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 MADELEINE 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 PAȘCA 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, PAȘCA 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 Purmorphamine (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; Paşca 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**).

282 TIENG and co-workers did not show the presence of DAT in their engineered nervous tissues
283 (ENTs), but they were able to prove the synthesis of DA via high-performance liquid
284 chromatography (HPLC) after cell lysis (Tieng et al., 2014). Also JO and KIM also applied the
285 same method to assess the DA content within the hMOs (Jo et al., 2016; Kim et al., 2019). To
286 verify that the mDANs were not only able to produce DA, but actually able to release the
287 neurotransmitter, the supernatant of the 3D cultures can be analysed with an enzyme-linked
288 immunosorbent assay (ELISA). SMITS et al. detected a DA release that increased as the hMOs
289 matured (Smits, Reinhardt, et al., 2019). An interesting observation was reported for the first
290 time by JO et al., where they detected insoluble, dark-coloured deposits in their hMOs after
291 approximately 60 days (Jo et al., 2016). Using a Fontana-Masson staining they confirmed that
292 these granules were neuromelanin (NM), which is a by-product of DA biosynthesis and
293 accumulates postnatally in the *substantia nigra pars compacta* (SNc) of the human brain (Kim
294 et al., 2019; Pasca, 2018; Zecca et al., 2003). Other hMO culture protocols have also stimulated
295 the production of NM (Monzel et al., 2017; Smits, Reinhardt, et al., 2019) (**Table 2**). The
296 presence of NM is a unique feature of the primate brain (Pasca, 2018). It is neither apparent in
297 mice brains nor murine midbrain-specific organoids (Dawson et al., 2010; Fedorow et al.,
298 2005; Jo et al., 2016; Marton & Paşca, 2016). Wild-type mDANs, derived and cultured in
299 monolayer conditions, only produce NM after an artificial inductions of progerin expression,
300 which is associated with premature ageing (Miller et al., 2013; Sterneckert et al., 2014).
301 Membrane-bound, dense pigmented NM was also detected in long-term cultures of
302 homozygous DJ-1 mutant and idiopathic PD patient-derived mDANs (Burbulla et al., 2017).
303 Currently, it is unknown whether NM has a protective or damaging effect on the cell survival,
304 however it is proven that NM-containing mDANs of the SNc are especially vulnerable during
305 the course of PD (Jo et al., 2016; Marton & Paşca, 2016; Zecca et al., 2003). Therefore, NM
306 containing hMOs have a great potential to be used for *in vitro* PD modelling, possibly revealing
307 specific phenotypes that are not present in wild-type 2D cultures or murine models. Moreover,
308 hMOs provide the basis for future studies about the role of NM in PD (Marton & Paşca, 2016;
309 Michel et al., 2016). Since mDANs are essential to model the human midbrain, hMO research
310 has so far focused mainly on this specific type of neurons. Nevertheless, detailed *in vivo* studies
311 have described that the released DA diffuses into synaptic regions of glutamatergic and
312 GABAergic synapses and directly affects other striatal cell types, including the neurons
313 forming the island-/striosome GABA pathway, striatal cholinergic interneurons and the striatal
314 GABA interneurons, all possessing DA receptors (Borroto-Escuela et al., 2018; Calabresi et
315 al., 2014). Furthermore, *substantia nigra* (SN) dopaminergic neurons are directly controlled by
316 GABAergic input (Tepper & Lee, 2007). Evidence from these studies suggest that the presence
317 of other neuronal subtypes is important to be able to model multifactorial disease like PD. A
318 first transcriptional characterisation of hMO was performed by JO and co-workers, showing
319 that aspects of prenatal midbrain gene expression profiles were found in the organoids in
320 contrast to the conventional 2D-derived mDANs (Jo et al., 2016; Lin et al., 2016; The GTEx
321 Consortium, 2015). For a further validation of the genetic expression profile during the course
322 of hMO development they suggested conducting a single-cell transcriptome analysis, as it has
323 been shown before for cerebral organoids (Camp et al., 2015; Kageyama et al., 2018). In a
324 recent study, single-cell transcriptomic data from hMOs demonstrated that there is an increased
325 expression of neuronal- and stem cell-specific genes in 35 day- compared to 70 day-old hMOs,
326 whereas exclusively the gene-gene correlations between only neuron-specific genes increased
327 considerably at day 70 (Smits, Magni, et al., 2019). This signifies an increasing commitment
328 of cells towards the neuronal cellular fate during the course of organoid development and
329 further supports the finding of a progressive maturation of post-mitotic neurons. The
330 identification of these neuron-specific genes revealed that the genes up-regulated at the earlier
331 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 (Chaboub & 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 (Faivre-Sarrailh & 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 neurons within the hMOs, in addition to the functional DA receptors present. While neurons do not exist in isolation in the CNS but rather form functional networks with other neurons and non-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

432 dopaminergic network complexity in the patient-derived TH-positive neurons was identified
433 which is also known to occur in PD patients' brain (Bernheimer et al., 1973; Kordower et al.,
434 2013; Smits, Reinhardt, et al., 2019). Based on another published hMO protocol, KIM and co-
435 workers also derived organoids carrying the *LRRK2*-G2019S mutation (Jo et al., 2016; Kim et
436 al., 2019). In line with the findings of SMITS and co-workers, KIM et al. discovered that the
437 mDANs within the *LRRK2*-G2019S organoids reveal a decreased neurite length in comparison
438 to the mDANs within the control organoids. They further assessed an overall decreased
439 expression level of mDANs-specific markers, such as TH, aromatic l-amino acid
440 decarboxylase (AADC), and DAT in their engineered *LRRK2*-G2019S hMOs (Kim et al.,
441 2019). They achieved a partial recovery of those gene's expression levels after treating the
442 *LRRK2*-G2019S mutant organoids with the *LRRK2* kinase inhibitor GSK2578215A. This
443 *LRRK2* inhibition suggests a positive impact on mDAN cell death and additionally proves that
444 this 3D cell culture system is susceptible to investigating therapeutic strategies against PD
445 (Kim et al., 2019). KIM and co-workers also determined hMOs carrying the *LRRK2*-G2019S
446 mutation exhibit an abnormal localisation of α-synuclein that is phosphorylated at serine 129
447 (pS129). They claim that pS129-α-synuclein is aberrantly expressed in mutated hMOs and they
448 detected a *LRRK2*-G2019S mutation-dependent increase of thioflavin T-positive deposits in
449 TH-positive neurons over time, even though the overall α-synuclein expression did not appear
450 to increase (Kim et al., 2019). Surprisingly, these detected thioflavin T-positive deposits
451 appeared to be extracellular and were not clearly overlapping with the TH signal. Whether
452 these findings are reproducible with another analytical method, still needs to be explored
453 further. Nevertheless, the assessment of PD-associated pathologies, such as the
454 synucleinopathies, in human-specific advanced cell culture models is crucial due to the
455 inherent differences between human and mouse mDAN vulnerability and as existing murine
456 transgenic models have not been efficient in developing an accurate representation of the
457 underlying disease mechanisms (Burbulla et al., 2017; Byers et al., 2012; Hemmer et al., 2018;
458 Koh et al., 2018). In the study of SMITS et al., a significant increase of FOXA2-positive
459 progenitor cells in the patient-specific organoids was demonstrated (Smits, Reinhardt, et al.,
460 2019). Since FOXA2 is required for the generation of mDANs, it is hypothesised that this
461 might be a compensatory response to an impaired specification of mDANs promoted by the
462 mutated *LRRK2* gene (Sasaki et al., 1997), additionally it might be a results of a decreased
463 differentiation potential of the progenitor cells. Similar compensatory mechanisms have been
464 described in PD before and might represent an attempt to counteract neurodevelopmental
465 defects induced by PD-specific mutations (Blesa et al., 2017). While introducing also isogenic
466 control hMOs, it was also confirmed that the introduction of the *LRRK2*-G2019S mutation
467 caused deleterious effects on the complexity of mDANs within a healthy background. On the
468 contrary, *LRRK2*-G2019S gene correction within a PD patient background is not sufficient to
469 rescue this effect. As *LRRK2*-G2019S is not fully penetrant and the probability of developing
470 PD individually varies among the carriers, it is suggested that its pathological function
471 comprises of additional pathways (Goldwurm et al., 2007; Smith et al., 2006). In this context,
472 a permissive genetic background, due to cumulative genetic variants, might mediate and either
473 enhance or diminish the *LRRK2*-induced neurodegeneration (Bolognin et al., 2019).
474 Remarkably, in the analysis of all studied features, the PD patient-derived lines cluster together,
475 independently of the presence or absence of the mutation (Smits, Reinhardt, et al., 2019). This
476 indicates that the genetic background of the PD patients, regardless of gene editing, accounts
477 for most of the differences between the studied cell lines and seems to be a major discriminating
478 factor. Thus, not the *LRRK2*-G2019S mutation but rather the genetic background of the patients
479 constitutes the strongest contribution to the phenotypes and supports the hypothesis that the
480 genetic background of PD patients can influence the degeneration of mDANs (Bolognin et al.,
481 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).

