [22, 23] are all being discussed as candidates involved in the underlying neurobiology of ASD.

The basis of all organizational events in the human brain is the process of self-assembling and self-organization - the formation of complex structures from units of less complexity by local internal interactions [24, 25]. The physical properties of the cells, such as shape, cell-cell adhesion, and polarity, can modify gene expression and vice-versa [26]. Stochastic
fluctuations of intrinsic and environmental factors can induce additional migration processes and alter the position of a cell in the 3D cellular cluster to fit its gene expression profile. This in turn may change the entire architecture of a larger region of the developing brain [26]. Therefore, we propose, that disturbances in the self-organization process that occur during brain development may result in ASD pathology.

ASD is a highly “humanized” condition meaning that investigations in animal models face many challenges. Murine models of ASD provide genetic homogeneity and to some extent allow the study of behavioral deviations [27]. However, the existing genetic differences between species and the specificity of the human brain structures complicates extrapolation of experimental results from animals to humans. One challenge in the comparison is the less complex pattern of organization in the mouse cortex compared to the human cortex [28, 29]. The neocortex of humans contains significantly higher numbers and more diverse forms of \( \gamma \)-aminobutyric acidergic (GABAergic) interneurons than that of rodents [28, 30, 31, 32, 33, 34, 35, 36]. It has been demonstrated that the morphology of dendrites and the number of dendritic spines is disrupted in autistic patients [37]. However, the dendrite morphology in humans is more complex and the number of dendritic spines is higher compared to mice [32, 38]. Anatomical differences between human and rodents in the sub-ventricular zone (SVZ) have also been observed [39]. The human SVZ is divided into inner (iSVZ) and outer (oSVZ) SVZ whereas rodents lack the outer SVZ [40]. Thus, a more relevant \textit{in vitro} model for recapitulating early human brain development and related pathology is highly needed.

\textbf{Modeling ASD in “a dish” – what we learned from human induced pluripotent stem cells (iPSC)}

Before venturing into further considerations, it is important to address several challenges related to modeling neurodevelopmental disorders in “a dish”. Firstly, it is important to assess
the timing of changes during brain development that lead to the disease. In addition to that, it is important to understand how to translate this into a viable in vitro model displaying a comparable “age stage” (Figure 1). It is also important to consider the particular brain region that should be targeted as well as the genes and their developmental and cell type trajectories.

Normally, a transient increase in the number of cortical neurons is observed during the second trimester of a healthy pregnancy [41, 42]. This growth spurt is reported to slows down by the time of birth or several months after [43, 44]. This period of active neurogenesis is critical for maturation in the cortical laminar development as well as the development of the cortical circuitry [45]. Interestingly, one of the most replicated findings in patients with autism is the increased number of neurons in the prefrontal cortex as well as increased head size, that phenomenon seems to remain stable during the first year of life, and then the numbers of neurons decrease to the normal amount and the growth goes back to the normal percentile [46, 47, 48, 49, 50].

Despite the complexity and heterogeneity of ASD, genetic studies, post-mortem brain analyses, and functional imaging studies all point to an abnormal regulation of specific processes including cell proliferation, differentiation, and migration in the brain as key mechanism involved in the pathogenesis of autism [51, 52, 53, 54, 55, 56]. Two independent studies, applying different analytical approaches, identify spatial and temporal convergence in genes involved in neurodevelopmental disorders [57, 58]. Integrative functional genomic analyses demonstrate that de novo ASD gene variants are grouped in gene “modules” responsible for neurodevelopmental processes, including early transcriptional regulation and synaptogenesis. ASD genes variants seems to be enriched in superficial cortical layers and glutamatergic projection neurons [57]. ASD gene “modules” are involved in the migration and differentiation of inhibitory and excitatory neuronal populations which are essential in
cortical development [57]. These processes occur in the brain stem cell niche - a complex and dynamic structure, with components that synchronically interact and mediate the balanced response of stem cells to the need of the organism (Figure 2A). [59]. Throughout life, the mammalian brain harbors neuronal stem cells (NSCs) mainly in the subventricular zone (SVZ) and subgranular zone (SGZ)[60]. The SVZ is the largest neurogenic region, maintaining the capacity to generate new neurons which functionally integrate in the brain [60]. Aberrations in this region would have a drastic influence on brain development/organization [54, 61]. Genes regulating cell proliferation, differentiation, and migration in the SVZ have been reported to be dysregulated in young autistic patients when compared to healthy controls [54, 61]. Individuals with autism have different DNA methylation profiles for genes related to cell migration and brain development, suggesting an abnormal epigenetic regulation of key neurodevelopmental genes in the autistic brain [62, 63]. Altogether, this suggests a critical dysregulation of molecular and epigenetic mechanisms in the SVZ region of the brain in individuals with autism [62, 63].

In recent years, increasing scientific attention has been focused on induced pluripotent stem cells (iPSC) as a potent tool to study the neuronal differentiation and interactions. iPSC are derived from somatic cells via the overexpression of a certain set of reprogramming factors like Oct4, Sox2, Klf4, and c-Myc [64, 65, 66, 67]. IPSC derived from patients bring their specific genetic signature and derived neurons show their typical disease morphology [68].

