Midbrain organoids: A new tool to investigate Parkinson's disease

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Abstract

The study of human 3D cell culture models not only bridges the gap between traditional 2D in vitro experiments and in vivo animal models, it also addresses processes that cannot be recapitulated by either of these traditional models. Therefore, it offers an opportunity to better understand complex biology including brain development. The brain organoid technology provides a physiologically relevant context, which holds great potential for its application in modelling neurological diseases.

Here, we compare different methods to obtain highly specialised structures that resemble specific features of the human midbrain. Regionally patterned neural stem cells were utilised to derive such human midbrain-specific organoids. The resulting neural tissue exhibited abundant neurons with midbrain dopaminergic neuron identity, as well as astroglia and oligodendrocyte differentiation. Within the midbrain organoids, neurite myelination and the formation of synaptic connections were observed. Regular neuronal fire patterning and neural network synchronicity were determined by multielectrode array recordings. In addition to electrophysiologically functional neurons producing and secreting dopamine, responsive neuronal subtypes, such as GABAergic and glutamatergic neurons were also detected. In order to model disorders like Parkinson’s disease in vitro, midbrain organoids carrying a disease specific mutation were derived and compared to healthy control organoids to investigate relevant neurodegenerative pathophysiology. In this way midbrain-specific organoids constitute a powerful tool for human-specific in vitro modelling of neurological disorders with a great potential to be utilised in advanced therapy development.
Introduction

The development of the organoid methodology counts today as a major technological breakthrough in stem cell research. It enabled immense advances in the application of human induced pluripotent stem cells (hiPSCs) and was even celebrated as the ‘Method of the Year’ in 2017 (“Method of the Year 2017: Organoids,” 2018).

Cerebral organoids

Initial experiments on self-organisation of pluripotent stem cells (PSCs) under 3D conditions were performed more than ten years ago. Yet, it were the findings of MADELINE LANCASTER and co-workers that actually launched a new era in research on the human brain by introducing the “cerebral organoids” in 2013 (Kelava & Lancaster, 2016b; Lancaster et al., 2013). Supporting the conclusions of initial 3D culture experiments, LANCASTER et al. took advantage of the PSCs’ intrinsic self-organisation and derived neuroepithelium under 3D conditions (Kadoshima et al., 2013). To avoid limitations on specific brain region identities, they did not add patterning growth factors. Instead, after aggregation, the cells were embedded in Matrigel, a surrogate matrix that was previously introduced for the generation of intestinal organoids (Sato et al., 2009). This serves as a structural support, inducing the correct polarity stimulus to promote the complex outgrowth of large, apicobasal neuroepithelial buds (Lancaster & Knoblich, 2014c, 2014a; Wang et al., 2018). These buds expand during the course of culture and acquire not only various brain identities but also fluid-filled lumina reminiscent of brain ventricles. To improve nutrient supply and oxygen exchange, the floating cerebral organoids were cultured in spinning bioreactors or on orbital shaker plates, allowing organoids to grow up to 4 mm in diameter (Kelava & Lancaster, 2016b; Lancaster & Knoblich, 2014a). These optimised growth conditions combined with the intrinsic self-organising capabilities of PSCs resulted in the formation of a variety of brain regions within a single organoid, including hindbrain, midbrain, forebrain, and even retinal tissue identities (Lancaster & Knoblich, 2014b; Lancaster et al., 2013; Renner et al., 2017; Wang et al., 2018). Remarkably, a detailed study about the patterning events during the course of cerebral organoid development and differentiation indicates that spatial and temporal patterning events are reminiscent of those determining the human brain development (Renner et al., 2017).

Modification of cerebral organoids

The classic cerebral organoid protocol, describing the generation of general whole-brain organoids, has been modified by many research groups in the last years and has resulted in the formation of more regionally specific 3D cell cultures (Lancaster et al., 2016; Lancaster & Knoblich, 2014a). In the study of ANCA PAŞCA and co-workers, both bone morphogenetic protein (BMP) and transforming growth factor β (TGF-β) signalling pathways were inhibited by the small molecules Dorsomorphin (DM) and SB-431542 (SB) to achieve an effective neural induction (Paşca et al., 2015). This dual-SMAD inhibition, in combination with fibroblast growth factor (FGF) 2, epidermal growth factor (EGF) and the absence of extracellular scaffolding, gave rise to neural progenitors expressing the dorsal telencephalic markers paired box protein 6 (PAX6) and forkhead box protein G1 (FOXG1). Further neuronal differentiation was promoted by replacing FGF2 and EGF with brain-derived neurotrophic factor (BDNF) and neurotrophic factor 3 (NT3) and led to the generation of various neural and glial identities of the dorsal cortex within each spheroid, including superficial and deep cortical layer neurons (Kelava & Lancaster, 2016b; Paşca et al., 2015). With this, PAŞCA et al. described a method that gave rise to a 3D culture specific of a brain subregion; a culture that exhibited a reduced number of ectodermal derivatives compared to the original cerebral organoid protocol. A similar approach of using a dual-SMAD inhibition was published by QIAN et al. in 2016.
While maintaining the basis of the LANCASTER protocol, such as Matrigel embedding and agitation, they demonstrated that it is possible to culture organoids in 3D printed miniaturised bioreactors, thus enabling more feasible, scaled-up productions of neural 3D cultures (Qian et al., 2016). Another advantage of the self-engineered multi-well spinning device is the possibility of comparing numerous different culture conditions in parallel. PAȘCA et al. aimed to reduce the tissue heterogeneity of the cerebral organoids and therefore pre-patterned the embryoid bodies (EBs) to obtain specific brain regions. The inhibition of TGF-β signalling by SB and activation of Wnt signalling by glycogen synthase kinase 3 (GSK-3β) inhibitor CHIR-99021 (CHIR) within the first two weeks of culture, resulted in forebrain organoids organised in defined, multi-layered progenitor zones, including homologues to the ventricular zone (VZ), the inner and outer subventricular zones (SVZ). Moreover, neuronal types of all six cortical layers could be detected within these forebrain-specific organoids. With the help of the mini-bioreactors, QIAN and co-workers also developed a method to derive hypothalamic-specific organoids. After a dual-SMAD inhibition with SB and LDN-193189 (LDN), they patterned the neuroectodermal cells to a hypothalamic fate by activating Wnt and sonic hedgehog (SHH) signalling, applying WNT3a, SHH and Pumorphamine (PMA) to the culture. 40 days later, these organoids contained cell populations expressing markers specific of hypothalamic neuronal lineages (Qian et al., 2016).

