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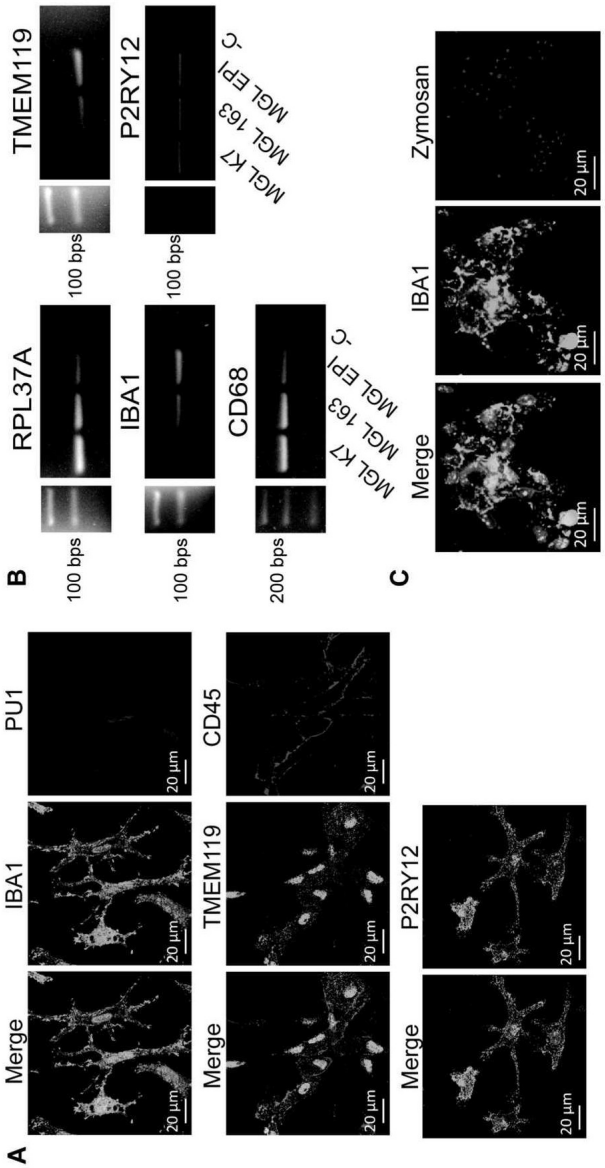
(54) **Means and methods for generating immunocompetent midbrain assembloid.**

(57) The present invention relates to a method of generating a midbrain assembloid, as well as a midbrain assembloid obtainable by said method. Furthermore, the invention relates to a method of testing a compound of interest for its ability to elicit a cellular response, by contacting the midbrain assembloid with the compound of interest.



Means and methods for generating immunocompetent midbrain assembloid

TECHNICAL FIELD OF THE INVENTION

[001] The present invention relates to a method of generating a midbrain assembloid, as well as midbrain assembloid obtainable by said method. Furthermore, the invention relates to a method of testing a compound of interest for its ability to elicit a cellular response.

BACKGROUND OF THE INVENTION

[002] The human brain is a highly complex organ in terms of structure, molecular and cellular composition, making it a challenging target for research. Three-dimensional (3D) brain models have been recently developed to better mimic the spatial and functional complexity of the human brain. Over the last decade, different protocols were developed to generate either whole brain organoids (Lancaster et al., 2013; Lindborg et al., 2016) or discrete regions of the brain (Birey et al., 2017; Monzel et al., 2017; Qian et al., 2016; Shi et al., 2012). These models have proven to be suitable to model neurological disorders, including microcephaly (Lancaster et al., 2013), Batten disease (Gomez-Giro et al., 2019), Parkinson's disease (PD) (Smits et al., 2019) and others (Choi et al., 2014; Qian et al., 2016). In fact, to better model PD recent efforts have focused on the development of PD patient specific midbrain organoids (Kim et al., 2019; Smits et al., 2019). Midbrain organoids contain spatially patterned groups of dopaminergic neurons, making them a suitable model to study PD. They consist of differentiated and functional neurons, which contain active synapses, as well as astroglia and oligodendrocytes (Monzel et al., 2017; Smits et al., 2019). Moreover, they are able to recapitulate cardinal features of PD, including loss of dopaminergic neurons (Smits et al., 2019), and protein aggregation (Kim et al., 2019).