One common finding is an abnormal maturation and differentiation of neuronal progenitor cells (NPC) derived from ASD iPSC (Supplement table 1): Shortened cell cycle and decreased levels of neuronal maturation [69, 70, 71, 72]. A dysregulation of the β-catenin/BRN2 transcriptional cascade leads to increased cell proliferation [73]. It has been confirmed in the mouse model that the dysregulation of β-catenin/Brn2/Tbr2 transcriptional
Ilieva et al.

A cascade mediates a transient embryonic brain enlargement during the deep layer cortical neuron formation. This is the result of an abnormal expansion of the basal NPC of the developing brain and is associated with adult social and repetitive behavioral abnormalities [74]. This mechanism links fetal brain development and adult behavior, demonstrating a fetal origin for social and repetitive behavior deficits seen in disorders such as autism.

Reduced number of synapses and reduced spine density, have also been observed in neurons originated from autism patients derived iPSC [75, 76, 77]. A similar pattern is seen in neurons differentiated from iPSC from patients with Phelan-McDermid syndrome [78], a syndrome which arises from 22q13 deletion or mutation in SHANK3 gene giving rise to a broad array of medical and behavioral symptoms, including autistic features. In this syndrome, defects in excitatory synaptic transmission result from both a failure to form correct number of excitatory synapses and a reduction in the expression of glutamatergic neurons and receptors [79]. Inhibitory synaptic transmission is, however, not affected. This leads to an imbalance between excitatory and inhibitory neuronal populations in the brain of the patients.

Altered neuronal morphology as reduced soma size and reduced neurite length [80, 81, 82, 83], electrophysiological defects [84, 85], altered Ca^{2+} signaling [77, 84, 86] are also common findings in the neurons derived from ASD iPSC.

Astrocytes derived from Rett syndrome iPSC have accelerated formation and differentiation [87]. The condition media from those astrocytes has negative effect on morphology and function of healthy neurons [88]. Thus, astrocyte dysfunction may play a role in the aberration of neuronal circuitry in ASD.

However, in vitro approaches applied in stem cell research are based on the analyses of single homogeneous cellular populations grown in 2D and neglect the fact that all tissues are
integrated 3D structures of multiple cell types and extracellular matrix. 2D cell cultures lack the means of spatial and temporal signaling as well as the interactions of the cells in a natural environment. Purified primary cells can lose their phenotype when cultured in 2D and demonstrate other gene expression patterns than they would in 3D [89, 90, 91, 92].

**Brain organoids – it is all about space and timing**

The generation of organoids is based on two major concepts – region-specific organoids and (whole) brain organoids. The first methodology employs guided neuronal differentiation by extrinsic morphogens and growth factor patterning to form region-specific organoids; for example: forebrain [93], cortical organoids [94, 95], midbrain [96] and hypothalamic organoids [97]. These region specific organoids are highly dependant on the morphogene induction and specific growth factors used and result in the generation of cell types specific for a particular brain region. In contrast to that, the whole brain organoid approach relies on intrinsic mechanisms of self-organization and recapitulates complex whole-brain structures with extracellular scaffold embedding, without the use of growth factors or inhibitors [98, 99].

A timeline overview of the different methodologies for generating cortical and cerebral organoids is shown in table 1.

The most commonly used method to guide iPSC into forebrain neuronal differentiation in 2D as well as in 3D is dual inhibition of the transcription factor SMAD via the signal transduction pathways of TGFβ and bone morphogenic protein (BMP) using the signal molecules NOGGIN and SB435142 (small molecule inhibitor of the type I TGF-β receptor) [100]. Further, neuronal differentiation could be guided to anterior or posterior fate, with various morphogenes, for example fibroblast growth factor 8 (FGF8) and retinoic acid [101]. Targeted differentiation of interneurons [102, 103] is also of great interest for ASD research,
since interneuron migration has been suggested to be abnormal in post-mortem tissue samples from ASD patients [69, 104, 105]. (Figure 2 B).

The first link between 2D and 3D culturing is the serum free culture of embryoid body-like aggregates (SFEBq) with quick re-aggregation technique [94, 106]. Embryoid bodies cultured under serum-free conditions and in the presence of inductive factors can generate forebrain NPC. NPC can differentiate and guide to the generation of a number of individual brain regions including cortex, pituitary gland, and retina in a temporal and spatially coordinated manner [107].

Several protocols for the generation of forebrain/cortical organoids have been developed based on the SFEBq method [79, 80, 93, 108]. They use forebrain inductive signals and the addition of dissolved matrigel that provides extracellular matrix proteins and growth factors. Thus, this procedure is capable of recapitulating the self-organizing aspects of human corticogenesis: spontaneous development of intracortical polarity and cortical folding with complex zone separations. A multilayered structure, in the same apical-basal order that is seen in the human fetal cortex in the early second trimester, has been observed [97]. Furthermore, utilizing this protocol, the outer SVZ contains basal progenitors that share characteristics with outer radial glia, abundantly found in the human fetal brain [93].