531 Future development of cell culture technologies will further improve brain-specific organoid
532 models and will support both the investigation of more complicated interactions in the human
533 brain and the modelling of a larger range of neurological disorders (Di Lullo & Kriegstein,
534 2017; Y. Wang et al., 2018). Despite the current limitations, we can conclude that the hMO
535 system presented here, along with other models, may be a first step toward a more human
536 patient-specific, probably even personalised, era of advanced disease modelling and therapy
537 development.
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540 **Table 1: Comparison of hMO derivation protocols:** Overview of applied compounds to derive
541 midbrain-specific neuralectoderm by neural induction. hMO protocols from TIENG (2014) and QIAN
542 (2016) are based on 2D experiments by Kriks et al. (2011); hMO protocols from JO (2016) and KIM
543 (2019) are based on 2D experiments by Chambers et al. (2009); hMO protocols from MONZEL (2017)
544 and SMITS (2019) are based on 2D experiments by Reinhardt et al. (2013).
545

	TIENG (2014)	QIAN (2016)	JO (2016)	MONZEL (2017)	KIM (2019)	SMITS (2019)
Dual–SMAD inhibition						
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	3 μM	3 μM	3 μM	3 μM	3 μM	3 μM
SHH activation						
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	100 ng/ml	100 ng/ml	100 ng/ml	—	100 ng/ml	—

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570 **Table 2: Comparison of characteristics of different hMO derivation protocols:** Overview of
571 hMO-specific features reported in Tieng et al. 2014, Qian et al. 2016, Jo et al. 2016, Monzel et al.
572 2017, Kim et al. 2019, and Smits, Reinhardt et al. 2019
573 (•different methods applied to determine and calculate TH content, °different methods applied to
574 determine DA content).
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	Tieng (2014)	Qian (2016)	Jo (2016)	Monzel (2017)	Kim (2019)	Smits (2019)
mDANs						
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	n/a	yes
serotonergic	no	n/a	n/a	n/a	n/a	yes

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601 **Table 3: Comparison of NSC derived hMO models:** Overview of selected
602 features of hMOs approaches published in Monzel et al. 2017 and Smits, Reinhardt et al. 2019.
603 Neuronal subtypes refers to the analysis whether neurons other than dopaminergic neuros are
604 determined. As starting cell population, small molecule neural precursor cells (smNPCs) are used in
605 Monzel et al., 2017, while Smits et al., 2019 starts with midbrain floor plate neural progenitor cells
606 (mfNPCs).
607 (•different methods applied to determine and calculate TH content).
608

	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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615 # References

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617 Abe-Fukasawa, N., Otsuka, K., Aihara, A., Itasaki, N., & Nishino, T. (2018). Novel 3D Liquid Cell
618 Culture Method for Anchorage-independent Cell Growth, Cell Imaging and Automated Drug
619 Screening. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-21950-5

620 Abeliovich, A., & Hammond, R. (2007). Midbrain dopamine neuron differentiation: Factors and fates.
621 *Developmental Biology*, *304*, 447–454. https://doi.org/10.1016/j.ydbio.2007.01.032

622 Abud, E. M., Ramirez, R. N., Martinez, E. S., Carson, M. J., Poon, W. W., Blurton-jones, M., Abud,
623 E. M., Ramirez, R. N., Martinez, E. S., Healy, L. M., Nguyen, C. H. H., & Newman, S. A.
624 (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron*,
625 *94*(2), 278-293.e9. https://doi.org/10.1016/j.neuron.2017.03.042

626 Arlotta, P. (2018). Organoids required! A new path to understanding human brain development and
627 disease. *Nature Methods*, *15*(1), 27–29. https://doi.org/10.1038/nmeth.4557

628 Benson, D. L., & Huntley, G. W. (2019). Are we listening to everything the PARK genes are telling
629 us? In *Journal of Comparative Neurology* (Vol. 527, Issue 9, pp. 1527–1540). Wiley-Liss Inc.
630 https://doi.org/10.1002/cne.24642

631 Berger, E., Magliaro, C., Paczia, N., Monzel, A. S., Antony, P., Linster, C. L., Bolognin, S.,
632 Ahluwalia, A., & Schwamborn, J. C. (2018). Millifluidic culture improves human midbrain
633 organoid vitality and differentiation. *Lab on a Chip*, *18*(20), 3172–3183.
634 https://doi.org/10.1039/c8lc00206a

635 Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., & Seitelberger, F. (1973). Brain
636 dopamine and the syndromes of Parkinson and Huntington Clinical, morphological and
637 neurochemical correlations. *Journal of the Neurological Sciences*, *20*(4), 415–455.
638 https://doi.org/10.1016/0022-510X(73)90175-5

639 Bian, S., Repic, M., Guo, Z., Kavirayani, A., Burkard, T., Bagley, J. A., Krauditsch, C., & Knoblich,
640 J. A. (2018). Genetically engineered cerebral organoids model brain tumor formation. *Nature*
641 *Methods*, *15*(8), 631–639. https://doi.org/10.1038/s41592-018-0070-7