**Disease modelling with brain organoids**

Similar characteristics on a molecular, cellular and physiological basis between human brain organoids and the actual human brain justify their increasing application in studying brain biology and modelling neurological disorders (Wang, Zhu et al. 2018). Already LANCASTER and co-workers discovered the potential of cerebral organoids as a model to detect impaired neurodevelopment and they derived organoids carrying a mutation that causes microcephaly (Lancaster et al., 2013). It was also suggested that a Zika virus (ZIKV) infection was causing microcephaly in neonates. In 2016, the World Health Organization (WHO) declared the ZIKV and its associated complications an emergency of public health (Dutta et al., 2017; Qian et al., 2016). This activation of the global research community led to an accelerated development of vaccines and treatments, with many studies based on cerebral organoids, which were able to recapitulate features of human cortical development in vitro (Cugola et al., 2016; Dang et al., 2016; Garcez et al., 2016; Miner & Diamond, 2016; Nowakowski et al., 2016; Qian et al., 2016; Wells et al., 2016; Xu et al., 2016). The results of these studies indicate that a ZIKV infection affects the neurogenesis, disrupts the cortical layers of the organoids, and, in a similar manner, causes microcephalic-like deficits in cortical development (Dutta et al., 2017). Due to the specific embryonic formation of human brains, only a human-specific 3D cell culture model exhibiting advanced organisational features could have led to the reported discoveries. Neither murine nor 2D cell culture were able to address the potential link between ZIKV and microcephaly (Dutta et al., 2017; Qian et al., 2016; Setia & Muotri, 2019). In addition to this successful application, brain organoids have proven useful to study other neurological disorders. Recently, so-called “tumouroids” have been established from human glioblastoma, the most common and aggressive brain cancer (Dutta et al., 2017). The hypoxic gradients and stem cell heterogeneity found in these tumouroids cannot be recreated via conventional culture methods. Therefore, glioblastoma organoids offer a unique opportunity for their application in brain cancer diagnostics and therapeutics (Bian et al., 2018; Dutta et al., 2017; Hubert et al., 2016). Furthermore, two different approaches using 3D human neural cell culture systems were reported to recapitulate Alzheimer's disease (AD) phenotypes in vitro (Choi et al., 2014; Raja et al., 2016). These 3D cultures provide an environment that promotes the formation of amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs), pathological events that could not
have been serially linked before by using 2D cultured human neurons (Choi et al., 2014, 2016; D’Avanzo et al., 2015; Raja et al., 2016). This confirms that the evolving brain organoid methodology facilitates the development of more precise human cellular models that can support the research of neurodegenerative disorders. The technology of more complex 3D cell culture systems not only bridges the gap between traditional 2D in vitro experiments and in vivo animal models, but also addresses processes that cannot be recapitulated by these traditional models. For example, drug failure or unanticipated side-effects upon translation to humans can be a result of the different metabolisms of humans and animals. Therefore, human organoids offer an opportunity to unravel complex biological processes, such as the development of the human brain, where conventional models have not proven successful. The establishment of stem cell-derived brain organoids allows the modelling of key aspects of human brain development in vitro, by utilising the enormous differentiation potential of PSCs and their ability to self-organise with a specific spatial orientation. Overall, this novel technology provides a physiologically relevant context, such as interactions between glia cells and neurons in a spatially organised microenvironment, which holds great potential for its application in modelling neurological diseases.