The second methodological approach – generation of brain organoids [98], does not employ growth factors and morphogens to drive a particular brain region signature. Instead, EBs are embedded in Matrigel, which induces the growth of neuroepithelial cells. Those develop into complex brain structures based on self-organization. Those structures are not propagating randomly, but demonstrate similar patterns and regionalization as in the in vivo developing brain. During the developmental process, the expression of hindbrain markers is down-regulated, mimicking the normal expansion of the forebrain tissue during brain development.
Cell populations expressing hippocampal and ventral forebrain markers have also been detected, but those regions are not morphologically organized as *in vivo*.

Cortical and brain organoids demonstrate regional connectivity *via* interneurons and they also display something that is unique for the human brain development; a progenitor zone organization [98, 99, 109].

Thus, the organoids follow the *in vivo* timeline development. They also have the ability to recreate the self-organizing complexity of the brain’s developmental stages [99]. Organoids are therefore a relevant and valuable model of neurodevelopment and the related pathology, such as e.g. microcephaly [98] and lissencephaly [110]. Moreover, organoids can generate a broad diversity of cells in the human brain [111]. In addition to this, SVZ-like regions are also well defined. All this makes organoids a very promising model for this specific brain region and can help to elucidate the role of SVZ in pathogenesis of ASD as it was previously discussed [97]. (Figure 2, C).

Li et al. [112] demonstrate that the activation of the PTEN-AKT pathway – a key signaling cascade of the brain development, triggered by various growth factors, can enhance the proliferation of NPC in the organoids and thus its expansion in size and surface cortical folding [112].

Forebrain organoids recapitulate saltatory migration of interneurons in the fetal forebrain - after migration, interneurons functionally integrate with glutamatergic neurons and form cortical circuits [109]. Moreover, organoids derived from Timothy syndrome patients demonstrated that interneurons display abnormal migratory saltations due to mutation in CaV1.2 channels [109].
On gene expression level, organoid cultures can replicate both early (8-10 GW) [93, 98] and late mid-fetal (22-24/35 GW) development [95, 97, 108]. A growing amount of single-cell RNA sequencing studies report that most of the cells in organoids have remarkably similar to human fetal brain gene expression program. [93, 111, 113].

Moreover, a significant overlap in expression of risk genes for schizophrenia and ASD (such as synaptic transmission, cell-cell signaling, ion transport, etc.), that has previously been observed in post-mortem brain tissue of patients, has also been found during organoid development [97].

Finally, organoids recapitulate most of the epigenomic features of the mid-fetal brain development [114]. Accumulation of early non-CG methylation sites at super enhancers regions of the genes has been detected in organoids as well as in the developing brain which points future transcriptional repression in mature brain. This finding is of great importance since the epigenetics play a critical role in the pathogenesis of ASD.

The reports investigating organoids derived from patients with ASD, are still sparse. However, an up-regulation in the expression of the transcription factor FOXG1 was detected in the cortical organoids (corresponding to the first gestation trimester) derived from idiopathic ASD patients [115]. This genetic upregulation results in a shortened cell cycle length, an abnormal cell proliferation, and an unbalanced differentiation of inhibitory neurons. This leads to an unbalanced ratio of inhibitory and excitatory neurons. The phenomenon was observed only in early progenitor cells, but not in later stages of the cortical development, suggesting that a compensatory mechanism is involved, which is likely to restrict extensive neuronal production [115]. All those findings could explain the accelerated brain growth in the early neurodevelopment in patients with ASD and later compensation, which has been described earlier [46-50].
Challenges in modeling ASD with iPSC and brain organoids

It still remains unclear how the process of reprogramming affects the genetic and epigenetic signature of differentiated neurons and thus might influence the experimental results. Genome-wide studies were applied to quantify the similarity between in vitro neural stem cell models and brain development in vivo [116]. The primary human neural progenitors derived from human fetal brains show remarkable preservation of in vivo gene expression and network architecture. In contrast to that, neurons derived from iPSC represent an immature, fetal state. They differ in the gene expression pattern of the in vivo development. It was identified a genes “module” related to chromatin remodeling that is not presented in iPSC-derived neuronal stem cells and contains genes involved in the generation of lower cortical layer neurons. Furthermore, genes specific to neurodevelopmental processes related to ASD pathogenesis are not preserved in iPSC derived neurons. It has been suggested, that optimizing the differentiation protocols might help to better in vitro models, e.g. brain organoids, and to overcome this challenge. [116].

Per se reprogramming can introduce mutations of the genomic DNA as well as exogenous reprogramming genes [117]. However, this challenge could be overcome by “transdifferentiation”. This is an alternative approach, in which one somatic cell type is converted to another somatic cell type, avoiding the pluripotent stage and related genetic reorganizations. For example, skin fibroblasts can be either reprogrammed into functional neurons within three weeks by forced expression of neurogenin-2 [118] or they can be converted directly into induced neural progenitor cells, by timely restricted expression of Sox2 [119], Klf4 and c-Myc and limited and very strictly controlled expression of Oct4 [120].

