The possibilities of organoid research in neurodegenerative diseases

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Abstract

Neurodegenerative diseases are characterized by progressive neuronal loss in the central nervous system and peripheral nervous system but have been hard to study due to the inaccessibility of the brain *in vivo*. However, new *in vitro* models of the developing brain such as the new 3 dimensional brain organoids offer an unprecedented opportunity to study aspects of human brain development and disease. This relatively new technology has been used to investigate the neurological condition microcephaly as well as some neuropsychiatric disorders as autism and schizophrenia but has barely been studied in neurodegenerative diseases. This thesis demonstrates that combining different organoid-techniques could generate new possibilities and potential future models for organoid research in neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease (PD).

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Introduction

Containing billions of neurons and glia cells and a extensive and determined pattern of circuitry, the human brain is one of the most complex organs of the body. Its inaccessibility *in vivo* and the limitations of post mortem studies limits scientific research possibilities [1].
The mouse has been a widely used in human cortical neurogenesis-, development- and disease studies. However, because of its evolutionary distance, divergent cerebral physiology and pathology, it is debatable how this model could recapitulate the human brain studies [2]. Stem cell technology offers a broad spectrum of opportunities, but working with a singular-cell-type *in vitro* culture (2d culture) has its limitations concerning the lack of different cell types and their natural signalling complexity. This could explain how outcome of drug screenings in those classic 2D culture models often do not reproduce the setting *in vivo* [3].

Stem cells (SC) could be divided and subdivided into different categories. Embryonic SC (ESCs), which could be isolated from the inner cell mass of a blastocyst and can give rise to all 3 germ layers. Multi- and toti potent stem cells have more differentiated state and can be isolated in later stages of embryonic development. Compared to pluripotent stem cells (PSCs) the last-mentioned SC are more defined and limited to their differential potentials. Among different pluripotent genes, ESCs express also the 4 Yamanaka factors that nowadays is being used to generate (induce) PSC from somatic cells. By overexpressing those factors in the somatic cell it induced the endogenous (cell own) expression of pluripotent genes and converts "resets" the cell state from somatic -into pluripotent state. However it should be mentioned that those cells have different epigenetic landscape compared to ESC [3].

IPSCs and ESCs are both pluripotent, which means that they can differentiate into most cell types of the body [3]. IPSCs and ESCs can self-assemble with the help of the right matrix and scaffold to grow into organ-like structures with different cell types and orientation also seen in the normal organs. Because of their ability to differentiate into diverse range of somatic cell types, using the 3D culture scaffold, they can mimic the *in vivo* environment by culturing the SC in small organ-like tree-dimensional structures called " Organoids" [4].

Cerebral organoids are being generated in the similar manner by using PSC and growing them into tree-dimensional structures under certain growth conditions to generate small brain-like structures. They recapitulate cortical development and organization in early human brain development while they possess the characteristics of different brain regions. So far, brain organoids have been used to study neural progenitor dysfunction that occurs at early stages of the brain development. A couple of these studies include microcephaly disorder, a group of neurodevelopmental diseases that result in the same clinical feature of a small brain [5].

The underlying mechanism that causes the characteristics of common neurodegenerative diseases with a huge social impact like Parkinson and Alzheimer are still unknown. Alzheimer disease (the majority of the 24 million people suffering from dementia [7]) is extremely difficult to imitate in animal models and drug testing often shows a different outcome when tested *in vivo*, which results in a dismal success rate of clinical trails and single-cell models have been inefficient due to the extracellular features of the disease [8].
7-10 million people worldwide develop Parkinson disease (PD) [6]. Parkinson is a disorder in which the dopaminergic neurons of the midbrain degrade causing classical PD physical symptoms for the patients such as tremor, shaking, stiffness and mobility difficulties. In most cases, the trigger to the degradation of those neurons is unknown and even though PD is considered to be an age-related disease notions are rising that there is a strong neurodevelopmental component that effects/defines the susceptibility to develop the disease [9].

So far, the cerebral organoids have been used to study neurodevelopmental diseases as microcephaly [5, 20] and neuropsychological diseases as autism [22] and schizophrenia [1],

but have barely been used as a model for progressive neurodegenerative diseases. This group of diseases has a huge impact on daily lives of millions and organoid research offers great possibilities to discover causes, mechanism and drug compound development, since a lot of insights are still undiscovered. In this thesis, multiple articles and branches of research from different sources will be combined to create an insight in cerebral organoid technology and it's development and the possible applications in the research of common neurodegenerative disorders as Alzheimer and Parkinson. Because, like *M. A. Lancaster* [5] said after the discovery of the first cerebral organoid; *'A primary goal in neuroscience is to understand the roots of human neurological disease'*.