642 Blesa, J., Trigo-Damas, I., Dileone, M., del Rey, N. L. G., Hernandez, L. F., & Obeso, J. A. (2017).
643 Compensatory mechanisms in Parkinson's disease: Circuits adaptations and role in disease
644 modification. *Experimental Neurology*, *298*(October), 148–161.
645 https://doi.org/10.1016/j.expneurol.2017.10.002

646 Bolognin, S., Fossépré, M., Qing, X., Jarazo, J., Ščančar, J., Moreno, E. L., Nickels, S. L., Wasner,
647 K., Ouzren, N., Walter, J., Grünewald, A., Glaab, E., Salamanca, L., Fleming, R. M. T., Antony,
648 P. M. A., & Schwamborn, J. C. (2019). 3D Cultures of Parkinson's Disease-Specific
649 Dopaminergic Neurons for High Content Phenotyping and Drug Testing. *Advanced Science*,
650 *6*(1), 1800927. https://doi.org/10.1002/advs.201800927

651 Borroto-Escuela, D. O., Perez De La Mora, M., Manger, P., Narváez, M., Beggiato, S., Crespo-
652 Ramírez, M., Navarro, G., Wydra, K., Díaz-Cabiale, Z., Rivera, A., Ferraro, L., Tanganelli, S.,
653 Filip, M., Franco, R., & Fuxe, K. (2018). Brain Dopamine Transmission in Health and
654 Parkinson's Disease: Modulation of Synaptic Transmission and Plasticity Through Volume
655 Transmission and Dopamine Heteroreceptors. *Frontiers in Synaptic Neuroscience*, *10*(July).
656 https://doi.org/10.3389/fnsyn.2018.00020

657 Braak, H., & Del Tredici, K. (2004). Poor and protracted myelination as a contributory factor to
658 neurodegenerative disorders. In *Neurobiology of Aging* (Vol. 25, Issue 1, pp. 19–23). Elsevier
659 Inc. https://doi.org/10.1016/j.neurobiolaging.2003.04.001

660 Bu, L.-L., Yang, K., Xiong, W.-X., Liu, F.-T., Anderson, B., Wang, Y., & Wang, J. (2016). Toward
661 precision medicine in Parkinson's disease. *Annals of Translational Medicine*, *4*(2), 26.

662 https://doi.org/10.3978/j.issn.2305-5839.2016.01.21

663 Bunk, E. C., Ertaylan, G., Ortega, F., Pavlou, M. A., Gonzalez Cano, L., Stergiopoulos, A., Safaiyan,
664 S., Völs, S., van Cann, M., Politis, P. K., Simons, M., Berninger, B., del Sol, A., &
665 Schwamborn, J. C. (2016). Prox1 Is Required for Oligodendrocyte Cell Identity in Adult Neural
666 Stem Cells of the Subventricular Zone. *STEM CELLS*, *34*(8), 2115–2129.
667 https://doi.org/10.1002/stem.2374

668 Burbulla, L. F., Song, P., Mazzulli, J. R., Zampese, E., Wong, Y. C., Jeon, S., Santos, D. P., Blanz, J.,
669 Obermaier, C. D., Strojny, C., Savas, J. N., Kiskinis, E., Zhuang, X., Krüger, R., Surmeier, D. J.,
670 & Krainc, D. (2017). Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in
671 Parkinson's disease. *Science*, *357*(6357), 1255–1261. https://doi.org/10.1126/science.aam9080

672 Byers, B., Lee, H., & Reijo Pera, R. (2012). Modeling Parkinson's Disease Using Induced Pluripotent
673 Stem Cells. *Current Neurology and Neuroscience Reports*, *12*, 237–242.
674 https://doi.org/10.1007/s11910-012-0270-y

675 Calabresi, P., & Di Filippo, M. (2015). The changing tree in Parkinson's disease. *Nature*
676 *Neuroscience*, *18*(9), 1196–1198. https://doi.org/10.1038/nn.4092

677 Calabresi, P., Picconi, B., Tozzi, A., Ghiglieri, V., & Di Filippo, M. (2014). Direct and indirect
678 pathways of basal ganglia: A critical reappraisal. *Nature Neuroscience*, *17*(8), 1022–1030.
679 https://doi.org/10.1038/nn.3743

680 Camp, J. G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Bräuninger, M., Lewitus, E.,
681 Sykes, A., Hevers, W., Lancaster, M., Knoblich, J. a, Lachmann, R., Pääbo, S., Huttner, W. B.,
682 & Treutlein, B. (2015). Human cerebral organoids recapitulate gene expression programs of
683 fetal neocortex development. *Proceedings of the National Academy of Sciences of the United*
684 *States of America*, *112*(51), 15672–15677. https://doi.org/10.1073/pnas.1520760112

685 Chaboub, L. S., & Deneen, B. (2013). Astrocyte Form and Function in the Developing Central
686 Nervous System. *Seminars in Pediatric Neurology*, *20*(4), 230–235.
687 https://doi.org/10.1016/J.SPEN.2013.10.003

688 Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., & Studer, L.
689 (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of
690 SMAD signaling. *Nature Biotechnology*, *27*(3), 275–280. https://doi.org/10.1038/nbt.1529

691 Choi, S. H., Kim, Y. H., Hebisch, M., Sliwinski, C., Lee, S., D'Avanzo, C., Chen, H., Hooli, B.,
692 Asselin, C., Muffat, J., Klee, J. B., Zhang, C., Wainger, B. J., Peitz, M., Kovacs, D. M., Woolf,
693 C. J., Wagner, S. L., Tanzi, R. E., & Kim, D. Y. (2014). A three-dimensional human neural cell
694 culture model of Alzheimer's disease. *Nature*, *515*(7526), 274–278.
695 https://doi.org/10.1038/nature13800

696 Choi, S. H., Kim, Y. H., Quinti, L., Tanzi, R. E., & Kim, D. Y. (2016). 3D culture models of
697 Alzheimer's disease: a road map to a "cure-in-a-dish." *Molecular Neurodegeneration*, *11*(1), 75.
698 https://doi.org/10.1186/s13024-016-0139-7

699 Chung, W.-S., Welsh, C. A., Barres, B. A., & Stevens, B. (2015). Do glia drive synaptic and cognitive
700 impairment in disease? *Nature Neuroscience*, *18*(11), 1539–1545.
701 https://doi.org/10.1038/nn.4142

702 Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. *Cell*, *154*(2), 274.
703 https://doi.org/10.1016/j.cell.2013.07.004

704 Clevers, H. (2016). Modeling Development and Disease with Organoids. *Cell*, *165*(7), 1586–1597.
705 https://doi.org/10.1016/j.cell.2016.05.082

706 Cooper, O., Seo, H., Andrabi, S., Guardia-Laguarta, C., Graziotto, J., Sundberg, M., McLean, J. R.,
707 Carrillo-Reid, L., Xie, Z., Osborn, T., Hargus, G., Deleidi, M., Lawson, T., Bogetofte, H., Perez-

708 Torres, E., Clark, L., Moskowitz, C., Mazzulli, J., Chen, L., … Isacson, O. (2012).
709 Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with
710 familial Parkinson's disease. *Science Translational Medicine*, *4*(141), 141ra90.
711 https://doi.org/10.1126/scitranslmed.3003985

712 Cugola, F. R., Fernandes, I. R., Russo, F. B., Freitas, B. C., Dias, J. L. M., Guimarães, K. P.,
713 Benazzato, C., Almeida, N., Pignatari, G. C., Romero, S., Polonio, C. M., Cunha, I., Freitas, C.
714 L., Brandaõ, W. N., Rossato, C., Andrade, D. G., Faria, D. D. P., Garcez, A. T., Buchpigel, C.
715 A., … Beltrao-Braga, P. C. B. B. (2016). The Brazilian Zika virus strain causes birth defects in
716 experimental models. *Nature*, *534*(7606), 267–271. https://doi.org/10.1038/nature18296

717 Cullen, D. K., Wolf, J. A., Vernekar, V. N., Vukasinovic, J., & LaPlaca, M. C. (2012). Neural Tissue
718 Engineering and Biohybridized Microsystems for Neurobiological Investigation In Vitro (Part
719 1). *Critical Reviews in Biomedical Engineering*, *39*(3), 201–240.