[003] A major feature of neurological disorders is chronic inflammation (Bradburn et al., 2019; Tu et al., 2019). However, the current midbrain organoid system derived from neuroepithelial stem cells lacks certain brain structures thereby presenting major disadvantages for the understanding of the physiological brain. Additionally, neuro-inflammation related disease modeling is not possible in the currently available systems.

[004] The technical problem underlying the present application is to comply with this need. The solution to said technical problem is the provision of means and methods for generating midbrain assembloids as reflected in the claims, described herein, illustrated in the Figures and exemplified in the Examples of the present application.

SUMMARY OF THE INVENTION

[005] In a first aspect the present invention relates to a method of generating a midbrain assembloid, comprising: contacting a midbrain organoid, which is cultured in a three-dimensional cell culture comprising a matrix, with a co-culture medium comprising macrophage precursor cells, thereby obtaining a midbrain assembloid.

[006] The present invention may also relate to methods as defined elsewhere herein, wherein the midbrain organoid is produced from neural epithelial stem cells (NESC).

[007] The present invention may also relate to methods as defined elsewhere herein, wherein said midbrain organoid has been produced by: i) contacting the neural epithelial stem cells with a maintenance medium; ii) contacting the cells of step i) with patterning medium; and iii) contacting the cells of step ii) with organoid maturation medium, thereby obtaining the midbrain organoid.

[008] The present invention also envisages methods as defined elsewhere herein, wherein a) the cells are kept in maintenance medium for 1, 2, 3, 4 days, preferably for 2 days; b) the cells are kept in patterning medium for 2, 4, 6, 8 days, preferably for 6 days; c) the cells are kept in maturation medium for 3, 6, 9, 12 days, preferably for 9 days.

[009] The present invention may also relate to methods as defined elsewhere herein, wherein the midbrain organoid is cultured in organoid maturation medium until day 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 of dopaminergic neuron differentiation before being contacted with the co-culture medium, preferably the midbrain organoid is cultured in organoid maturation medium until day 15 of dopaminergic neuron differentiation before being contacted with co-culture medium.

[0010] The present invention may also relate to methods as defined elsewhere herein, wherein the midbrain organoid is kept under static culturing conditions until day 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 of dopaminergic neuron differentiation, preferably the midbrain organoid is kept under static culturing conditions until day 15 of dopaminergic neuron differentiation.

[0011] Additionally, the present invention also envisages methods as defined elsewhere herein, wherein the midbrain organoid is an early or late midbrain organoid. Furthermore, it is envisaged that said midbrain organoid comprises dopaminergic neurons and does not comprise microglia.

[0012] Furthermore, the present invention may also relate to methods as defined elsewhere herein, wherein the organoid maturation medium which comprises TGF- β3 and cAMP.

[0013] The present invention also relates to a method wherein the macrophage precursor cells have been produced from induced pluripotent stem cells (iPSC).

[0014] The present invention also relates to a method as defined elsewhere herein, wherein the co-culture medium comprises: i) at least two different cytokines, ii) at least two different

neurotrophins, iii) a notch pathway inhibitor, iv) an activator of activin/transforming growth factor β (TGF- β) signaling pathway, and v) macrophage precursor cells.

[0015] The present invention may also relate to methods as defined elsewhere herein, wherein the at least two cytokines are selected from the group consisting of IL-34, GM-CSF, IL-1, IL-2, IL-7, IL-10, IL-18, wherein preferably the at least two cytokines are IL-34 and GM-CSF.