Brain development

In vivo, the development of the brain starts with the formation of the neuronal tube, a closed sheet of epithelium with neuronal progenitors called neuroectoderm [3] or neuroepithelium [10]. This neuronal tube subdivides into 4 regions that later form the forebrain, the hindbrain, the midbrain and the spinal chord. The forebrain consists of the two cerebral hemispheres, thalamus and hypothalamus. The midbrain is associated with functions like vision, hearing and motor control. The hindbrain consists of the cerebellum and the pons, and the precursor cells for the spinal chord are located in the last region [5].

Neurons and RGCs can be visualized with immunocytochemistry techniques [8]. With this technique, you can visualize neuronal markers by using primary antibodies against those markers (proteins expressed on the cell surface or in the nucleus) and secondary antibodies, which have fluorescent signal and can be visualized by certain laser wave length when attached to the primary antibodies.

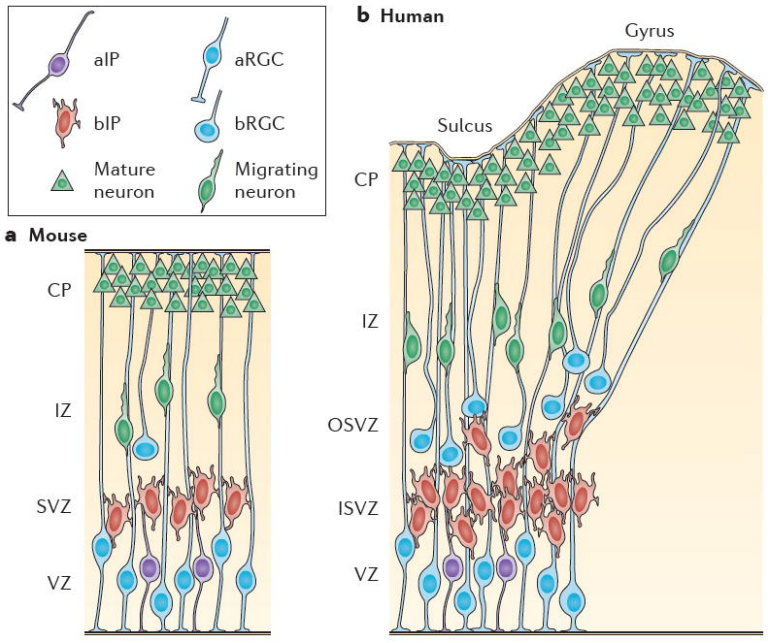

Figure 1: schematic representation of neuroepithelium during human and mouse cortex development.
Apical of the VZ is a layer called the subventricular zone (SVZ), this layer is populated by IPs and RGCs. The SVZ is histologically separated into the inner subventricular zone (iSVZ) and the outer subventricular zone (oSVZ). The SVZ is less developed in mice and the separation of oSVZ and iSVZ is completely absent. Migration of the neuronal progenitors from apical and basal IPs to RGCs to mature neurons is clearly visible in the developing brains from both mice and humans. The neurons migrate through the intermediate zone (IZ) to eventually form a layered structure in the cortical plate (CP).

Original image: Growth and folding of the mammalian cerebral cortex: from molecules to malformations. P218, figure 1 [37]

The ventricular zone or pre-cortex consist of multiple layers of functionally different cell types Neuronal stem cells, also called intermediate progenitors (IPs) first mature into radial glial cells (RGCs), and next, they develop into neurons (*figure 1*, [37]). The first stage of the development of the neuroepithelium starts with the formation of the ventricular zone (VZ). The oSVZ contains a large proportion of the RGCs and contribute to neocortical expansion by increasing the number of neural progenitor cells [12]. The oSVZ is separated from the iSVZ by an inner fibre layer. This fibre is, together with the oSVZ, completely absent in mice (*figure 1*, [37]), and only present to a limited degree in rodents. The oSVZ is considered pivotal to the evolutionary increase of human cortex size and complexity [5].

Organoids, history and diversity

'An organoid is now defined as a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organizes trough cell sorting and spatially restricted lineage commitment '
H. Clevers (2016) [13]

The key to the development of the modern organoid technology was the discovery of the LGR5+ cells by Sato et al. working at the Clevers lab in 2009 [14]. He and his team identified a gene, called LGR5, that was expressed specifically in the self-renewing stem cells near the bottom of the intestinal crypts. They found that if these LGR5-stem cells were provided with the appropriate growth factors and endogenous niche signals they could divide into a polarized epithelium. The cells in the epithelium did differentiate and self-organize according to their natural (hierarchical and functional) role into the formation of a small intestinal crypt-like structure, which can also be called intestinal glands. Later, structures consisting up to 40 crypt-domains were developed. Those domains were named organoids and had a villus-like epithelium surrounding a lumen, resembling the situation of the intestine *in vivo*. These organoids were self-organized structures, had a basic organ-like physiology and RNA expression similar to freshly isolated small-intestinal crypts. These new organoids were also genetically and phenotypically stable on long term (up to 8 months) and were amenable to standard experimental manipulations [15]. This system was subsequently adapted to generate other intestinal organoids that use LGR5+ stem cells, such as the stomach, the colon and the liver [3].