720 D'Avanzo, C., Aronson, J., Kim, Y. H., Choi, S. H., Tanzi, R. E., & Kim, D. Y. (2015). Alzheimer's
721 in 3D culture: Challenges and perspectives. *BioEssays*, *37*(10), 1139–1148.
722 https://doi.org/10.1002/bies.201500063

723 Dang, J., Tiwari, S. K., Lichinchi, G., Qin, Y., Patil, V. S., & Eroshkin, A. M. (2016). Zika Virus
724 Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate
725 Immune Receptor TLR3. *Stem Cell*, *19*(2), 258–265. https://doi.org/10.1016/j.stem.2016.04.014

726 Dawson, T. M., Ko, H. S., & Dawson, V. L. (2010). Genetic Animal Models of Parkinson's Disease.
727 In *Neuron* (Vol. 66, Issue 5, pp. 646–661). https://doi.org/10.1016/j.neuron.2010.04.034

728 Di Lullo, E., & Kriegstein, A. R. (2017). The use of brain organoids to investigate neural
729 development and disease. *Nature Reviews Neuroscience*, *18*, 573–584.
730 https://doi.org/10.1038/nrn.2017.107

731 Doi, D., Samata, B., Katsukawa, M., Kikuchi, T., Morizane, A., Ono, Y., Sekiguchi, K., Nakagawa,
732 M., Parmar, M., & Takahashi, J. (2014). Isolation of human induced pluripotent stem cell-
733 derived dopaminergic progenitors by cell sorting for successful transplantation. *Stem Cell*
734 *Reports*, *2*(3), 337–350. https://doi.org/10.1016/j.stemcr.2014.01.013

735 Drost, J., & Clevers, H. (2017). Translational applications of adult stem cell-derived organoids. *The*
736 *Company of Biologists*, *144*, 968–975. https://doi.org/10.1242/dev.140566

737 Dutta, D., Heo, I., & Clevers, H. (2017). Disease Modeling in Stem Cell-Derived 3D Organoid
738 Systems. *Trends in Molecular Medicine*, *23*(5), 393–410.
739 https://doi.org/10.1016/j.molmed.2017.02.007

740 Faivre-Sarrailh, C., & Devaux, J. J. (2013). Neuro-glial interactions at the nodes of Ranvier:
741 implication in health and diseases. *Frontiers in Cellular Neuroscience*, *7*, 196.
742 https://doi.org/10.3389/fncel.2013.00196

743 Fatehullah, A., Tan, S. H., & Barker, N. (2016). Organoids as an in vitro model of human
744 development and disease. *Nature Publishing Group*, *18*(3), 246–254.
745 https://doi.org/10.1038/ncb3312

746 Fedorow, H., Tribl, F., Halliday, G., Gerlach, M., Riederer, P., & Double, K. L. (2005). Neuromelanin
747 in human dopamine neurons: Comparison with peripheral melanins and relevance to Parkinson's
748 disease. *Progress in Neurobiology*, *75*(2), 109–124.
749 https://doi.org/10.1016/j.pneurobio.2005.02.001

750 Garcez, P. P., Loiola, E. C., Da Costa, R. M., Higa, L. M., Trindade, P., Delvecchio, R., Nascimento,
751 J. M., Brindeiro, R., Tanuri, A., & Rehen, S. K. (2016). Zika virus impairs growth in human
752 neurospheres and brain organoids. *Science*, *352*(6287). https://doi.org/10.1126/science.aaf6116

753 Garcia-Dominguez, M. (2003). Ebf gene function is required for coupling neuronal differentiation and

754 cell cycle exit. *Development*, *130*(24), 6013–6025. https://doi.org/10.1242/dev.00840

755 Garcia-Reitboeck, P., Anichtchik, O., Dalley, J. W., Ninkina, N., Tofaris, G. K., Buchman, V. L., &
756 Spillantini, M. G. (2013). Endogenous alpha-synuclein influences the number of dopaminergic
757 neurons in mouse substantia nigra. *Experimental Neurology*, *248*, 541–545.
758 https://doi.org/10.1016/J.EXPNEUROL.2013.07.015

759 Giandomenico, S. L., & Lancaster, M. A. (2017). Probing human brain evolution and development in
760 organoids. *Current Opinion in Cell Biology*, *44*, 36–43.
761 https://doi.org/10.1016/j.ceb.2017.01.001

762 Goldwurm, S., Zini, M., Mariani, L., Tesei, S., Miceli, R., Sironi, F., Clementi, M., Bonifati, V., &
763 Pezzoli, G. (2007). Evaluation of LRRK2 G2019S penetrance: relevance for genetic counseling
764 in Parkinson disease. *Neurology*, *68*(14), 1141–1143.
765 https://doi.org/10.1212/01.wnl.0000254483.19854.ef

766 Grealish, S., Diguet, E., Parmar, M., Bjo, A., Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B.,
767 Heuer, A., & Bramoulle, Y. (2014). Human ESC-Derived Dopamine Neurons Show Similar
768 Preclinical Efficacy and Potency to Fetal Neurons when Grafted in a Rat Model of Parkinson ' s
769 Clinical Progress Human ESC-Derived Dopamine Neurons Show Similar Preclinical Efficacy
770 and Potency to Fetal Neur. *Cell Stem Cell*, *15*, 653–665.
771 https://doi.org/10.1016/j.stem.2014.09.017

772 Haenseler, W., Zambon, F., Lee, H., Vowles, J., Rinaldi, F., Duggal, G., Houlden, H., Gwinn, K.,
773 Wray, S., Luk, K. C., Wade-martins, R., James, W. S., & Cowley, S. A. (2017). Excess α -
774 synuclein compromises phagocytosis in iPSC-derived macrophages. *Scientific Reports*, *April*, 1–
775 11. https://doi.org/10.1038/s41598-017-09362-3

776 Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., Yow, A., Soldner, F.,
777 Hockemeyer, D., Hallett, P. J., Osborn, T., Jaenisch, R., & Isacson, O. (2010). Differentiated
778 Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and
779 reduce motor asymmetry in Parkinsonian rats. *Proceedings of the National Academy of Sciences*
780 *of the United States of America*, *107*(7), 15921–15926. https://doi.org/10.1073/pnas.1010209107