Discussion

The lack of advanced experimental in vitro models that truly recapitulate the complexity of the human brain is one of the main limitations in neuroscience and in the field of disease modelling. Current in vitro approaches to model physiology and pathology of human neurons are primarily based on cultures of neurons grown under 2D conditions. While the resulting monolayer cell cultures have proven useful as a tool to study disease mechanisms and to identify potential neuroprotective compounds (Cooper et al., 2012; Nguyen et al., 2011; Qing et al., 2017; Reinhardt, Schmid, et al., 2013; Ryan et al., 2013; Sánchez-Danés et al., 2012; Spathis et al., 2017), these culture conditions do not model several characteristics which are relevant to the human brain. Features such as cell-cell interactions and cytoarchitecture might be crucial to predict the effectiveness of in vitro tested compounds in clinical trials (Abe-Fukasawa et al., 2018). In this case, the in vitro human brain organoid technology is a valuable tool, it allows to opportunity to understand complex biology in a physiologically relevant context and also enables advances in translational applications (Fatehullah et al., 2016). Originally, brain organoid approaches relied on the endogenous capacity of PSCs to self-organise under 3D conditions, intrinsically following early steps of the brain development (Arlotta, 2018). These approaches resulted in ectodermal derivatives with complex cytoarchitectures beyond what is possible with 2D PSC derivatives (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015). Since neurons form functional networks with other neurons and non-neuronal cells in the brain, it is essential to expand the research of neurodegenerative diseases by exploiting 3D models that are able to reproduce these interactions. In general, 3D conditions are able to more closely mimic in vivo environments and therefore enable an accelerated neuronal differentiation and network formation in vitro (D’Avanzo et al., 2015; Haycock, 2011). Moreover, it has been shown that neurons developed in a 3D environment express a more representative range of neuronal genes than neurons derived in 2D conditions (Seidel et al., 2012). A monolayer of neurons cannot provide as many connections between individual cells as a 3D neuronal culture, and the smaller synaptic distances in a 3D neuronal network promotes functional signal transduction (Cullen et al., 2012; D’Avanzo et al., 2015). By creating a third dimension, neurons develop in an environment that is closer to nature and actually relevant to human physiology, consequently gaining morphological and physiological properties similar to those in vivo.
Specifically, the generation and characterisation of a novel midbrain-specific 3D cell culture system provides an advanced in vitro model to study neurodevelopmental processes as well as neurodegenerative diseases of the human midbrain. In order to achieve the formation of these highly specialised structures, distinctively resembling the human midbrain, organoids have been derived from regionally patterned neural stem cells. This particular starting population, already committed to the ventral neural tube fate of the mesencephalon with further application of spatio-temporal specific signalling under 3D culture conditions, has led to the establishment of novel human midbrain-specific organoids (hMO). Here, we compare and evaluate newly derived hMO methods that create a powerful tool for human-specific in vitro disease modelling of neurological disorders.

Derivation of midbrain-specific organoids

To achieve the in vitro derivation of the human midbrain, additional stimuli of specific pathways along with the 3D PSC culture are required. To date, six 3D cell culture approaches have been published by different research groups for deriving tissue that resembles features of the human midbrain (Jo et al., 2016; Kim et al., 2019; Monzel et al., 2017; Qian et al., 2016; Smits, Reinhardt, et al., 2019; Tieng et al., 2014). All of these approaches can be attributed to previous 2D cell culture experiments, which explored the fundamental principles for the generation and characterisation of midbrain fate-specific cells, derived from PSC via exogenous patterning cues (Kirkeby et al., 2012; Kriks et al., 2011; Reinhardt, Glatza, et al., 2013). For instance, QIAN et al. were inspired by 2D experiments performed by KRIKS et al. and also initial midbrain-like tissue experiments reported by TIENG and co-workers (Kriks et al., 2011; Qian et al., 2016; Tieng et al., 2014). In the same year, another protocol describing the generation of hMOs was published (Jo et al., 2016). Jo and co-workers and later Kim et al. based their derivation on the findings of CHAMBERS et al. (Chambers et al., 2009). Additionally, hMO protocols described by MONZEL et al. and SMITS et al. are based on 2D experiments by REINHARDT et al. (Monzel et al., 2017; Reinhardt, Glatza, et al., 2013; Smits, Reinhardt, et al., 2019).

The initial step for the derivation of hMOs is the formation of embryoid bodies and induction of the neuroectoderm via dual-SMAD inhibition. In all hMO protocols compared here, SB was used to inhibit the Activin/TGF-β signalling pathway (Table 1). The combination with BMP pathway inhibitors enabled a full neural conversion of the PSCs. The use of BMP antagonists Noggin, LDN, and DM has been shown in 2D (Chambers et al., 2009; Kriks et al., 2011; Reinhardt, Glatza, et al., 2013) and applied in 3D stem cell cultures (Table 1). To control the specification of the neural progenitor cells, CHIR, a potent chemical inhibitor of GSK-3β, was used to dose-dependently activate the WNT signalling pathway (Kirkeby et al., 2012). The final patterning, towards midbrain floor plate precursors, requires a treatment with small molecule activators of the SHH signalling pathway, such as recombinant SHH, PMA or smoothened agonist (SAG) (Kriks et al., 2011; Smits, Reinhardt, et al., 2019). This composition of exogenous patterning cues results in neural progenitors that can give rise to authentic, midbrain-specific dopaminergic neurons (mDANs) (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Nolbrant et al., 2017).