The first cellular diversity in the embryo is seen when the cells differentiate into one of the 3 germ layers; the endoderm, the mesoderm and the ectoderm. To create the endoderm, the factors FGF4 and WNT3A have to be present to promote hindgut and intestinal fate [16]. Those cells can be steered into different types of tissues; for example, the cells are differentiated into the hepatocyte lineage by inhibition the Notch- [3] and Tgf-β signalling [13]. Mature hepatocyte organoids show the same expression patterns as the primary foetal liver. Transplantation of these liver organoids into immunodeficient mice resulted in the maturation of the organoids into adult like liver tissue by connecting to hosts' circulation. Demonstrating the human-specific drug metabolism proofed the functionality of the organoids inside the mice's body. Also, transplantation of those hepatic organoids into mice with induced liver failure improved the survival rate [17]. To create mesoderm organoids, GSK3β and FGF signalling pathways have to be modulated to form precursors of the renal foetal state [3]. After guiding the cells into the specific epithelial renal physiology, they can be used as a model for certain (genetic) renal diseases. For example: genetic polycystic kidney disease, where patients generate cyst formation in renal tubules structures [17]. The final germ layer is the ectoderm. Ectodermal stem cells can, when steered into the right direction, differentiate into precursor neuronal tissue that eventually can be formed into cerebral organoids.

Development of cerebral organoids

The technique to fabricate brain organoids as we know them today was developed by *Lancaster at all.* in 2013 [5] (*figure 2* [5]). Those cerebral organoids were made from iPSCs derived from skin fibroblasts of a patient with microcephaly, a neurodevelopmental disorder which results in a reduced brain size. Instead of focussing on adding lots of (growth) factors and thereby pushing the cells into differentiating, they used the iPSCs' enormous self-organizing capacity. Eventually, they succeeded to trigger the intrinsic cues to start the development by providing the necessary environment. [5]

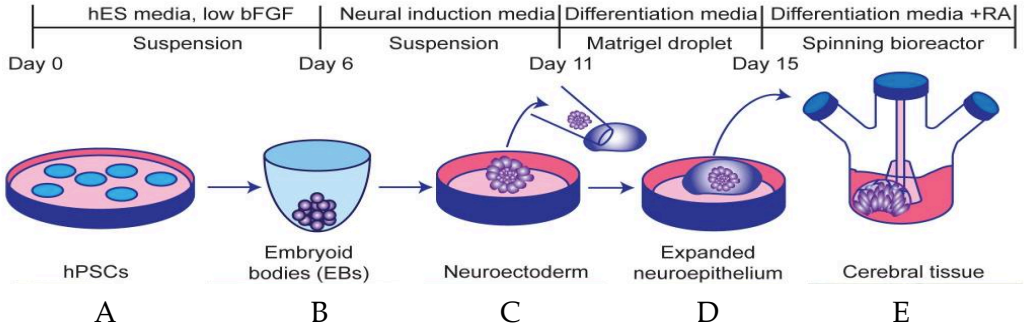

Figure 2. Schematic of the traditional brain organoid culture system.
A| Organoids are cultured from (human) pluripotent stem cells
B| PSCs become ectoderm by embedding them in an embryonic stem cell medium that contains less basic fibroblast growth factor. Fibroblast growth factor is a signalling molecule that inhibits stem cell differentiation and keeps the cells in the original state. In this medium the cells become ectoderm aggregates, called embryonic bodies (EBs) [23]
C| The EBs are suspended into a neural induction medium were the formation of neuroectoderm starts. [23]
D| To get to the formation of an organized epithelia the neuroectoderm is embedded in in Matrigel, a hydrogel with extracellar matrix proteins to give chemical and structural support. [23]
E| Earlier, the growth was stagnated by the limits of stationary diffusion of oxygen and nutrients. *Lancaster et all* [5] was the first to use a spinning bioreactor to promote diffusion and enhance nutrient absorption. This bioreactor can be used for the long-term culture [4]
Original image: Cerebral organoids model human brain development and microcepaly [5] p374 fig1A.

In 2016, *Qian et al [*19] developed a new type of bioreactor and a method to develop brain-region-specific organoids to increase homogeneity and create opportunities for more specific research. He created organoids that were forebrain- midbrain- or hypothalamic- specific. His forebrain organoids showed all 6 cortical layers and the neurons were able of firing action potentials. The forebrain organoids also showed a well-developed oSVZ populated with outer radial glia cells, which is considered pivotal to the evolutionary increase in size and complexity of the human cortex [19].