The main limitation of the studies on ASD in iPSC is the small number of patient’s derived cell lines. Most of the studies involve cell lines derived from one to five patients (table 1).
Only one single study [121] describes a bio-bank with more than 200 cell lines incl. fibroblasts, iPSC, neural stem cells and glia from wide range of patients diagnosed with idiopathic autism and Fragile X-syndrome. This points to the necessity of doing further studies including a higher number of cases.

Another main concern in the previous studies, involving patients derived iPSC, is the lack of selection of well characterized clinical populations, meaning that the patients groups have not been well characterized. One of the largest weaknesses of most of the studies is the restricted and limited description of the clinical presentation and the precise selection criteria for the single patients. This makes the results of the studies very difficult to interpret and to eventually correlate the cellular findings with the clinical symptoms.

Since autism is considered a spectrum of disorders, we propose to either model the disease based on a single patient with a rare genetic mutation with a clear clinical manifestation and observe the differences in the cellular and molecular signature in comparison to the findings from a respectively matched healthy individual, or based on a big group of patients with idiopathic ASD where pathological findings in the organoids are difficult to distinguish from the healthy controls. Moreover, including a heterogeneous patient group with a disparate disorder aetiology could compromise data interpretation. Thus, common well-defined inclusion criteria together with clinically characterized patient cohort is an indispensable consideration. Nevertheless, it remains subject to discussion and needs to be elucidated further, whether a “global” or an “individualized” model of the disease will give a more relevant or a more complex picture.

An important question in modeling ASD is how to correlate the molecular and cellular findings in vitro with the clinical presentation. Pathology based on a single known mutation provides valuable information regarding molecular and cellular disturbances, but it is difficult
to correlate the in vitro findings with the clinical symptoms in the patient. Additionally, it is important to find out which of those findings are the key characteristics and how do we define an “autistic brain” in vitro? Therefore, the question of what is the appropriate readout to conclude that the model is relevant needs to be addressed. Taking all the findings together, we suggest that one potential criteria could be a correlation between brain volume (head circumference) and cell proliferation index [73] or organoid size [115]. Another promising correlation could be drug response (e.g. Risperidone) or presence of seizures in the patient and changes in receptor expression and synaptic activity in patient derived organoids (electrophysiology).

To draw relevant conclusions, it is necessary to generate stable and reproducible organoids. Currently, the organoid techniques suffer from significant variability issues, so called “batch syndrome”, in which different batches of organoids demonstrate significant variability in the quality of the brain regions they produce [99]. Furthermore, standardization of culture conditions and using a critical marker set for analysis are required.

Although the brain organoids recapitulate the highly coordinated generation of a cortex-specific repertoire of neuronal cell types, the reconstruction of the precise 3D-location of the multiple cell populations in the whole brain has not been achieved yet. This is due to the lack of knowledge in major developmental cues necessary to form a mature brain, and when these should be applied. It also depends on the absence of a vascular system that could distribute a sufficient amount of nutrients and oxygen inside organoids. Organoids also lack major developmental cues necessary to form a mature brain [93, 98]. Thus, the organoids-based models recapitulate single or partial components of the brain tissue.

Both neurogenesis and proper maturation of progenitor cells in the SVZ in later brain development is highly dependent on oxygen and nutrients delivery. A lack of vascularization
within the brain organoids leads to hypoxia and malnutrition. Furthermore, it results in necrosis in the center of the organoids [98]. Spinning bioreactors have successfully been employed to increase the oxygen exchange [97, 98]. Controlled speed of stirring and adding growth factors (BDNF) could also help solve this challenge [122]. Advancing knowledge in the field of nanotechnology and future tissue engineering may thus solve the challenge of inner necrosis, co-cultures of endothelial cells and the creation of artificial vascularization connected to a microfluidic system [97, 123]. The main advantages and disadvantages of 2D vs 3D culturing are presented in table 2.

**Perspectives and conclusion**

Although the first promising results have demonstrated that the organoid technology is very encouraging in modeling neurodevelopment and its diseases, it is necessary to go a long way before one can fully assess the value of 3D cell cultures. The field is developing very fast and efforts are now focused on optimizing the methodology and on understanding how well organoids relate to the human brain in respect of structure, function, genetic and epigenetic signature.

For further understanding of the molecular links between gene functions and ASD, the emerging technology of genome editing could be applied. By using “knock out” or “knock in” methods to diminish or induce certain genes in iPSC, one could mimic the loss-of-function and gain-of-function status, that exists in the developing human embryo prior to neuronal differentiation. CRISPR/Cas 9 technique provides the ability to introduce a variety of genetic alterations, ranging from single-nucleotide modifications to whole gene addition or deletion, all with a high degree of target specificity [124]. This will allow investigators to assess the importance of genetic modifiers on disease manifestations. The experimental design could be to insert a disease mutation into a wild-type cell line – thereby testing sufficiency of the
mutation for disease development – and correct the respective (disease) mutation in a patient-specific iPSC line, e.g. treat a disease mutation – thereby testing for necessity of the mutation for disease [124].