The further development of these protocols allowed the derivation of 3D cultured hMO. In contrast to 2D monolayer cultures, hMOs can recapitulate complex interactions of mDANs with other cell types of the central nervous system (CNS) in a 3D environment. Cluster analysis, comparing the differentially expressed genes of hMOs, 2D-cultured mDANs, and human prenatal midbrain samples indicated that hMOs share features of gene expression profiles of the prenatal midbrain, which cannot be recreated via the conventional 2D derivation method for mDANs (Jo et al., 2016). This demonstrates that the specific cellular structure and
heterogeneity of the midbrain-specific organoid cultures allows biological aspects to be modelled, which cannot be mimicked with current 2D stem cell cultures. Additionally, enormous amounts of disease-relevant mDANs can be produced in a rapid and reproducible way, which is required for disease modelling and drug discovery in the field of Parkinson’s disease (PD). Numerous published protocols describe the generation of ventral mDANs from human PSCs in 2D by replicating mDAN’s in vivo-specification in vitro (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Nolbrant et al., 2017). Although current protocols are based on the generation of LMX1A/FOXA2 positive midbrain floorplate progenitors, differentiations starting from PSCs are time-consuming and typically result in cultures containing various neuronal identities (Grealish et al., 2014; Hargus et al., 2010; Kirkeby et al., 2012; Kriks et al., 2011). Typically, brain organoids are generated from PSCs by exploiting developmental processes (Clevers, 2016; Lancaster et al., 2013) or by creating an environment favouring specific stem cell niches (Jo et al., 2016; Kim et al., 2019; Qian et al., 2016; Tieng et al., 2014). However, the utilisation of neural stem cells (NSCs) as a starting population for hMOS has the advantage that already patterned cells differentiate more efficiently into the desired structures. Other adult stem cell-derived organoid cultures have been established for instance, for the generation of intestinal or lung organoids. They contain cell types that are present in the organ from which they were derived and recapitulate some degree of its spatial organisation (Clevers, 2013; Drost & Clevers, 2017; Huch & Koo, 2015). Similarly, the presence of midbrain-specific stem cell niches and clusters of mDANs was shown in a NSC-derived hMO model and approaches to efficiently differentiate mDANs within hMOS by starting from expandable neural precursor cells (NPCs) were reported (Monzel et al., 2017; Smits, Magni, et al., 2019; Smits, Reinhardt, et al., 2019) (Table 3). In these approaches NPCs that received already some patterning towards the midbrain have been used. Both hMO models have been already used in other studies (Berger et al., 2018; Jan et al., 2018; Jarazo et al., 2019). Among the published hMO protocols, different approaches have been presented to estimate the number of mDANs that arise during the organoid development (Table 2). Tieng and Qian dissociated their 3D structures and cultured the resulting single cells as a monolayer (Qian et al., 2016; Tieng et al., 2014). Their quantification resulted in more than 60% TH-positive cells in 21 day-old cultures (Tieng et al., 2014) and around 55% TH-positive cells after 65 days (Qian et al., 2016). A more straightforward method to quantify the mDAN population is flow cytometry via fluorescence-activated cell sorting (FACS), which also requires dissociated hMOS. By doing so, it has been shown that after 61 days of culture around 64% cells were triple-positive for the mDAN markers TH, LMX1A, and FOXA2 (Monzel et al., 2017). Surprisingly, with the same method, Jo and co-workers identified only around 22% of MAP2-positive neurons co-expressing TH in their hMOS after 60 days of culture (Jo et al., 2016). A third approach to estimate the percentage of mDANs within hMOS has been described recently (Bolognin et al., 2019; Smits, Reinhardt, et al., 2019). Here, image analysis algorithms enabled the automated segmentation of nuclei and neurons, with around 62% TH-positive cells after 35 days and around 54% TH-positive cells after 70 days of 3D culture were quantified. The application of high-content image-analysis allows entire organoid sections to be examined, rather than dissociated single cells, which preserves the original morphology and cell-cell interactions of the mDANs. TH is a marker commonly used for mDAN detection, as it is a rate-limiting enzyme for DA biosynthesis. However, it is also expressed in other catecholaminergic cell types and does not represent a unique marker specifically for mDANs, as in the case of the dopamine transporter (DAT) for example or the actual presence of the neurotransmitter DA (Abeliovich & Hammond, 2007). DAT expression has been reported in almost every hMO model (Jo et al., 2016; Kim et al., 2019; Monzel et al., 2017; Qian et al., 2016; Smits, Reinhardt, et al., 2019) (Table 2).