Wonzel et al. (2017) [9] created more specialized midbrain organoids by using neuroepithelial stem cells (NESCs) instead of the normally used PSCs. The used NESCs were already patterned toward midbrain/hindbrain identity. The NESC-generated human midbrain organoids had highly differentiated midbrain specific neurons including synaptic connections. Astroglia and differentiated oligodendrocytes were found in those specific structures. The differentiation of oligodendrocytes has been inefficient in most stem-cell based protocols and the robust oligodendrocytes that were created in the present approach proofed their efficiency by showing a high degree of myelination [9].

Microcephaly

Microcephaly is a group of neurodevelopmental disorders that affect the size of the brain during pregnancy and is commonly defined as: *'significant reduction in the occipital-frontal head circumference compared with age and sex''* [24]. Primary microcephaly is caused by a decrease of the amount of neurons in the brain and results in a smaller head size from around week 32 of the gastrulation period. Patients with microcephaly condition suffer from severe congenital defects and other problems depending on the cause of the disorder and severity of their microcephaly [25].
Even though genes that can cause microcephaly have been identified, it hasn't been possible to recapitulate the severe reduced brain size as seen in humans in mice models, giving rise to the suspicion that the disorder causes a problem in the development of the human specific oSVZ. This has led to the development of the cerebral organoid to investigate neuronal morphology during the development in human cells.

Organoid research in microcephaly

Primary microcephaly was modelled in cerebral organoids by using patient-derived iPSCs from a patient with severe microcephaly. The patient suffered from a mutation in the CDK5RAP2 gene, which causes a loss of the equally named protein that localizes the spindle poles during mitosis in radial glial stem cells (RGSCs) (*Figure 3* [1]).[26].

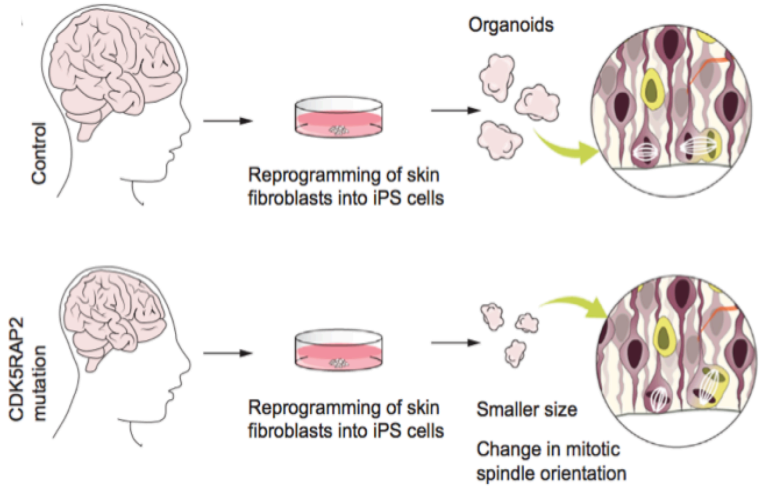

Figure 3. Cerebral organoids with the CDK5RAP2 mutation
The diseased organoids were significantly smaller compared with a non-microcephaly wild type on day 22. This observance was reminiscent with the reduced brain size in patients with the disorder. Subsequently, they examined the spindle orientation in the diseased organoids and observed that those organoids displayed many skew- and vertical oriented spindles where as the wild type organoids showed the normal horizontal orientations [5] .
Original article: Developmental biology 420 (2016) 203 figure 3a [1]

The horizontal orientation of the spindles is necessary to create two proliferative daughter cells, both RGSCs, attached to apical and basal surfaces. Skew or vertical spindles lead to asymmetric divisions that result in only one proliferative daughter cell, which means that only one cell is able to divide and expand, and one cell is differentiated/non-proliferative daughter cell (*figure 4*). So at first; the wrong spindle orientation results in an increase in the amount of cortical neurons as the progenitor cells divide into one post mitotic neuron or glia cell. But as the progenitor pool population reduces early in cortical development, the total number of cortical neurons decreases. The same observation can be made in organoids,

where at day 22 the controls showed large neuroepithelial tissues with RGSCs while patient derived organoids only exposed occasional regions with those progenitor cells but had an increased amount of neurons [5] (*figure 4*). With the help of the organoid model, there is concluded that people with a CDK5RAP2-gene mutation suffer from microcephaly through premature neural differentiation. A lot of intermediate progenitors are embedded in the oSVZ; this explains why it had been so hard to recapitulate this disorder with the severity of the human form in mice [5].