781 Haycock, J. W. (2011). 3D Cell Culture: A Review of Current Approaches and Techniques. *Methods*
782 *Molecular Biologyl Biol*, *695*, 1–5. https://doi.org/10.1007/978-1-60761-984-0_1

783 Hemmer, K., Smits, L. M., Bolognin, S., & Schwamborn, J. C. (2018). In vivo Phenotyping of Human
784 Parkinson's Disease-Specific Stem Cells Carrying the LRRK2-G2019S Mutation Reveals
785 Increased a-Synuclein Levels but Absence of Spreading. *Opera Med Physiol*, *4*(2), 71–77.
786 https://doi.org/10.20388/omp2018.001.0057

787 Hillje, A.-L., & Schwamborn, J. C. (2016). Utilization of stem cells to model Parkinson's disease –
788 current state and future challenges. *Future Neurology*, *11*(2), 171–186.
789 https://doi.org/10.2217/fnl.16.7

790 Hubert, C. G., Rivera, M., Spangler, L. C., Wu, Q., Mack, S. C., Prager, B. C., Couce, M., McLendon,
791 R. E., Sloan, A. E., & Rich, J. N. (2016). A Three-Dimensional Organoid Culture System
792 Derived from Human Glioblastomas Recapitulates the Hypoxic Gradients and Cancer Stem Cell
793 Heterogeneity of Tumors Found In Vivo. *Cancer Research*, *76*(8), 2465–2477.
794 https://doi.org/10.1158/0008-5472.can-15-2402

795 Huch, M., & Koo, B. (2015). Modeling mouse and human development using organoid cultures.
796 *Development (Cambridge, England)*, *142*(18), 3113–3125. https://doi.org/10.1242/dev.118570

797 Illes, S., Jakab, M., Beyer, F., Gelfert, R., Couillard-Despres, S., Schnitzler, A., Ritter, M., & Aigner,
798 L. (2014). Intrinsically active and pacemaker neurons in pluripotent stem cell-derived neuronal
799 populations. *Stem Cell Reports*, *2*(3), 323–336. https://doi.org/10.1016/j.stemcr.2014.01.006

800 Jablonska, B., Aguirre, A., Raymond, M., Szabo, G., Kitabatake, Y., Sailor, K. A., Ming, G.-L., Song,
801 H., & Gallo, V. (2010). Chordin-induced lineage plasticity of adult SVZ neuroblasts after
802 demyelination. *Nature Neuroscience*, *13*(5), 541–550. https://doi.org/10.1038/nn.2536

803 Jan, A., Jansonius, B., Delaidelli, A., Bhanshali, F., An, Y. A., Ferreira, N., Smits, L. M., Negri, G.
804 L., Schwamborn, J. C., Jensen, P. H., Mackenzie, I. R., Taubert, S., & Sorensen, P. H. (2018).
805 Activity of translation regulator eukaryotic elongation factor-2 kinase is increased in Parkinson
806 disease brain and its inhibition reduces alpha synuclein toxicity. *Acta Neuropathologica*
807 *Communications*, *6*(1). https://doi.org/10.1186/s40478-018-0554-9

808 Jarazo, J., Barmpa, K., Rosety, I., Smits, L. M., Arias-Fuenzalida, J., Walter, J., Gomez-Giro, G.,
809 Monzel, A. S., Qing, X., Cruciani, G., Boussaad, I., Jäger, C., Rakovic, A., Berger, E., Bolognin,
810 S., Antony, P. M. A., Klein, C., Krüger, R., Seibler, P., & Schwamborn, J. C. (2019).
811 Parkinson's disease phenotypes in patient specific brain organoids are improved by HP-β-CD
812 treatment. *BioRxiv*, 813089. https://doi.org/10.1101/813089

813 Jo, J., Xiao, Y., Xuyang Sun, A., King Tan, E., Shawn Je, H., Ng, H., Jo, J., Xiao, Y., Sun, A. X.,
814 Tan, E. K., Je, H. S., Ng, H., Tan, ng K., Je, H. S., & Ng, H. (2016). Midbrain-like Organoids
815 from Human Pluripotent Stem Cells Contain Functional Dopaminergic and Neuromelanin-
816 Producing Neurons. *Cell Stem Cell*, *19*(2), 248–257. https://doi.org/10.1016/j.stem.2016.07.005

817 Kadoshima, T., Sakaguchi, H., Nakano, T., Soen, M., Ando, S., & Eiraku, M. (2013). Self-
818 organization of axial polarity, inside-out layer pattern , and species-specific progenitor dynamics
819 in human ES cell-derived neocortex. *Proceedings of the National Academy of Sciences*, *110*,
820 20284–20289. https://doi.org/10.1073/pnas.1315710110/-
821 /DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1315710110

822 Kageyama, J., Wollny, D., Treutlein, B., & Camp, J. G. (2018). ShinyCortex: Exploring single-cell
823 transcriptome data from the developing human cortex. *Frontiers in Neuroscience*, *12*(MAY),
824 10–13. https://doi.org/10.3389/fnins.2018.00315

825 Kelava, I., & Lancaster, M. A. (2016a). Dishing out mini-brains : Current progress and future
826 prospects in brain organoid research. *Developmental Biology*, *420*(2), 199–209.
827 https://doi.org/10.1016/j.ydbio.2016.06.037

828 Kelava, I., & Lancaster, M. A. (2016b). Stem Cell Models of Human Brain Development. *Cell Stem*
829 *Cell*, *18*(6), 736–748. https://doi.org/10.1016/j.stem.2016.05.022

830 Kim, H., Park, H. J., Choi, H., Chang, Y., Park, H., Shin, J., Kim, J., Lengner, C. J., Lee, Y. K., &
831 Kim, J. (2019). Modeling G2019S-LRRK2 Sporadic Parkinson's Disease in 3D Midbrain
832 Organoids. *Stem Cell Reports*, *12*(3), 518–531. https://doi.org/10.1016/J.STEMCR.2019.01.020

833 Kirkeby, A., Grealish, S., Wolf, D. a, Nelander, J., Wood, J., Lundblad, M., Lindvall, O., & Parmar,
834 M. (2012). Generation of regionally specified neural progenitors and functional neurons from
835 human embryonic stem cells under defined conditions. *Cell Reports*, *1*(6), 703–714.
836 https://doi.org/10.1016/j.celrep.2012.04.009

837 Koh, Y. H., Tan, L. Y., & Ng, S. (2018). Patient-Derived Induced Pluripotent Stem Cells and
838 Organoids for Modeling Alpha Synuclein Propagation in Parkinson ' s Disease. *Frontiers in*
839 *Cellular Neuroscience*, *12*(November), 1–12. https://doi.org/10.3389/fncel.2018.00413

840 Kordower, J. H., Olanow, C. W., Dodiya, H. B., Chu, Y., Beach, T. G., Adler, C. H., Halliday, G. M.,
841 & Bartus, R. T. (2013). Disease duration and the integrity of the nigrostriatal system in
842 Parkinson's disease. *Brain*, *136*(8), 2419–2431. https://doi.org/10.1093/brain/awt192