TIENG and co-workers did not show the presence of DAT in their engineered nervous tissues (ENTS), but they were able to prove the synthesis of DA via high-performance liquid chromatography (HPLC) after cell lysis (Tieng et al., 2014). Also Jo and Kim also applied the same method to assess the DA content within the hMOs (Jo et al., 2016; Kim et al., 2019). To verify that the mDANs were not only able to produce DA, but actually able to release the neurotransmitter, the supernatant of the 3D cultures can be analysed with an enzyme-linked immunosorbent assay (ELISA). SMITS et al. detected a DA release that increased as the hMOs matured (Smits, Reinhardt, et al., 2019). An interesting observation was reported for the first time by Jo et al., where they detected insoluble, dark-coloured deposits in their hMOs after approximately 60 days (Jo et al., 2016). Using a Fontana-Masson staining they confirmed that these granules were neuromelanin (NM), which is a by-product of DA biosynthesis and accumulates postnatally in the substantia nigra pars compacta (SNc) of the human brain (Kim et al., 2019; Pasca, 2018; Zecca et al., 2003). Other hMO culture protocols have also stimulated the production of NM (Monzel et al., 2017; Smits, Reinhardt, et al., 2019) (Table 2). The presence of NM is a unique feature of the primate brain (Pasca, 2018). It is neither apparent in mice brains nor murine midbrain-specific organoids (Dawson et al., 2010; Fedorow et al., 2005; Jo et al., 2016; Marton & Paşca, 2016). Wild-type mDANs, derived and cultured in monolayer conditions, only produce NM after an artificial inductions of progerin expression, which is associated with premature ageing (Miller et al., 2013; Sterneckert et al., 2014). Membrane-bound, dense pigmented NM was also detected in long-term cultures of homozygous DJ-1 mutant and idiopathic PD patient-derived mDANs (Burbulla et al., 2017). Currently, it is unknown whether NM has a protective or damaging effect on the cell survival, however it is proven that NM-containing mDANs of the SNc are especially vulnerable during the course of PD (Jo et al., 2016; Marton & Paşca, 2016; Zecca et al., 2003). Therefore, NM containing hMOs have a great potential to be used for in vitro PD modelling, possibly revealing specific phenotypes that are not present in wild-type 2D cultures or murine models. Moreover, hMOs provide the basis for future studies about the role of NM in PD (Marton & Paşca, 2016; Michel et al., 2016). Since mDANs are essential to model the human midbrain, hMO research has so far focused mainly on this specific type of neurons. Nevertheless, detailed in vivo studies have described that the released DA diffuses into synaptic regions of glutamatergic and GABAergic synapses and directly affects other striatal cell types, including the neurons forming the island-/striosome GABA pathway, striatal cholinergic interneurons and the striatal GABA interneurons, all possessing DA receptors (Borroto-Escuela et al., 2018; Calabresi et al., 2014). Furthermore, substantia nigra (SN) dopaminergic neurons are directly controlled by GABAergic input (Tepper & Lee, 2007). Evidence from these studies suggest that the presence of other neuronal subtypes is important to be able to model multifactorial disease like PD. A first transcriptional characterisation of hMO was performed by Jo and co-workers, showing that aspects of prenatal midbrain gene expression profiles were found in the organoids in contrast to the conventional 2D-derived mDANs (Jo et al., 2016; Lin et al., 2016; The GTEx Consortium, 2015). For a further validation of the genetic expression profile during the course of hMO development they suggested conducting a single-cell transcriptome analysis, as it has been shown before for cerebral organoids (Camp et al., 2015; Kageyama et al., 2018). In a recent study, single-cell transcriptomic data from hMOs demonstrated that there is an increased expression of neuronal- and stem cell-specific genes in 35 day- compared to 70 day-old hMOs, whereas exclusively the gene-gene correlations between only neuron-specific genes increased considerably at day 70 (Smits, Magni, et al., 2019). This signifies an increasing commitment of cells towards the neuronal cellular fate during the course of organoid development and further supports the finding of a progressive maturation of post-mitotic neurons. The identification of these neuron-specific genes revealed that the genes up-regulated at the earlier time point are particularly relevant in the processes of neurogenesis, neuronal migration and
differentiation (for example, early B-cell factor 3 (EBF3), (Garcia-Dominguez, 2003) and L1 cell adhesion molecule (L1CAM), (Patzke et al., 2016)), whereas the up-regulated genes at the later time point have been implicated in vivo in a modulatory contribution to neurite extension (for example, repulsive guidance molecule B (RGMB), Ma et al. (2011)). This indicates a higher commitment of the cells toward their intended fate and a progressive maturation of post-mitotic neurons within the hMOs. Since the presence of neuronal subtypes, glutamatergic and GABAergic neurons have been reported in hMOs before (Jo et al., 2016; Tieng et al., 2014), the residence of specific neuronal subtypes has been addressed with the high-resolution single-cell analysis (Smits, Magni, et al., 2019). The expression of genes typical for dopaminergic, glutamatergic, GABAergic, and serotonergic neurons have been investigated and their presence further confirmed by immunohistochemistry staining for the respective neurotransmitters. This allows dopaminergic, glutamatergic and GABAergic neurons and a few serotonergic neurons to be robustly detected within hMOs (Table 2). So far, detailed analysis of the neuronal subtype’s function and their interaction has not been addressed. However, with regards to the fact that mDANs physiologically synapse in the striatum and not in the midbrain in vivo, hMOs are potentially limited as an in vitro model in this case.