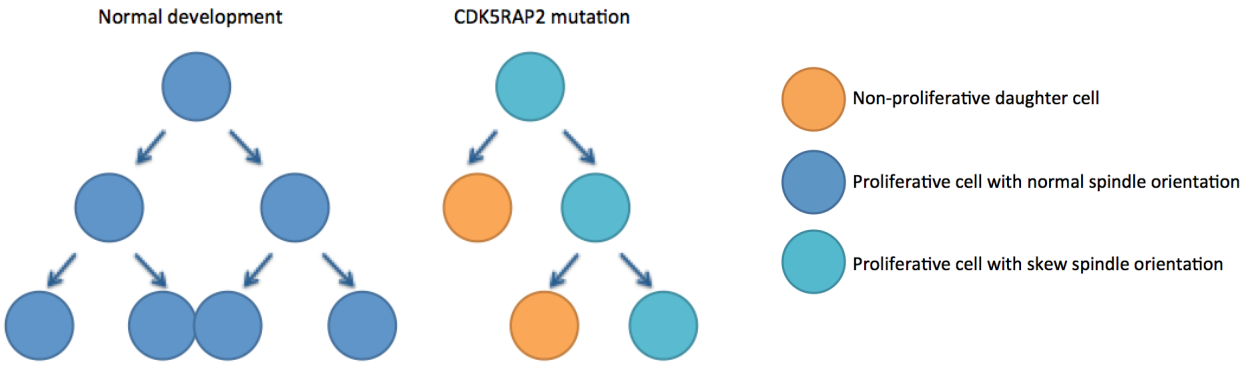

Figure 4. Division of proliferative cells in the oSVZ: CDK5RAP2 mutation compared to wild type.
First | The amount of mature cells in the brain tissue rises (due to the maturation of the non-proliferative daughter cells). While the amount of proliferative cells (RGSCs) decreases, just as the total amount of cells in this layer.
Later | As the wild-type proliferative cells mature, the diseased oSVZ turns out smaller and thinner compared to the wild type, which results in the phenotype of microcephaly.

Alzheimer's disease (AD)

Alzheimer disease (AD) is a progressive neurodegenerative disorder that causes cognitive impairment, memory loss, behavioural changes and difficulty learning due to the loss of neurons in the brain. There are two main pathological hallmarks that characterize AD; the extracellular deposits of amyloid plaques and the intracellular accumulation of neurofibrillary tangles. Amyloid plaques are created by the cleavage of the amyloid precursor protein (APP) into Aβ peptides by α-, β- and γ-secretase. The pattern of cleavage and subunits of the secretase influences the ratio of different isoforms of Aβ peptides; Aβ1-42 and Aβ1-40. The Aβ1-42 peptide aggregates more rapidly than the Aβ1-40 subtype due to differences in solubility. The other pathological characteristic of AD is the formation of neurofibrillary tangles, which is caused by aggregation of a microtubule-associated protein called tau. Normally, tau binds to the microtubules but in the case of AD, it can detach and form insoluble aggregates. Those aggregates stick together and can disrupt the structure and the function of the neuron in such extend that it can damage and in most extreme cases even the death of the cell [7].

In most cases, the cause of the onset of Alzheimer is unknown but in a small amount of patients the AD is caused by the inheritance of a gene that speeds up the progression of the disease. Most common are mutations in the genes coding for APP, and genes called PSEN1 and PSEN2, coding for γ-secretase subunits. Mutations in those subunits cause a different cleavage. This cleavage can have an impact on the length of the Aβ peptide, which influences the solubility and thereby the vulnerability to form aggregates [27]. The best-studied risk factor is the gene for late-onset AD is the apolipoprotein type 4 (ApoE ε4) [28]. The ApoE gene is associated with Aβ clearance and is primarily expressed in astrocytes [7]. The ε4

isoform is less effective than the ε2- or ε3-type and the cell is thereby more susceptible for Aβ plaques [28].

Even though AD is one of the most common age-related neurodegenerative disorders, a lot is still unknown. Just like the cause/trigger of the disease, the role of the phosphorylation of tau and the link between tau and Aβ [7]. It is also unclear why sometimes some healthy individuals also show Aβ plaques, [27] and how the Apoe protein helps with their clearing. Neurological conditions as AD are difficult to study because of the limited accessibility of human brain tissue and imitating the disease in mouse or rodent models has been challenging. This is due to the need to carry multiple transgenes to show Aβ aggregation and another pathology related gene to induce tau pathology. A lot of potential treatments have shown promising results in animal models but have failed to prevent the symptoms of Alzheimer in late phase clinical trials. Human neurons derived from AD patients failed to show fully developed plaques or tangles, but only an elevated level of Aβ and phosphorylated tau [29]. Cultured neonatal cells didn't imitate the disease [30] and one of the most important phenotype of AD, extracellular protein aggregation or amyloid plaques, is lost when using a single-cell culturing method. This emphasized the need for an alternative tree-dimensional model while using cells from human origin that imitate the *in vivo* situation. [8]