843 Korotkova, T. M., Ponomarenko, A. A., Brown, R. E., & Haas, H. L. (2004). Functional diversity of
844 ventral midbrain dopamine and GABAergic neurons. *Molecular Neurobiology*, *29*(3), 243–259.
845 https://doi.org/10.1385/MN:29:3:243

846 Kriks, S., Shim, J.-W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., Carrillo-Reid, L., Auyeung,
847 G., Antonacci, C., Buch, A., Yang, L., Beal, M. F., Surmeier, D. J., Kordower, J. H., Tabar, V.,
848 & Studer, L. (2011). Dopamine neurons derived from human ES cells efficiently engraft in
849 animal models of Parkinson’s disease. *Nature*, *480*(7378), 547–551.
850 https://doi.org/10.1038/nature10648

851 Lancaster, M. A., Corsini, N. S., Burkard, T. R., & Knoblich, J. A. (2016). Guided self-organization
852 recapitulates tissue architecture in a bioengineered brain organoid model. *BioRxiv*, 049346.
853 https://doi.org/10.1101/049346

854 Lancaster, M. A., & Knoblich, J. A. (2014a). Generation of cardiomyocytes from human pluripotent
855 stem cells. *Nature Protocols*, *9*(10), 1–18. https://doi.org/10.1038/nprot.2014.158

856 Lancaster, M. A., & Knoblich, J. A. (2014b). Organogenesis in a dish: modeling development and
857 disease using organoid technologies. *Science (New York, N.Y.)*, *345*, 1247125.
858 https://doi.org/10.1126/science.1247125

859 Lancaster, M. A., & Knoblich, J. A. (2014c). Generation of cerebral organoids from human
860 pluripotent stem cells. *Nature Protocols*, *9*, 2329. https://doi.org/10.1038/nprot.2014.158

861 Lancaster, M. A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L. S., Hurles, M. E., Homfray, T.,
862 Penninger, J. M., Jackson, A. P., & Knoblich, J. A. (2013). Cerebral organoids model human
863 brain development and microcephaly. *Nature*, *501*(7467), 373–379.
864 https://doi.org/10.1038/nature12517

865 Le Grand, J. N., Gonzalez-Cano, L., Pavlou, M. A., & Schwamborn, J. C. (2015). Neural stem cells in
866 Parkinson’s disease: a role for neurogenesis defects in onset and progression. *Cellular and*
867 *Molecular Life Sciences*, *72*(4), 773–797. https://doi.org/10.1007/s00018-014-1774-1

868 Lin, L., Göke, J., Cukuroglu, E., Dranias, M. R., VanDongen, A. M. J., & Stanton, L. W. (2016).
869 Molecular Features Underlying Neurodegeneration Identified through In Vitro Modeling of
870 Genetically Diverse Parkinson’s Disease Patients. *Cell Reports*, *15*(11), 2411–2426.
871 https://doi.org/10.1016/j.celrep.2016.05.022

872 Luhmann, H. J., Sinning, A., Yang, J., & Reyes-puerta, V. (2016). Spontaneous Neuronal Activity in
873 Developing Neocortical Networks : From Single Cells to Large-Scale Interactions. *Frontiers in*
874 *Neural Circuits*, *10*(May), 1–14. https://doi.org/10.3389/fncir.2016.00040

875 Marton, R. M., & Paşca, S. P. (2016). Neural Differentiation in the Third Dimension: Generating a
876 Human Midbrain. *Cell Stem Cell*, *19*(2), 145–146. https://doi.org/10.1016/j.stem.2016.07.017

877 Method of the Year 2017: Organoids. (2018). *Nature Methods*, *15*, 1.
878 https://doi.org/10.1038/nmeth.4575

879 Michel, P. P., Hirsch, E. C., & Hunot, S. (2016). Understanding Dopaminergic Cell Death Pathways
880 in Parkinson Disease. *Neuron*, *90*(4), 675–691. https://doi.org/10.1016/j.neuron.2016.03.038

881 Miller, J. D., Ganat, Y. M., Kishinevsky, S., Bowman, R. L., Liu, B., Tu, E. Y., Mandal, P. K., Vera,
882 E., Shim, J. W., Kriks, S., Taldone, T., Fusaki, N., Tomishima, M. J., Krainc, D., Milner, T. A.,
883 Rossi, D. J., & Studer, L. (2013). Human iPSC-based modeling of late-onset disease via
884 progerin-induced aging. *Cell Stem Cell*, *13*(6), 691–705.
885 https://doi.org/10.1016/j.stem.2013.11.006

886 Miner, J. J., & Diamond, M. S. (2016). Understanding how zika virus enters and infects neural target
887 cells. In *Cell Stem Cell* (Vol. 18, Issue 5, pp. 559–560).
888 https://doi.org/10.1016/j.stem.2016.04.009

889 Molofsky, A. V, Krencik, R., Krenick, R., Ullian, E. M., Ullian, E., Tsai, H., Deneen, B., Richardson,
890 W. D., Barres, B. A., & Rowitch, D. H. (2012). Astrocytes and disease: a neurodevelopmental
891 perspective. *Genes & Development*, *26*(9), 891–907. https://doi.org/10.1101/gad.188326.112

892 Monzel, A. S., Smits, L. M., Hemmer, K., Hachi, S., Moreno, E. L., van Wuellen, T., Jarazo, J.,
893 Walter, J., Brüggemann, I., Boussaad, I., Berger, E., Fleming, R. M. T. T., Bolognin, S., &
894 Schwamborn, J. C. (2017). Derivation of Human Midbrain-Specific Organoids from
895 Neuroepithelial Stem Cells. *Stem Cell Reports*, *8*(5), 1–11.
896 https://doi.org/10.1016/j.stemcr.2017.03.010

897 Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., Bakiasi, G., Tsai, L. H.,
898 Aubourg, P., Ransohoff, R. M., & Jaenisch, R. (2016). Efficient derivation of microglia-like
899 cells from human pluripotent stem cells. *Nature Medicine*, *22*(11), 1358–1367.
900 https://doi.org/10.1038/nm.4189

901 Nguyen, H. N., Byers, B., Cord, B., Shcheglovitov, A., Byrne, J., Gujar, P., Kee, K., Schüle, B.,
902 Dolmetsch, R. E., Langston, W., Palmer, T. D., & Pera, R. R. (2011). LRRK2 mutant iPSC-
903 derived DA neurons demonstrate increased susceptibility to oxidative stress. *Cell Stem Cell*,
904 *8*(3), 267–280. https://doi.org/10.1016/j.stem.2011.01.013

905 Nolbrant, S., Heuer, A., Parmar, M., & Kirkeby, A. (2017). Generation of high-purity human ventral
906 midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation.
907 *Nature Protocols*, *12*(9), 1962–1979. https://doi.org/10.1038/nprot.2017.078

908 Nowakowski, T. J., Pollen, A. A., Di Lullo, E., Sandoval-Espinosa, C., Bershteyn, M., & Kriegstein,
909 A. R. (2016). Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in
910 Neural Stem Cells. *Cell Stem Cell*, *18*(5), 591–596. https://doi.org/10.1016/j.stem.2016.03.012