Besides the detailed characterisation of the neuronal population, also the analysis of astroglia and oligodendrocyte differentiation is also crucial for accurately modelling the human midbrain. The presence of astrocytes is essential for the formation of synapses and regular neuronal activity (Chung et al., 2015). Astrocytes are defined later than neurons during development, and their immunoreactivity is only detectable in hMOs after 35 days of cultivation (Chabou & Deneen, 2013; Molofsky et al., 2012; Monzel et al., 2017) (Table 2). Fast information transmission between neurons depends on axonal myelination, which is achieved by oligodendrocytes in the CNS. In most stem cell-based differentiation protocols, the differentiation into oligodendrocytes is extremely inefficient (Bunk et al., 2016; Jablonska et al., 2010). However, differentiation into oligodendrocytes and detected myelination of non-dopaminergic neurons has been achieved in hMOs (Monzel et al., 2017). Some neurites in these hMOs were ensheathed by oligodendrocytes and even structures such as the nodes of Ranvier became apparent, which are of critical importance for electrochemical transmission of signals in axons (Fauvres-Sarraillh & Devaux, 2013). The feature of unmyelinated or thinly myelinated neurons is particularly well described for SNc mDANs, and explains why only about 30% of βIII Tubulin (TUJ1) and myelin basic protein (MBP) overlapping cells have been quantified in the hMO system (Braak & Del Tredici, 2004; Monzel et al., 2017; Orimo et al., 2011; Sulzer & Surmeier, 2013). To allow for future applications and improve the impact of hMOs in pathophysiological and pharmacological studies, the electrical activity and functional maturity of the midbrain-specific 3D cultures have been assessed. The presence or rather the co-localisation of the presynaptic marker SYNAPTOPHYSIN and the postsynaptic marker PSD95 indicated direct contact between a pre- and a postsynapse (Monzel et al., 2017). The exact morphology of hMO-derived synapses until now has not been addressed in detail, although other hPSC-derived brain organoids already reflect many aspects of human synapse formation and function (Wilson & Newell-Litwa, 2018). Whole-cell patch recordings have been performed with sliced hMOs sections (Jo et al., 2016) or with neurons obtained from hMOs (Smits, Reinhardt, et al., 2019). This is an established, but invasive method that allow specific neuronal subtypes to be identified and analysed, however, the continuous development of an individual organoid cannot yet be followed in such detail. Alternatively, non-invasive recordings of extra cellular field potentials can be achieved by a MEA systems and allow insights into physiological properties of in vitro cultures and chronological analysis (Luhmann et al., 2016). Tieng and co-workers detected spontaneous and evoked electrophysiological activities in their ENTs (Tieng et al., 2014). Furthermore, in the study of Monzel et al., spikes occurred closely in time on multiple electrodes, which indicates neuronal network...
synchronicity, were detected (Monzel et al., 2017). To specifically determine the activity of different neuronal receptors within the organoid, the response to chemical compounds can be examined. The functionality of DA receptors has been tested with the application of quinpirole, a specific D2/D3 receptor agonist. Importantly, mDANs express D2 autoreceptors. This has previously been used in several studies and shown to effectively suppress the firing in hMOs (Jo et al., 2016; Monzel et al., 2017; Smits, Magni, et al., 2019). Together with the reported DA production and release, this strongly suggests that TH-positive neurons, developed in hMOs, exhibit electrophysiological and biochemical qualities of mature mDANs and express functional, quinpirole-responsive, DA receptors. To further isolate and attribute the recorded signals to neuronal subtypes, inhibitory and excitatory communication was additionally blocked with specific drugs following an established experimental design by Illes and co-workers. Drugs like, Gabazine an antagonist of GABA receptors which result in a disinhibition of target neurons of GABAergic neurons, as well as NMDA-receptor and AMPA/Kainate-receptor antagonists inhibiting glutamatergic excitatory communication were used (Illes et al., 2014). Together with the characteristic hallmarks of synapse formation, consisting of a direct contact between pre- and postsynapses and composing the prerequisite for electrophysiological and neuronal network functionality, these experiments confirmed functional GABAergic and glutamatergic neuronal cells, it is important to expand our research of neurodegenerative diseases using 3D models that are able to recapitulate cell autonomous as well as non-cell autonomous aspects. Utilising 3D cell culture models that comprise a variety of neuronal subtypes could lead to new insights into the selective vulnerability, which is observed in neurodegeneration. Evidence suggests that specific regulation of the excitability of mDANs by other neuronal subtypes in the midbrain might explain their selective vulnerability in PD (Calabresi et al., 2014; Calabresi & Di Filippo, 2015; Korotkova et al., 2004). This underlines the importance and the enormous potential for future disease models that utilise hMOs, as they contain functionally connected heterogeneous neuronal populations.