Organoid research and Alzheimer disease

[29]. Organoids are, compared to this 3D culture model, less labour-intensive and possess a more natural phenotype without the need to genetically manipulate or add exogenous toxins [8]. *Raja et al* was the first in 2016 to use the organoid technology to study an age-related disease by creating organoids from human iPSCs derived from familiar AD patients and compared them with healthy control iPSC organoids. They used a neuronal marker and a marker for neuronal progenitor cells to observe the maturation of organoids with dense neuron-rich tissue. In the AD organoids, they observed the presence of spontaneous Aβ aggregation and the formation of intracellular hyperphosphorylated tau. To test their model, they inhibited Aβ generation with secretase inhibitors. Besides the attenuated Aβ pathology, they found a decreased amount of tau. This finding demonstrated the connection between the Aβ and tau pathology in this AD model. Also, susceptibility for drug testing in this type of organoid model was revealed. [8]

Potential organoid research in Alzheimer disease

Astrocytes are a type of glial cells that play a central role in brain development and function, such as maintaining the optimal environment for development, energy supply, removal of toxins and debris, synapse formation and many others. They are also important for the formation and maintenance of the blood-brain-barrier, which separates the extracellular fluid in the brain from the circulating blood and forms a semi-permeable/selective barrier. They are involved in vessel dilation and permeability through the activation of tight junction proteins. [32]

In AD, astrocytes play an important role with the degradation of the amyloid and thereby preventing the formation of Aβ plaques [31]. ApoE is expressed in astrocytes and is essential to attract, and degrade Aβ deposits in brain sections [30]. The ε4 subtype causes a risk factor for early-onset non-familial AD because it is found to be less efficient than the other subtypes (ε2 and ε3) in clearing Aβ [32]. The ApoE protein doesn't bind directly to the Aβ, but through a chain of receptors/transporters in the cell. The mechanism behind this process is not fully understood yet [33].

Parkinson disease

PD is a progressive neurodegenerative disorder in which loss of dopaminergic neurons in certain parts of the brain associated with the voluntary movement pattern (*figure 5a*). Damage in this voluntary movement pattern (*figure 5b*) causes the classic PD symptoms including tremors, difficulty of movement and deliberated motor function. Besides a small group of genetic mutations associated with early-onset PD, concrete causes for this neuronal degradation have not been discovered yet. The symptoms are the result of progressive cell death of dopaminergic neurons in parts of the basal ganglia, a set of structures that is part of the midbrain, such as the striatum and the substantia nigra. The neurons of the striatum extend to form the direct and indirect pathway, which are both causing a decrease of the impulses to the thalamus in normal situation. This mechanism results in the inhabitation and filtering of the motor impulses and therefore causing smoother and more coordinated movement (*figure 5c*). When this system fails, like in PD, the thalamus receives excessive inhibitory input, which causes excitation of the thalamus-cortical-spinal pathway (*figure 5d*). This disturbed mechanism causes most of the characteristics of the PD phenotype.

The second characteristic of PD is the presence of Lewy bodies. Lewy bodies are protein aggregates mainly made of α-synucelin fibres and other abnormal insoluble proteins originating from the cytosol of neurons. [6]

Potential organoid research in Parkinson disease

There are currently no organoid models for PD developed, but two different studies have recently been published concerning midbrain-specific organoids. In *2016, Jo et al* [18] developed the first midbrain specific organoids, which had functional dopaminergic neuron. After 2 months, they detected neuromelanin, insoluble black/brown pigments that normally accumulate in pars compata, the part of the substantia nigra that is characterized by the dopaminergic neuron death during PD. Neuromelanin hasn't been detected in any sigle-cell *in vitro* model or in normal (non-specific) human cerebral organoids [18]. The interest in neuromelanin has risen the past years because the presence of this polymer in neurons that are vulnerable to Parkinson-related cell death, and its age related accumulation [34]. Currently is the most widely accepted hypothesis that neuromelanin serves as a by-product of dopamine synthesis, this is support for this theory is that the amount of neuromelanin rises after adding the dopamine precursor (L-DOPA) in midbrain specific organoids [18]. Even though neuromelanin is hypothesized not to be involved in the primarily initiation of PD, it could contribute to the progression of the neuronal degeneration [34].

By combining the organoid technology *Raja et al* [t], who used familiar mutations for Alzheimer disease to create organoids that showed a pathology close to the *in vivo* situation, with the midbrain organoids technology of *Jo et al* [18] an organoid model for Parkinson disease could be created. Genetic defects that have been associated with PD in genes such as *LRRK2*, *PARK2*, *PARK7*, *SNCA*, and *PINK1* genes could serve as mutations to initiate the PD pathology in organoids with developed dopaminergic neurons to observe the pathology.