Despite the limitations of using organoid techniques, investigations of brain organoids at different time points in their development can give a wider and more detailed picture of the disease dynamic and thus the development of therapeutic and prevention strategies. It is a tool that can be used for screening of chemical compounds as potential drugs (“in spheroid” drug testing) [125, 126, 127].

Acknowledgments and Disclosures

This work was supported by Odense Center for Applied Neuroscience BRIDGE (University of Southern Denmark).

We would like to thank Dr. Abigail Sheldrick for comments and careful reading of the manuscript.

The authors report no biomedical financial interests or potential conflicts of interest.
References


60. Luis C. Fuentealba, Kirsten Obernier, Arturo Alvarez-Buylla. Adult Neural Stem Cells Bridge Their Niche, Cell Stem Cell 10, June 14, 2012


72. Sheridan SD, Theriault KM, Reis SA, Zhou F, Madison JM, Daheron L, et al. (2011): Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in


Figures descriptions

**Figure 1** Cerebral organoids follow the *in vivo* timeline and can recapitulate the early (8-10 gestation week) to mid-fetal (22-24/35 gestation week) human brain development.

**Figure 2**

A. **Brain stem cell niche** - The elements of the local environment that participate in the balance between quiescence and cell division and mediate the balanced response of stem cells to the need of the organism include: 1) the constraints of the architectural space; 2) mechanical forces in three dimensions; 3) physical engagement of the cell membrane with molecules on neighboring cells or extracellular matrix; 4) signaling interactions at the interface of stem cells and niche or descendant cells; 5) paracrine and endocrine signals from local or distant sources; 6) neural input; 7) metabolic products of tissue activity and oxygen. Disturbances in the interactions between those elements may lead to disbalance in cell proliferation, differentiation and migration and contribute to development of ASD pathology.

B. **Signaling pathways for cortical neurons differentiation** - iPSC can be guided into neural progenitors fate in three ways: culturing embryoid bodies (EB) in suspension and further isolation of neural cell from the adherent culture of EB, SFEBq method, and dual-SMAD inhibition method. Differentiation into telencephalic progenitors can be triggered by inhibition of the Wnt pathway during induction process. Further, neural progenitors can be guided either to dorsal fate by inhibition of sonic hedgehog (SHH) signaling or treatment with retinoic acid (RA), or to ventral fate by activation of SHH signaling together with Wnt inhibition.

C. **Comparison of neurodevelopment in mice (a), human (b) and in vitro – organoids (c)**. Neuronal stem cells (radial glia) reside in ventricular zone and are the source for more differentiated cells like neurons, intermediate progenitors, and basal radial glia. Intermediate progenitors and basal radial glia reside in subventricular zone (SVZ). In human and organoids SVZ is separated into an inner and outer SVZ. Neurons migrate via basal processes through the intermediate zone (IZ) to the cortical plate (CP). CP in human is extremely well developed comparing to mice. Human cortex is folded into numerous gyri and sulci. In contrast mouse brain is smooth. Organoids differ from human developing brain in the size of the cortical wall and complexity of neural progenitor population. Development of vascular system in organoids is missing.

VZ-ventricular zone, SVZ-subventricular zone, iSVZ – inner SVZ, oSVZ – outer SVZ, IZ – intermediate zone, CP – cortical plate, aIP – apical intermediate
progenitors, bIP – basal intermediate progenitors, aRG – apical radial glia, bRG – basal radial glia, BV – blood vessels.