[0016] The present invention may also relate to methods as defined elsewhere herein, wherein the at least two different neurotrophins comprised in the co-culture medium are selected from the group consisting of BDNF, GDNF, NGF, NT-3, NT-4, CNTF, preferably the at least two neurotrophins are GDNF and BDNF.

[0017] Additionally, the invention envisages that said notch pathway inhibitor comprised in the co-culture medium is selected from the group consisting of DAPT, and RO4929097, preferably the notch pathway inhibitor is DAPT.

[0018] The invention further envisages the activator of activin/transforming growth factor β (TGF- β) signaling pathway is selected from the group consisting of TGFβ1, TGFβ2, activin A, activin B, activin AB and nodal, preferably the activator of activin/TGF-β signaling pathway is activin A.

[0019] The present invention may also relate to methods as defined herein wherein the co-culture medium comprises: i) IL-34, ii) GM-CSF, iii) BDNF; iv) GDNF,v) DAPT,vi) activin A, and vii) macrophage precursor cells.

[0020] The present invention may also relate to methods as defined elsewhere herein wherein the co-culture medium does not comprise TGF-β3 and cAMP.

[0021] The present invention may also envisage the methods as defined elsewhere herein, comprising: i) contacting the midbrain organoid as defined herein with the co-culture medium as defined herein, ii) culturing the midbrain organoid with the co-culture medium for 20 days, 40 days, 60 days or for 70 days, preferably culturing the midbrain organoid for 20 days wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain assembloid.

[0022] The present invention may further envisage the methods as defined elsewhere herein, wherein the midbrain assembloid is embedded in an extracellular matrix five days after the addition of macrophage precursors.

[0023] The present invention may also envisage the methods as defined elsewhere herein, wherein the midbrain assembloid comprises dopaminergic neurons and microglia.

[0024] Additionally, said midbrain assembloid may further comprise a) radial glia, b) midbrain specific neural epithelial stem cells, c) neuronal progenitors, d) neuroblasts, e) young dopaminergic neurons, f) young cholinergic neurons, g) mature dopaminergic neurons, h)

GABAergic neurons, i) glutamatergic neurons, j) mature dopaminergic neurons, and/or k) serotonergic neurons.

[0025] The present invention may also envisage the methods as defined elsewhere herein, wherein the dopaminergic neurons are characterized by the expression of the markers selected from the group consisting of FOXA2, TH, CALB1, ADCYAP1, GIRK2 and SLC6A3.

[0026] The present invention may also relate to methods as defined elsewhere herein the microglia is characterized by an immune cell signature.

[0027] The present invention may also envisage methods as defined elsewhere herein, wherein the immune cell signature is characterized in that the microglia expresses the markers selected from the group consisting of IBA1, PU1, HLA-DMB, CSF1R, IL18, C1QC, TLR2, CLEC7A, MRC1, NAIP, ITGAM, CCl2, P2RX4.

[0028] The present invention may also relate to methods as defined elsewhere herein, wherein the immune cell signature is further characterized in that a higher amount of cytokines and chemokines is released in the medium of the midbrain assembloid when compared to a control.

[0029] The present invention also envisages that said control is a midbrain organoid as defined herein which is not contacted with a co-culture medium as defined herein.

[0030] The invention also relates to methods as defined herein, wherein the medium of the midbrain organoid is organoid maturation medium as defined in the methods herein disclosed.

[0031] The present invention may also envisage methods as defined elsewhere herein, wherein the microglia expresses phagocytic gene markers.

[0032] It is envisaged by the invention that said phagocytic gene markers are selected from the group consisting of HLA-A, HLA-B, HLA-C, TLR2, TLR4, ATG7, LAMP2, SQSTM1, and CYBB.

[0033] Further, the invention relates to methods wherein, the midbrain assembloid has a lower cell number compared to a control as defined herein.