911 Orimo, S., Uchihara, T., Kanazawa, T., Itoh, Y., Wakabayashi, K., Kakita, A., & Takahashi, H.
912 (2011). Unmyelinated axons are more vulnerable to degeneration than myelinated axons of the
913 cardiac nerve in Parkinson's disease. *Neuropathology and Applied Neurobiology*, *37*(7), 791–
914 802. https://doi.org/10.1111/j.1365-2990.2011.01194.x

915 Ormel, P. R., Vieira de Sa, R., Hol, E. M., Pasterkamp, R. J., Vieira de Sá, R., van Bodegraven, E. J.,
916 Karst, H., Harschnitz, O., Sneeboer, M. A. M., Johansen, L. E., van Dijk, R. E., Scheefhals, N.,
917 Berdenis van Berlekom, A., Ribes Martínez, E., Kling, S., MacGillavry, H. D., van den Berg, L.
918 H., Kahn, R. S., Hol, E. M., … Pasterkamp, R. J. (2018). Microglia innately develop within
919 cerebral organoids. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06684-2

920 Paşca, A. M., Sloan, S. a, Clarke, L. E., Tian, Y., Makinson, C. D., Huber, N., Kim, C. H., Park, J.-Y.,
921 O'Rourke, N. a, Nguyen, K. D., Smith, S. J., Huguenard, J. R., Geschwind, D. H., Barres, B. a,
922 & Paşca, S. P. (2015). Functional cortical neurons and astrocytes from human pluripotent stem
923 cells in 3D culture. *Nature Methods*, *12*(7). https://doi.org/10.1038/nmeth.3415

924 Pasca, S. P. (2018). The rise of three-dimensional human brain cultures. *Nature Review*, *553*(7689),
925 437–445. https://doi.org/10.1038/nature25032

926 Patzke, C., Acuna, C., Giam, L. R., Wernig, M., & Südhof, T. C. (2016). Conditional deletion of
927 L1CAM in human neurons impairs both axonal and dendritic arborization and action potential
928 generation. *Journal of Experimental Medicine*, *213*(4), 499–515.
929 https://doi.org/10.1084/jem.20150951

930 Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., Yao, B., Hamersky,
931 G. R., Jacob, F., Zhong, C., Yoon, K., Jeang, W., Lin, L., Li, Y., Thakor, J., Berg, D. A., Zhang,
932 C., Kang, E., Chickering, M., … Ming, G. (2016). Brain-Region-Specific Organoids Using
933 Mini- bioreactors for Modeling ZIKV Exposure. *Cell*, 1–17.
934 https://doi.org/10.1016/j.cell.2016.04.032

935 Qing, X., Walter, J., Jarazo, J., Arias-Fuenzalida, J., Hillje, A. L., & Schwamborn, J. C. (2017).
936 CRISPR/Cas9 and piggyBac-mediated footprint-free LRRK2-G2019S knock-in reveals neuronal
937 complexity phenotypes and α-Synuclein modulation in dopaminergic neurons. *Stem Cell*
938 *Research*, *24*, 44–50. https://doi.org/10.1016/j.scr.2017.08.013

939 Raja, W. K., Mungenast, A. E., Lin, Y., Ko, T., Abdurrob, F., Seo, J., & Tsai, L. (2016). Self-
940 Organizing 3D Human Neural Tissue Derived from Induced Pluripotent Stem Cells Recapitulate
941 Alzheimer ' s Disease Phenotypes. *PLoS One*, 1–18.
942 https://doi.org/10.1371/journal.pone.0161969

943 Reinhardt, P., Glatza, M., Hemmer, K., Tsytsyura, Y., Thiel, C. S., Hoing, S., Moritz, S., Parga, J. A.,
944 Wagner, L., Bruder, J. M., Wu, G., Schmid, B., Röpke, A., Klingauf, J., Schwamborn, J. C.,
945 Gasser, T., Scholer, H. R., & Sterneckert, J. (2013). Derivation and expansion using only small
946 molecules of human neural progenitors for neurodegenerative disease modeling. *PLoS One*,
947 *8*(3), e59252. https://doi.org/10.1371/journal.pone.0059252

948 Reinhardt, P., Schmid, B., Burbulla, L. F., Schöndorf, D. C., Wagner, L., Glatza, M., Höing, S.,
949 Hargus, G., Heck, S. A., Dhingra, A., Wu, G., Müller, S., Brockmann, K., Kluba, T., Maisel, M.,
950 Krüger, R., Berg, D., Tsytsyura, Y., Thiel, C. S., … Sterneckert, J. (2013). Genetic correction of
951 a lrrk2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent
952 changes in gene expression. *Cell Stem Cell*, *12*(3), 354–367.
953 https://doi.org/10.1016/j.stem.2013.01.008

954 Renner, M., Lancaster, M. A., Bian, S., Choi, H., Ku, T., Peer, A., Chung, K., & Knoblich, J. A.
955 (2017). Self-organized developmental patterning and differentiation in cerebral organoids. *The*
956 *EMBO Journal*, *36*, 1316–1329. https://doi.org/10.15252/embj.201694700

957 Roybon, L., Christophersen, N. S., Brundin, P., & Li, J.-Y. (2004). Stem cell therapy for Parkinson's
958 disease: where do we stand? *Cell and Tissue Research*, *318*, 261–273.
959 https://doi.org/10.1007/s00441-004-0946-y

960 Ryan, S. D., Dolatabadi, N., Chan, S. F., Zhang, X., Akhtar, M. W., Parker, J., Soldner, F., Sunico, C.
961 R., Nagar, S., Talantova, M., Lee, B., Lopez, K., Nutter, A., Shan, B., Molokanova, E., Zhang,
962 Y., Han, X., Nakamura, T., Masliah, E., … Lipton, S. A. (2013). Isogenic Human iPSC
963 Parkinson's Model Shows Nitrosative Stress-Induced Dysfunction in MEF2-PGC1α
964 Transcription. *Cell*, *155*(6), 1351–1364. https://doi.org/10.1016/j.cell.2013.11.009

965 Sánchez-Danés, A., Richaud-Patin, Y., Carballo-Carbajal, I., Jiménez-Delgado, S., Caig, C., Mora, S.,
966 Di Guglielmo, C., Ezquerra, M., Patel, B., Giralt, A., Canals, J. M., Memo, M., Alberch, J.,
967 López-Barneo, J., Vila, M., Cuervo, A. M., Tolosa, E., Consiglio, A., & Raya, A. (2012).
968 Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and
969 sporadic Parkinson's disease. *EMBO Molecular Medicine*, *4*(5), 380–395.
970 https://doi.org/10.1002/emmm.201200215

971 Sasaki, H., Hui, C., Nakafuku, M., & Kondoh, H. (1997). A binding site for Gli proteins is essential
972 for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro.
973 *Development (Cambridge, England)*, *124*(7), 1313–1322.
974 http://www.ncbi.nlm.nih.gov/pubmed/9118802

975 Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H.,
976 Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build cryptvillus
977 structures in vitro without a mesenchymal niche. *Nature*, *459*, 262.
978 https://doi.org/10.1038/nature07935