**Figure 3**

**Perspectives in psychiatric disease research based on patient’s derived iPSC and brain organoids.** The organoid platform could be employ in the fundamental and applied research of neurodevelopmental psychiatric disorders (autism, schizophrenia). Multi-omics and functional profiling of 3D brain organoids followed by big data analysis will help to identify sets of genes and sub-pathways relevant as potential drug targets, which correlate with clinical symptoms and genetic alterations as well as the phenotypic characteristics of the affected neuronal populations. Finally, organoids can be used as “in‐sphero” tool for screening of chemical compounds as therapeutic agents and thus develop a more effective therapeutics.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Procedure</th>
<th>Results</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eiraku et al. (2008), [94]</td>
<td>SFEBq – serum free floating culture of embryoid body-like aggregates with quick re-aggregation Growth factors patterning to stimulate neuronal differentiation – WNT inhibitor, Nodal/Activin/TGFβ inhibitor</td>
<td>Derivation of individual brain regions – retina, cerebral cortex, pituitary.</td>
<td></td>
</tr>
<tr>
<td>Mariani et al. (2012), [93]</td>
<td>Cortical like 3D culture based on SFEBq Small molecules, growth factors, rostral neuralizing factors (FGF2, inhibitors of BMP, Wnt/β catenin, TGFβ/activin), nodal pathway to induce forebrain fate</td>
<td>3D structures containing polarized radial glia, intermediate progenitors, and a spectrum of layer – specific cortical neurons reminiscent of their organization in vivo Forebrain neuronal gene expression program No hindbrain, posterior and ventral regions markers expression</td>
<td>Gene expression profile of the embryonic telencephalon – dorsal telencephalon correlation with early human cortical wall at 8-10 week</td>
</tr>
<tr>
<td>Kadoshima et al. (2013), [108]</td>
<td>Nodal/Activin/TGFβ inhibitor, BMP- inhibitor</td>
<td>Forebrain organoids self-forms multilayered structure including three neuronal zones (subplate, cortical plate, and Cajal-Retzius cell zones) and three progenitor zones (ventricular, subventricular, and intermediate zones)</td>
<td>recapitulates human fetal cortex in the early second trimester</td>
</tr>
<tr>
<td>Authors</td>
<td>Description</td>
<td>Characteristics</td>
<td>Recapitulating</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Lancaster et al. (2013), [98]</td>
<td>Cerebral organoids: Self-organizing capacity of iPSC, EB formation, Embedding in Matrigel, No growth factors patterning</td>
<td>Complex structures – cerebral organoids; Discrete brain regions – forebrain and hindbrain (decrease with time)</td>
<td>Recapitulate first trimester after conception</td>
</tr>
<tr>
<td>Pasca et al. (2015), [95]</td>
<td>None-adherent, no extracellular matrix, SMAD inhibitor, FGF2, EGF, BDNF, NT3</td>
<td>Cortical neurons and astrocytes from iPSC hCS – human cortical spheroids; only excitatory neurons of the dorsal telencephalon; laminated neocortex at 2.5 months recapitulate mid fetal prenatal brain (19-24 pcw)</td>
<td>Do not describe cell death</td>
</tr>
<tr>
<td>Schwartz et al. (2015), [123]</td>
<td>hESC-derived neural progenitors, endothelial cells, mesenchymal stem cells, microglia/macrophage precursors combined on chemically defined polyethylene glycol hydrogels</td>
<td>Screening of toxins</td>
<td></td>
</tr>
<tr>
<td>Qian et al. (2016), [97]</td>
<td>Miniaturized spinning bioreactor (Spin Ω) Pre-patterned embryoid bodies with SMAD inhibitors, embedded in Matrigel and treatment with GSK-3β inhibitor, WNT3A protein and SMAD inhibitor Sonic hedgehog (SHH), FGF8, SMAD inhibitors, GSK-3β inhibitor Pre-patterning with dual SMAD inhibitors – neuroectodermal fate WNT3A, SHH, Purmorphamine</td>
<td>Forebrain organoids with all six cortical layers, and GABAergic neuronal subtypes; oSVZ-like region with NPCs</td>
<td>Prefrontal cortex development: GW 22 at day 84 GW 14-24/35 at day 100 Multi-layer progenitor zone recapitulating human cortical development maintenance of the stem cell niche risk genes for schizophrenia and ASD show significant overlap comparing to post mortem patients tissue</td>
</tr>
</tbody>
</table>
Table 2 Advantages and Disadvantages of 2D vs 3D models

<table>
<thead>
<tr>
<th></th>
<th>2D cell culture:</th>
<th>3D cell culture:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advantages</td>
<td>Easier environmental control, cell manipulation, and imaging.</td>
<td>Cytoarchitecture similar to <em>in vivo</em></td>
</tr>
<tr>
<td></td>
<td>Homogenic</td>
<td>Variety of cellular populations</td>
</tr>
<tr>
<td></td>
<td>High reproducibility</td>
<td>Organ specific functions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Niche-like environment</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Inability to depict traits exhibited <em>in vivo</em> (e.g. gene expression)</td>
<td>Diffusional transport limitations (oxygen, nutrients).</td>
</tr>
<tr>
<td></td>
<td>Less compatibility with <em>in vivo</em> settings.</td>
<td>Technical challenges for manipulation and imaging.</td>
</tr>
<tr>
<td></td>
<td>Increased drug sensitivity (due to monolayer) and exposed surface.</td>
<td>Standardization and reproducibility issues – “batch syndrome”</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time and labor consuming</td>
</tr>
</tbody>
</table>
Figure 1

Brain developmental stages

<table>
<thead>
<tr>
<th>embryonic</th>
<th>early fetal</th>
<th>early mid fetal</th>
<th>late mid fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="embryonic brain organoid" /></td>
<td><img src="image" alt="early fetal brain organoid" /></td>
<td><img src="image" alt="early mid fetal brain organoid" /></td>
<td><img src="image" alt="late mid fetal brain organoid" /></td>
</tr>
</tbody>
</table>

Brain organoids
Figure 2
Figure 3

Cell and tissue engineering
Patient’s derived iPSC
Brain organoids

In vitro disease modeling

Defined cohort of ASD patients
New diagnostic markers

Prevention and therapy

Drug screening “in spher"o"