**Disease modelling in midbrain-specific organoids**

Organoids, specifically modelling the human midbrain, hold great promises for studying the human brain development and for modelling the neurodegenerative disorder PD. PD is the second most common degenerative neurological disorder after Alzheimer’s disease and is defined by the selective loss of mDANs in the SNc of the human midbrain. Intriguingly, after decades of research on PD, the molecular mechanisms underlying the initiation and progression of the neurodegenerative disease, commonly occurring as idiopathic form, have not been entirely revealed and remain largely elusive (Roybon et al., 2004). Therefore, the establishment of region-specific brain organoids offers new possibilities to study neuronal diseases that are linked to a specific part of the human brain, such as PD. Neurodegenerative disorders, such as PD, are typically considered to be age-associated diseases (Sepe et al., 2016; Xu et al., 2016). However, there is accumulating evidence that PD has a strong neurodevelopmental component that probably defines the susceptibility to develop the disease (Garcia-Reitboeck et al., 2013; Le Grand et al., 2015; Schwamborn, 2018). This finding supports the importance of human brain development models to investigate the disease’s underlying mechanisms. A recent publication provides a proof-of-principle study where either patient-derived or genetically modified hMOs harbouring the disease-associated G2019S mutation in the LRRK2 gene show PD-relevant phenotypes including reduced number of mDANs (Smits, Reinhardt, et al., 2019). By evaluating the number of links (branching) and nodes (dendrite bifurcation points) of the mDANs developed within the different hMO groups, a significant reduction of the...
dopaminergic network complexity in the patient-derived TH-positive neurons was identified which is also known to occur in PD patients’ brain (Bernheimer et al., 1973; Kordower et al., 2013; Smits, Reinhardt, et al., 2019). Based on another published hMO protocol, Kim and co-workers also derived organoids carrying the LRRK2-G2019S mutation (Jo et al., 2016; Kim et al., 2019). In line with the findings of Smits and co-workers, Kim et al. discovered that the mDANs within the LRRK2-G2019S organoids reveal a decreased neurite length in comparison to the mDANs within the control organoids. They further assessed an overall decreased expression level of mDANs-specific markers, such as TH, aromatic l-amino acid decarboxylase (AADC), and DAT in their engineered LRRK2-G2019S hMOs (Kim et al., 2019). They achieved a partial recovery of those gene’s expression levels after treating the LRRK2-G2019S mutant organoids with the LRRK2 kinase inhibitor GSK2578215A. This LRRK2 inhibition suggests a positive impact on mDAN cell death and additionally proves that this 3D cell culture system is susceptible to investigating therapeutic strategies against PD (Kim et al., 2019). Kim and co-workers also determined hMOs carrying the LRRK2-G2019S mutation exhibit an abnormal localisation of α-synuclein that is phosphorylated at serine 129 (pS129). They claim that pS129-α-synuclein is aberrantly expressed in mutated hMOs and they detected a LRRK2-G2019S mutation-dependent increase of thioflavin T-positive deposits in TH-positive neurons over time, even though the overall α-synuclein expression did not appear to increase (Kim et al., 2019). Surprisingly, these detected thioflavin T-positive deposits appeared to be extracellular and were not clearly overlapping with the TH signal. Whether these findings are reproducible with another analytical method, still needs to be explored further. Nevertheless, the assessment of PD-associated pathologies, such as the synucleinopathies, in human-specific advanced cell culture models is crucial due to the inherent differences between human and mouse mDAN vulnerability and as existing murine transgenic models have not been efficient in developing an accurate representation of the underlying disease mechanisms (Burbulla et al., 2017; Byers et al., 2012; Hemmer et al., 2018; Koh et al., 2018). In the study of Smits et al., a significant increase of FOXA2-positive progenitor cells in the patient-specific organoids was demonstrated (Smits, Reinhardt, et al., 2019). Since FOXA2 is required for the generation of mDANs, it is hypothesised that this might be a compensatory response to an impaired specification of mDANs promoted by the mutated LRRK2 gene (Sasaki et al., 1997), additionally it might be a results of a decreased differentiation potential of the progenitor cells. Similar compensatory mechanisms have been described in PD before and might represent an attempt to counteract neurodevelopmental defects induced by PD-specific mutations (Blesa et al., 2017). While introducing also isogenic control hMOs, it was also confirmed that the introduction of the LRRK2-G2019S mutation caused deleterious effects on the complexity of mDANs within a healthy background. On the contrary, LRRK2-G2019S gene correction within a PD patient background is not sufficient to rescue this effect. As LRRK2-G2019S is not fully penetrant and the probability of developing PD individually varies among the carriers, it is suggested that its pathological function comprises of additional pathways (Goldwurm et al., 2007; Smith et al., 2006). In this context, a permissive genetic background, due to cumulative genetic variants, might mediate and either enhance or diminish the LRRK2-induced neurodegeneration (Bolognin et al., 2019). Remarkably, in the analysis of all studied features, the PD patient-derived lines cluster together, independently of the presence or absence of the mutation (Smits, Reinhardt, et al., 2019). This indicates that the genetic background of the PD patients, regardless of gene editing, accounts for most of the differences between the studied cell lines and seems to be a major discriminating factor. Thus, not the LRRK2-G2019S mutation but rather the genetic background of the patients constitutes the strongest contribution to the phenotypes and supports the hypothesis that the genetic background of PD patients can influence the degeneration of mDANs (Bolognin et al., 2019). These findings show that 3D hMOs and the corresponding mDANs represent powerful
new tools for in vitro disease modelling. Importantly, in PD patients in the midbrain mainly the SN is affected while a neighbouring region, the ventral tegmental area, which also contains DNs is largely unaffected. Current hMO models so far have not addressed this different vulnerability sufficiently. However, this is a limitation that certainly will be investigated in the future. By deriving further hMOs that carry defects in genes that are known to cause PD we could broaden the understanding of disease-related abnormalities and the context in which they arise (Benson & Huntley, 2019). The patient-specific nature of these models also opens promising avenues for future personalised medicine approaches (Bu et al., 2016; Hillje & Schwamborn, 2016; Smits, Reinhardt, et al., 2019).

Perspectives

The generation and characterisation of midbrain-specific organoid protocols and thereby the provision of sophisticated in vitro models to study both neurodevelopmental processes and neurodegenerative diseases of the human midbrain have led to novel findings in the field of advanced 3D in vitro cell culture systems. Furthermore, the reported PD-relevant phenotypes in PD patient-derived hMOs have proved that these methods are a powerful tool for human-specific in vitro disease modelling of neurological disorders.

For such applications, an organoid model should be reproducible and stable for extended cultivation and manipulation. Even though the organoid technology is a powerful asset in the field of brain research, the hMO models show intrinsic disadvantages and limitations (Berger et al., 2018; Y. Wang et al., 2018). The lack of a natural body axis or supportive tissue prevents an organoid’s organisation that is identical to the pattern of the in vivo human brain (Kelava & Lancaster, 2016a; Lancaster et al., 2013; Y. Wang et al., 2018). The identification of a specific brain regions as well as the reproducibility might be imperfect, nevertheless it is unlikely to create the exact culture conditions found in the human brain in utero (Trujillo & Muotri, 2018).

A major limitation of the hMOs presented here, as well as other published brain organoid systems, is the absence of vasculature, which restricts the supply of oxygen and nutrition, especially in the inner part of the organoids (Kelava & Lancaster, 2016a; Y. Wang et al., 2018). It also might limit the growth of organoids beyond a certain size and increase the appearance of dead cells in the centre of the organoids (Berger et al., 2018; Giandomenico & Lancaster, 2017; Monzel et al., 2017). The cell number, and consequently the cell density within the organoid seem to play an important role and might be a target for improvement. The choice of the surrogate matrix, the starting point and duration of the differentiation are further aspects that can influence the fidelity of the 3D culture. Recently, brain organoids were successfully transplanted into a mouse brain and murine blood vessels could be detected in the grafts (Mansour et al. 2018). Even though the transplanted organoid mimicked more precisely the in vivo brain anatomy, this method bears the disadvantage of xenocontamination (Trujillo & Muotri, 2018; Y. Wang et al., 2018). The absence of microglia, the resident innate immune cells of the CNS, is another major disadvantage for disease modelling, as they are actively involved in the development and maturation of neurons. In the case of cerebral organoids, an adaptation for a microglia-containing organoid model has been recently published (Ormel et al., 2018). Since some hMOs are derived from the neuroectoderm and microglia originate developmentally from the mesoderm, there is only the possibility to integrate externally-derived microglia or their precursors to the developing hMO (Abud et al., 2017; Haenseler et al., 2017; Muffat et al., 2016; Trujillo & Muotri, 2018; Y. Wang et al., 2018).
Future development of cell culture technologies will further improve brain-specific organoid models and will support both the investigation of more complicated interactions in the human brain and the modelling of a larger range of neurological disorders (Di Lullo & Kriegstein, 2017; Y. Wang et al., 2018). Despite the current limitations, we can conclude that the hMO system presented here, along with other models, may be a first step toward a more human patient-specific, probably even personalised, era of advanced disease modelling and therapy development.
**Table 1: Comparison of hMO derivation protocols:** Overview of applied compounds to derive midbrain-specific neuralectoderm by neural induction. hMO protocols from TIENG (2014) and QIAN (2016) are based on 2D experiments by Kriks et al. (2011); hMO protocols from JO (2016) and KIM (2019) are based on 2D experiments by Chambers et al. (2009); hMO protocols from MONZEL (2017) and SMITS (2019) are based on 2D experiments by Reinhardt et al. (2013).