979 Schwamborn, J. C. (2018). Is Parkinson's Disease a Neurodevelopmental Disorder and Will Brain
980 Organoids Help Us to Understand It? *Stem Cells and Development*, *27*(14), 968–975.
981 https://doi.org/10.1089/scd.2017.0289

982 Seidel, D., Krinke, D., Jahnke, H. G., Hirche, A., Kloß, D., Mack, T. G. A., Striggow, F., & Robitzki,
983 A. (2012). Induced Tauopathy in a Novel 3D-Culture Model Mediates Neurodegenerative
984 Processes: A Real-Time Study on Biochips. *PLoS ONE*, *7*(11).
985 https://doi.org/10.1371/journal.pone.0049150

986 Sepe, S., Milanese, C., Gabriels, S., Derks, K. W. J., Payan-Gomez, C., van IJcken, W. F. J., Rijksen,

987 Y. M. A., Nigg, A. L., Moreno, S., Cerri, S., Blandini, F., Hoeijmakers, J. H. J., &
988 Mastroberardino, P. G. (2016). Inefficient DNA Repair Is an Aging-Related Modifier of
989 Parkinson's Disease. *Cell Reports*, *15*(9), 1866–1875.
990 https://doi.org/10.1016/j.celrep.2016.04.071

991 Setia, H., & Muotri, A. R. (2019). Brain organoids as a model system for human neurodevelopment
992 and disease. *Seminars in Cell & Developmental Biology*, *August 2018*, 0–1.
993 https://doi.org/10.1016/j.semcdb.2019.03.002

994 Smith, W. W., Pei, Z., Jiang, H., Dawson, V. L., Dawson, T. M., & Ross, C. A. (2006). Kinase
995 activity of mutant LRRK2 mediates neuronal toxicity. *Nature Neuroscience*, *9*(10), 1231–1233.
996 https://doi.org/10.1038/nn1776

997 Smits, L. M., Magni, S., Grzyb, K., Antony, P. M., Krüger, R., Skupin, A., Bolognin, S., &
998 Schwamborn, J. C. (2019). Single-cell transcriptomics reveals multiple neuronal cell types in
999 human midbrain-specific organoids. *BioRxiv*. https://doi.org/doi.org/10.1101/589598

1000 Smits, L. M., Reinhardt, L., Reinhardt, P., Glatza, M., Monzel, A. S., Stanslowsky, N., Rosato-Siri,
1001 M. D., Zanon, A., Antony, P. M., Bellmann, J., Nicklas, S. M., Hemmer, K., Qing, X., Berger,
1002 E., Kalmbach, N., Ehrlich, M., Bolognin, S., Hicks, A. A., Wegner, F., … Schwamborn, J. C.
1003 (2019). Modeling Parkinson's disease in midbrain-like organoids. *Npj Parkinson's Disease*,
1004 *5*(1), 5. https://doi.org/10.1038/s41531-019-0078-4

1005 Spathis, A. D., Asvos, X., Ziavra, D., Karampelas, T., Topouzis, S., Cournia, Z., Qing, X., Smits, L.
1006 M., Dalla, C., Rideout, H. J., Schwamborn, J. C., Tamvakopoulos, C., Fokas, D., & Vassilatis,
1007 D. K. (2017). Nurr1:RXRα heterodimer activation as monotherapy for Parkinson' s disease.
1008 *PNAS*, *114*(15), 3999–4004. https://doi.org/10.1073/pnas.1616874114

1009 Sterneckert, J. L., Reinhardt, P., & Schöler, H. R. (2014). Investigating human disease using stem cell
1010 models. *Nature Reviews. Genetics*, *15*(9), 625–639. https://doi.org/10.1038/nrg3764

1011 Sulzer, D., & Surmeier, D. J. (2013). Neuronal vulnerability, pathogenesis, and Parkinson's disease.
1012 In *Movement Disorders* (Vol. 28, Issue 1, pp. 41–50). https://doi.org/10.1002/mds.25095

1013 Tepper, J. M., & Lee, C. R. (2007). GABAergic control of substantia nigra dopaminergic neurons. In
1014 *Progress in Brain Research* (Vol. 160, pp. 189–208). Elsevier. https://doi.org/10.1016/S0079-
1015 6123(06)60011-3

1016 The GTEx Consortium. (2015). The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue
1017 gene regulation in humans. *Science*, *348*, 648–660. https://doi.org/10.1126/science.1262110

1018 Tieng, V., Stoppini, L., Villy, S., Fathi, M., Dubois-Dauphin, M., & Krause, K.-H. (2014).
1019 Engineering of midbrain organoids containing long-lived dopaminergic neurons. *Stem Cells and*
1020 *Development*, *23*(13), 1–32. https://doi.org/10.1089/scd.2013.0442

1021 Trujillo, C. A., & Muotri, A. R. (2018). Brain Organoids and the Study of Neurodevelopment. *Trends*
1022 *in Molecular Medicine*, *24*(12), 982–990. https://doi.org/10.1016/j.molmed.2018.09.005

1023 Wang, X., Allen, W. E., Wright, M. A., Sylwestrak, E. L., Samusik, N., Vesuna, S., Evans, K., Liu,
1024 C., Ramakrishnan, C., Liu, J., Nolan, G. P., Bava, F., & Deisseroth, K. (2018). Three-
1025 dimensional intact-tissue sequencing of single-cell transcriptional states. *Science*, *361*, 1–9.
1026 https://doi.org/10.1126/science.aat5691

1027 Wang, Y., Zhu, Y., & Qin, J. (2018). Human brain organoid-on-a-chip to model prenatal nicotine
1028 exposure. *Lab on a Chip*, *18*(6), 851–860. https://doi.org/10.1039/C7LC01084B

1029 Wells, M. F., Salick, M. R., Wiskow, O., Ho, D. J., Worringer, K. A., Ihry, R. J., Kommineni, S.,
1030 Bilican, B., Klim, J. R., Hill, E. J., Kane, L. T., Ye, C., Kaykas, A., & Eggan, K. (2016). Genetic
1031 Ablation of AXL Does Not Protect Human Neural Progenitor Cells and Cerebral Organoids
1032 from Zika Virus Infection. *Cell Stem Cell*, *19*(6), 703–708.

1033 https://doi.org/10.1016/j.stem.2016.11.011

1034 Wilson, E. S., & Newell-Litwa, K. (2018). Stem cell models of human synapse development and
1035 degeneration. In *Molecular Biology of the Cell* (Vol. 29, Issue 24, pp. 2913–2921). American
1036 Society for Cell Biology. https://doi.org/10.1091/mbc.E18-04-0222

1037 Xu, Y., Yang, J., & Shang, H. (2016). Meta-analysis of risk factors for Parkinson's disease dementia.
1038 *Translational Neurodegeneration*, *5*(1), 11. https://doi.org/10.1186/s40035-016-0058-0

1039 Zecca, L., Zucca, F. A., Wilms, H., & Sulzer, D. (2003). Neuromelanin of the substantia nigra: A
1040 neuronal black hole with protective and toxic characteristics. *Trends in Neurosciences*, *26*(11),
1041 578–580. https://doi.org/10.1016/j.tins.2003.08.009

1042