Gene editing

Cellular and functional characterization
Cell variety
Neuronal circuits
Synaptic connectivity
Electrophysiology

Multi-omics profiling
- Genomics
- Epigenomics
- Transcriptomics
- Proteomics
- Metabolomics

Bioinformatics and multidimensional Biomarker identification
- Computational biology
- Big data analysis
- Mathematical models

Biobanking

Cellular and functional characterization

Multi-omics profiling

Bioinformatics and multidimensional Biomarker identification

Drug screening “in spher"o"

Gene editing

In vitro disease modeling
Psychiatry in a Dish: Stem Cells and Brain Organoids Modeling Autism Spectrum Disorders

**Supplemental Information**

**Supplemental Table S1** – Finding in the studies of ASD iPSC derived neurons/glia

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diagnosis</th>
<th>N of patients</th>
<th>N of cell lines</th>
<th>Origin</th>
<th>Cell type differentiation</th>
<th>Findings</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marchetto et al. (2010), [1]</td>
<td>Rett syndrome – mutation in MeCP2 Missense Nonsense Frameshift Classical symptoms Controls</td>
<td>2</td>
<td>4</td>
<td>fibroblasts</td>
<td>neurons</td>
<td>Reduced synapses Reduced spine density Smaller soma size Altered Ca²⁺signaling Electrophysiological defects</td>
<td></td>
</tr>
<tr>
<td>Chamberline et al. (2010), [2]</td>
<td>Angelmann/Prader Willi AS-maternally inherited deletions of 15q11-q13 PWS paternal deletion of 15q11-q13</td>
<td>2</td>
<td>1</td>
<td>fibroblasts</td>
<td>neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheridan et al. (2011), [3]</td>
<td>Frigile X syndrome</td>
<td>3 male</td>
<td></td>
<td>fibroblasts</td>
<td>Post mitotic neurons and glia</td>
<td>Aberrant neuronal differentiation correlated with epigenetic modification of FMR1 gene and loss</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Diagnosis</td>
<td>N of patients</td>
<td>N of cell lines</td>
<td>Origin</td>
<td>Cell type differentiation</td>
<td>Findings</td>
<td>Remarks</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>--------</td>
<td>---------------------------</td>
<td>-----------------------------------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>Ananiev et al. (2011), [4]</td>
<td>Rett syndrome 5-years old T158M mut. 25-years old V247X mut. 8-years old R306C mut. 11-years old R294X mut. 28-years old health</td>
<td></td>
<td></td>
<td>fibroblasts</td>
<td>neurons</td>
<td>Smaller nuclear size</td>
<td></td>
</tr>
<tr>
<td>Pasça et al. (2011), [6]</td>
<td>Timothy syndrome Controls</td>
<td>2 3</td>
<td>36</td>
<td>fibroblasts</td>
<td>Cortical neurons</td>
<td>Defects in Ca(^{2+}) signaling and activity dependent gene expression Abnormal differentiation of cortical neurons Increased TH Increased norepinephrine and dopamine</td>
<td></td>
</tr>
<tr>
<td>Kim et al. (2011), [7]</td>
<td>Rett syndrome 3 (5 years old) 1 (8 years old)</td>
<td>10</td>
<td></td>
<td>fibroblasts</td>
<td>neurons</td>
<td>Mutation in methyl 1GpG binding protein 2 (MeCP2) Defect in neuronal</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Diagnosis</td>
<td>N of patients</td>
<td>N of cell lines</td>
<td>Origin</td>
<td>Cell type differentiation</td>
<td>Findings</td>
<td>Remarks</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>---------------------------------------------</td>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| De Rosa et al. (2012), [8]       | ADI-R diagnosis of autism  
Minimal IQ equivalent ≥35 or a minimal developmental level of 18 months as measured by the Vineland Adaptive Behavior Scales | 1 (25 years old) | 3               | Peripheral blood mononuclear cells          | GABA-ergic neurons        | maturation consistent with RTT phenotypes                                | Some neurons are mitotically active – in process of maturation         |
<p>| Liu et al. (2012), [9]           | 54 years old female FMR1 premutation carrier (30 and 94 CGG repeats)      | 1             | Multiple derivative lines – normal or expanded FMR1 allele | fibroblasts neurons       | Reduced post synaptic density protein 95 expression; Reduced synaptic puncta density; Reduced neurite length; Ca^{2+} signaling defects |                                                                 |
| Shcheglovitov et al. (2013), [10]| Phelan-McDermis SHANK3                                                     |               |                 | fibroblasts neurons                         | Impairment of excitatory neurons and synaptic transmission |                                                                 |
| Boissart et al. (2013), [12]     | ASD non-syndromic de novo mutation in SHANK3                              | 2             |                 | fibroblasts Cortical neurons - glutamatergic | Drug screening            |                                                                 |</p>
<table>
<thead>
<tr>
<th>Reference</th>
<th>Diagnosis</th>
<th>N of patients</th>
<th>N of cell lines</th>
<th>Origin</th>
<th>Cell type differentiation</th>
<th>Findings</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brick et al.</td>
<td>Fragile X (FXS) full mutation</td>
<td>5</td>
<td>More than 200</td>
<td>fibroblasts</td>