| Dual–SMAD inhibition | TIENG (2014) | QIAN (2016) | JO (2016) | MONZEL (2017) | KIM (2019) | SMITS (2019) |
|----------------------|--------------|-------------|-----------|--------------|------------|-------------|
| SB       | 10 μM        | 10 μM       | 10 μM     | 10 μM        | 10 μM      | 10 μM       |
| Noggin   | —            | —           | 200 ng/ml | —            | 200 ng/ml  | —           |
| LDN      | 100 nM       | 100 nM      | —         | —            | —          | 150 nM      |
| DM       | —            | —           | 1 μM      | —            | —          | —           |

| WNT activation | CHIR | CHIR | CHIR | CHIR | CHIR | CHIR |
|---------------|------|------|------|------|------|------|
|               | 3 μM | 3 μM | 3 μM | 3 μM | 3 μM | 3 μM |

| SHH activation | SHH | SHH | SHH | SHH | SHH | SHH |
|---------------|-----|-----|-----|-----|-----|-----|
|               | 100 ng/ml | 100 ng/ml | 100 ng/ml | —   | 100 ng/ml | —   |
| PMA           | 2 μM | 2 μM | —   | 0.5 μM | —   | —   |
| SAG           | —   | —   | —   | —   | —   | 0.5 μM |

| FGF8 activation | FGF8 | FGF8 | FGF8 | —   | FGF8 | —   |
|----------------|------|------|------|-----|------|-----|
|                | 100 ng/ml | 100 ng/ml | 100 ng/ml | —   | 100 ng/ml | —   |
Table 2: Comparison of characteristics of different hMO derivation protocols: Overview of hMO-specific features reported in Tieng et al. 2014, Qian et al. 2016, Jo et al. 2016, Monzel et al. 2017, Kim et al. 2019, and Smits, Reinhardt et al. 2019

(*different methods applied to determine and calculate TH content, °different methods applied to determine DA content).

|                   | TIENG (2014) | QIAN (2016) | JO (2016) | MONZEL (2017) | KIM (2019) | SMITS (2019) |
|-------------------|--------------|-------------|-----------|---------------|------------|-------------|
| TH°               | >60% (d21)   | 55% (d65)   | 22% (d60) | 64% (d61)     | n/a        | 54% (d70)   |
| DAT               | n/a          | yes         | yes       | yes           | yes        | yes         |
| DA°               | yes          | yes         | yes       | yes           | yes        | yes         |
| NM                | n/a          | n/a         | yes       | yes           | n/a        | yes         |
| glial cells       |              |             |           |               |            |             |
| oligodendrocytes  | yes          | n/a         | yes       | yes           | n/a        | n/a         |
| astrocytes        | yes          | yes         | yes       | yes           | n/a        | n/a         |
| neuronal subtypes |              |             |           |               |            |             |
| GABAergic         | no           | n/a         | yes       | n/a           | n/a        | yes         |
| glutamatergic     | yes          | n/a         | n/a       | n/a           | yes        |             |
| serotonergic      | no           | n/a         | n/a       | n/a           | yes        |             |
Table 3: Comparison of NSC derived hMO models: Overview of selected features of hMOs approaches published in Monzel et al. 2017 and Smits, Reinhardt et al. 2019. Neuronal subtypes refers to the analysis whether neurons other than dopaminergic neurons are determined. As starting cell population, small molecule neural precursor cells (smNPCs) are used in Monzel et al., 2017, while Smits et al., 2019 starts with midbrain floor plate neural progenitor cells (mfNPCs).

(*different methods applied to determine and calculate TH content).

|                      | MONZEL (2017) | SMITS (2019) |
|----------------------|---------------|--------------|
| **culture conditions** |               |              |
| used cell type       | smNPCs        | mfNPCs       |
| number of cells      | 9,000         | 3,000        |
| embedding            | yes           | no           |
| agitation            | yes           | no           |
| **mDANs**            |               |              |
| TH+ cells*           | ~64% (d61)    | ~54% (d70)   |
| regionalisation      | yes           | no           |
| A9/A10 specificity   | yes           | yes          |
| DAT                  | yes (d61)     | yes (d70)    |
| D2/D3 receptor responsive | yes     | yes          |
| DA release           | no            | yes          |
| NM                   | yes (>d149)   | yes (>d100)  |
| **other cell types** |               |              |
| oligodendrocytes     | yes (d61)     | n/a          |
| astrocytes           | yes (d61)     | n/a          |
| neuronal subtypes    | n/a           | yes          |
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