In 2017, CRISPR/Cas9 technology was used to investigate the 'guilty' gene variant in sporadic Parkinson [35]. Clustered regularly interspaced short palindromic repeats or shortly; CRISPR works together with the enzyme Cas9. It is originally a bacterium-derived immune system tool that finds and destroys viral DNA that has infected the bacteria's own genome. This system can be manipulated and used to cut the genome on a desired location and is able to cut and remove certain genetic combinations to the living DNA. [35,36]. The *SNCA* gene, coding for the protein α-synuclein, is a genetic risk factor for the development of Parkinson and is upregulated by certain SNPs. CRISPR/Cas9 technology was used to analyse variations in the candidate risk-associated SNP variants in the enhancers of the *SNCA* gene. The cells homozygous for the risk factor SNPs were compared with the controls and qRT-PCR revealed an increase in *SNCA* expression. [35] In the same year, CRISPR/Cas9

was used to create a knockout of a gene associated with autism in iPSCs. Those iPSCs were used to develop into cerebral organoids which could be used as a knockout model and could be compared it to a control organoid to characterize the transcriptional networks associated to the autism-coupled gene [36].

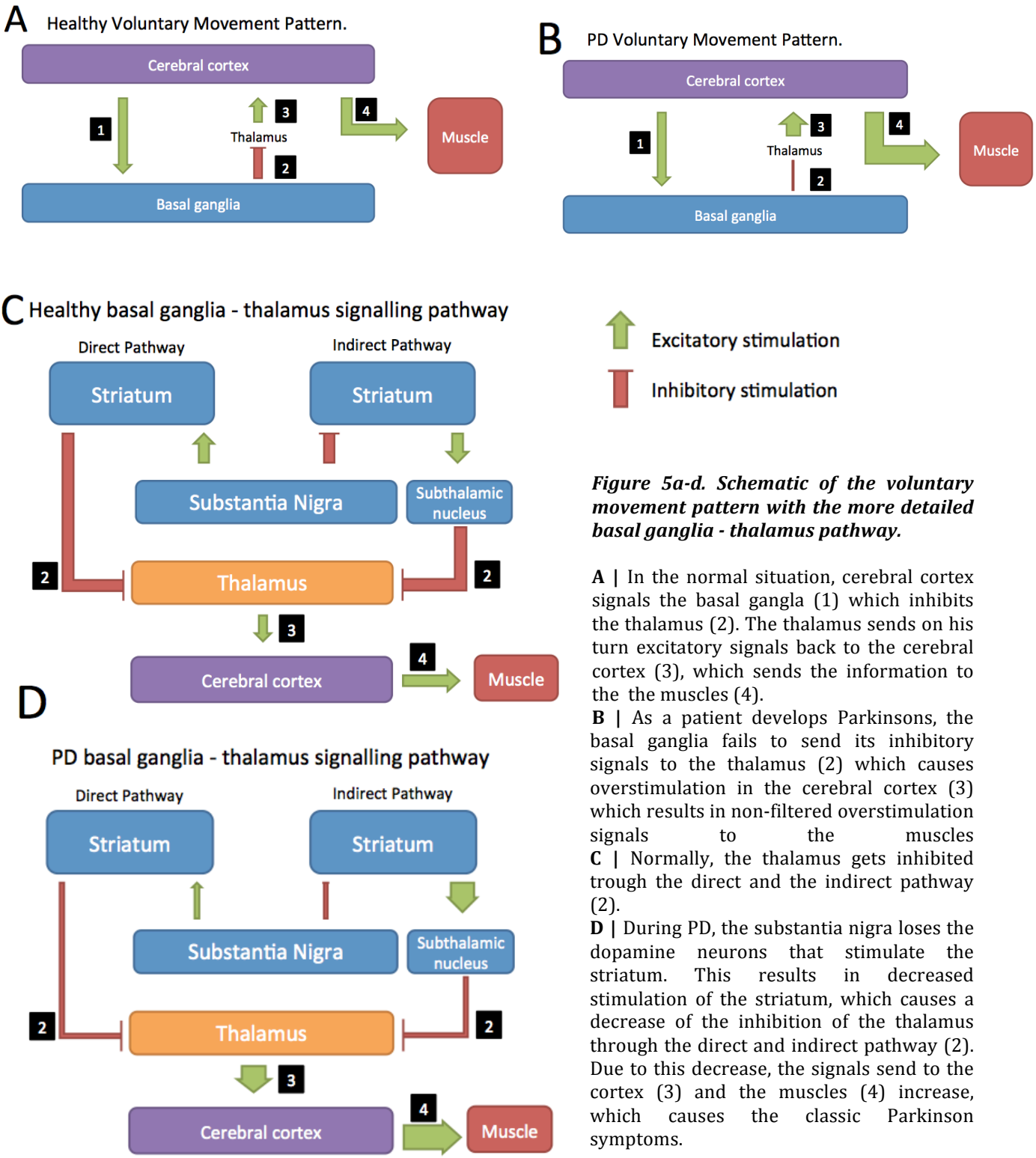

Figure 5a-d. Schematic of the voluntary movement pattern with the more detailed basal ganglia - thalamus pathway.