<td>Glutamatergic neurons</td>
<td>Biobank</td>
<td>Patients with FXS have genetic test CGG repeat number as a full mutation; Idiopathic autism – negative for FMR1 mutation; Age of the patients &gt;8 years; IQ of 40 or higher; Most are male (n=99); 6 female: Rett (1) Premutation (1) Idiopathic (3) Control (1); Controls are screened negative for ASD using Social Communication Questionnaire, IQ (WASI) and screened for CGG repeat size and/or FMR1 expression; Patients underwent ADOS-G, ADI-R and WASI</td>
</tr>
<tr>
<td></td>
<td>FXS with autism</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fragile X permutation</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FX permutation with autism</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ASD</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, head size &gt; 99%ile</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, head size 19%-90%ile</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, IQ ≥85</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, IQ ≤ 60</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, with seizures</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autism idiopathic, without seizures</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FXS, without seizures</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FXS with autism (idiopathic), without seizures</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Griesi-</td>
<td>8 years old male with</td>
<td>1</td>
<td></td>
<td>Dental pulp</td>
<td>Neurons</td>
<td>Disruption in axonal</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Diagnosis</td>
<td>N of patients</td>
<td>N of cell lines</td>
<td>Origin</td>
<td>Cell type differentiation</td>
<td>Findings</td>
<td>Remarks</td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>---------------------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Oliveira et al. (2014), [14]</td>
<td>balanced translocation 46 XY, t (3;11)(p21;q22) disruption of TRPC6 cation channel</td>
<td></td>
<td></td>
<td>stem cells</td>
<td></td>
<td>guidelines, dendritic spine growth, excitatory synapse formation and Ca(^{2+}) signaling</td>
<td></td>
</tr>
<tr>
<td>Belinsky et al. (2014), [15]</td>
<td>22q11.2 deletion and healthy individuals</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>Patch-clamp recordings, Electrophysiology and gene expression during neuronal development</td>
<td></td>
</tr>
<tr>
<td>Williams et al. (2014), [16]</td>
<td>Rett syndrome</td>
<td>3</td>
<td></td>
<td>Astroglial progenitors, GFAP+ astrocytes</td>
<td></td>
<td>Negative effect of condition media on neurons morphology and function</td>
<td></td>
</tr>
<tr>
<td>Doers et al. (2014), [17]</td>
<td>Fragile X syndrome 4-10 years old boys</td>
<td>3</td>
<td></td>
<td>fibroblasts</td>
<td>Forebrain neurons</td>
<td>Defects in neurite outgrowth</td>
<td></td>
</tr>
<tr>
<td>Andon-Noda et al. (2015), [18]</td>
<td>Rett syndrome – 10 years old RTT-monozygotic female tweens with mosaic expression of mutant X-linked methyl CpG-binding protein 2</td>
<td>2</td>
<td></td>
<td>2 sets wild type and mutant MECP2 expressing iPSC</td>
<td>astrocytes</td>
<td>Accelerated astrocyte formation, Increased astrocyte specific differentiation from multipotent neural stem cells</td>
<td></td>
</tr>
<tr>
<td>Mariani et al. (2015), [19]</td>
<td>Idiopathic Autism /macrocephalia head circumference &gt; 90%ile, males</td>
<td>4</td>
<td></td>
<td>fibroblasts</td>
<td>Organoids GABA-ergic neurons</td>
<td>Increased number of inhibitory neurons; Increased FOXG1 expression; In mature organoids proportion in proliferating cells no significant difference</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Diagnosis</td>
<td>N of patients</td>
<td>N of cell lines</td>
<td>Origin</td>
<td>Cell type differentiation</td>
<td>Findings</td>
<td>Remarks</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>-----------------</td>
<td>---------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ilieva et al.</td>
<td>Mothers</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td>Accelerated cell cycle – decreased cell cycle length; Increased synapse number</td>
<td>between ASD and controls</td>
</tr>
<tr>
<td></td>
<td>Fathers</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>brothers</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marcheto et al.</td>
<td>Idiopathic ASD – patients diagnosed with ASD and MRI scanning</td>
<td>8</td>
<td>5</td>
<td>Fibroblasts</td>
<td>Neuronal progenitor cells</td>
<td>Increased cell proliferation caused by dysregulation of β-catenin/BRN2 transcriptional cascade Abnormal neurogenesis Reduced synaptogenesis leading to functional defects in neuronal networks</td>
<td></td>
</tr>
<tr>
<td>(2016), [20]</td>
<td>Individuals with larger than normal average brain volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Behavior presentation DSM-IV; APA, Wecher Intelligence scale, the Autism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diagnostic observation Schedule, the Autism diagnostic interview, Revised</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and Vineland Adaptive behavior scales</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Supplemental References