A | In the normal situation, cerebral cortex signals the basal gangla (1) which inhibits the thalamus (2). The thalamus sends on his turn excitatory signals back to the cerebral cortex (3), which sends the information to the the muscles (4).
B | As a patient develops Parkinsons, the basal ganglia fails to send its inhibitory signals to the thalamus (2) which causes overstimulation in the cerebral cortex (3) which results in non-filtered overstimulation signals to the muscles
C | Normally, the thalamus gets inhibited trough the direct and the indirect pathway (2).
D | During PD, the substantia nigra loses the dopamine neurons that stimulate the striatum. This results in decreased stimulation of the striatum, which causes a decrease of the inhibition of the thalamus through the direct and indirect pathway (2). Due to this decrease, the signals send to the cortex (3) and the muscles (4) increase, which causes the classic Parkinson symptoms.

Discussion.

The cerebral organoid technology can be of great value for future medical science [1], the first brain organoid was developed only 3 and a half years ago [5] but since its discovery it has been used to model disorders like microcephaly [5], autism [22] and schizophrenia [1]. Cerebral organoid technology used to be really expensive and time invasive, but last year, a different spinning reactor was developed, which makes manufacturing of cerebral organoids faster and cheaper than before [19]. First the lack of the embryonic body axis resulted in the development of discrete, but unorganized brain regions [5]. A recent development in this direction is the establishment of region specific organoids for example forebrain [19], hippocampal [5], midbrain, and hypothalamic [18] specific structures.

The use of cerebral organoids the neurodegenerative diseases Alzheimer and Parkinson is still in its infancy. In the case of Alzheimer, one organoid model has been developed to recapitulate this disease. Familiar AD patients' iPSCs have been used to generate this age-related model, which showed the two main pathological hallmarks of Alzheimer, the amyloid plaques and the neurofibrillary tangles [8]. It has been clear that astrocytes play a big role in the clearance of one of these pathological hallmarks, the amyloid plaques, [7] using the ApoE protein. The specific mechanism of this system is unknown [33]. The cerebral organoid system would in this case be an ideal model to investigate the formation of those plaques and the role of the astrocyte Aβ clearance.

Astrocytes play a big role in preventing the formation of amyloid plaques, which manifests itself before the formation of tau, some hypothesize that it might even trigger its formation [7]. The ApoE protein helps with the Aβ clean-up through a mechanism which is not fully understood yet [33]. This gene, or the system that regulates it, could potentially be targeted to increase amyloid binding and therefore accelerate the removal of Aβ to stagnate the plaque formation or in best case even remove the existing Aβ-plaques. The AD organoid model [8] provides an in vitro system as close to in vivo as we can currently get, easy susceptible for manipulation and easy to create in large volumes. *Lancaster et al* [5] has developed an organoid of the hippocampal region, a region important in AD pathogenesis and in 2017, fully developed astrocytes were derived from cerebral organoids [4]. Combining all those techniques could create a model to investigate the role of astrocytes in AD, for example the never discovered mechanism of ApoE-Aβ binding.

No organoid model has been used yet to investigate Parkinson Disease, but there is a suitable candidate organoid to use if a model is needed. The midbrain specific organoids of *Jo et al* showed a group of developed dopaminergic neurons which displayed neuromelanin aggregation over time [18]. Neuromelanin indicates the presence of the substantia nigra, important for the clinical manifestation of PD. CRISPR-Cas9 has been used to overexpress and investigate the familiar PD-risk associated *SNCA* gene and its SCNA enhancing SNP variants in PSCs [35] It has also been proven that it is possible to use CRISPR manipulated PSCs to develop cerebral organoids [36]. Combining those techniques could potentially result in the creation of PD specific organoids, which can be used to track and investigate the development of the disease in vitro and could be used for drug testing. But also, more specifically, investigate the role of neuromelanin in PD or inquire the effect of the redundant α-synuclein (and the related *SNCA* risk-gene).

Even though huge improvements in the field of nutrients and oxygen supply for cerebral organoids have been made with the introduction of the bioreactor of *Qian et al* [19], one on the biggest limiting factors is still the lack of vascularisation in the structures [3]. Tissue has to receive a constant supply of nutrients and oxygen to stay alive, this is a huge growth-limiting factor in the unvascularized organoids which results in necrosis on the inside when the organoids get to big [8]. The immune system also has is part in the development of Alzheimer disease [7] as well as Parkinson disease [6], vascularisation would result in a bigger organoid, due to nutrient and oxygen supply, and a more accurate model due to the

involvement of the immune system. A method to vascularize an organoid could be a future aim to further optimize the organoid technology.

In general, this technology its on its way to become an even more valuable tool in future medical science as it offers personalized treatment, fast drug compound testing and possibilities in investigating the morphogenesis development of the foetal brain. But overall, this thesis outlines a couple of the great possibilities that this relatively new technology offers for the neurodegenerative diseases Alzheimer and Parkinson.

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