[0034] Accordingly, the invention envisages that the midbrain assembloid has a smaller size compared to a control as defined herein.

[0035] The present invention may also envisage methods as defined elsewhere herein, wherein the assembloid shows lower expression of genes involved in hypoxia and oxidative stress compared to a control as defined herein.

[0036] The present invention also relates to methods as defined herein, wherein the genes involved in hypoxia and oxidative stress are selected from the group consisting of COX1, SOD1, GPX4, MT-ATP6, GSTP1.

[0037] The present invention may also envisage methods as defined elsewhere herein, wherein higher amount of leucine, isoleucine, valine, phenylalanine and tyrosine is released in the medium of the midbrain assembloid when compared to a control as defined herein.

[0038] The present invention may also envisage methods as defined elsewhere herein, wherein the midbrain assembloid is further characterized by a) higher expression of immune response markers, b) lower expression of synaptic markers, and c) higher expression of action potential markers, when compared to a control as defined herein.

[0039] The present invention also relates to methods as defined herein, wherein the immune response markers are selected from the group consisting of: HLA-A, HLA-B, STAT2, STAT3, MRC2; the synaptic markers are selected from the group consisting of SYT1, SYP, ROBO1, DDC; the action potential markers are selected from the group consisting of CASK, CACNA1a, CACNA1e, CACNA1B, HCN1, KCNC3, KCND3.

[0040] The present invention also relates to methods as defined herein, wherein the midbrain assembloid shows increased glucose metabolism when compared to a control as defined herein.

[0041] The present invention may also envisage methods as defined elsewhere herein, wherein the increased glucose metabolism is characterized by a higher glucose and pyruvic acid uptake from the co-culture medium as defined in any one of claims 12-18 when compared to a control as defined herein.

[0042] The present invention further relates to methods as defined herein, wherein the midbrain assembloid shows an increased neuronal excitability when compared to a control as defined herein.

[0043] Accordingly, the present invention may relate to the methods as define herein, wherein the increased neuronal excitability is characterized by a lower threshold of action potential generation when compared to that of a control as defined herein.

[0044] In a second aspect the present invention relates to a midbrain assembloid obtainable by the methods envisaged by the invention and as defined elsewhere herein.

[0045] The invention thus further relates to a midbrain assembloid characterized by the features referred to by the methods disclosed herein.

[0046] In a third aspect the invention relates to a method of testing a compound of interest for its ability to elicit a cellular response comprising i) contacting the midbrain assembloid of claim 42 with said compound of interest; and ii) determining whether said compound elicits a cellular response.

[0047] The invention may thus envisage methods as disclosed herein, wherein said cellular response is promotion or inhibition of neuro-degenerative disease.

[0048] The present invention further relates to methods as defined herein, wherein said neurodegenerative disease is preferably Parkinson’s disease.

[0049] The present invention further relates to said compounds, wherein the compound is a drug, small molecule, peptide, protein, or a virus.

[0050] These aspects of the invention will be more fully understood in view of the following description, drawings and non-limiting examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0051] Figure 1. iPSC-derived microglia express specific markers, have phagocytosis ability and are compatible with the engineered co-culture medium. A. Immunofluorescence staining of microglia from line 163 for IBA1, PU.1 (upper panels), TMEM119 and CD45 (middle panels) and P2RY12. B. IBA1, CD68, TMEM119 and P2RY12 gene expression in microglia from the three used lines. C. Immunofluorescence staining of microglia from 163 line for IBA1 and Zymosan. D. Cell viability of microglia (MTT assay) after 10 days of treatment with midbrain organoid media (MOm) or microglia medium (MGLm) supplemented with neurotrophic factors. (n=3). E. Representative bright field images of the microglia morphology at day 0 and 7 of culture with the different media composition tested. F. Schematic diagram of the steps for the media optimization in assembloids and midbrain organoids. G. IBA1 positive (IBA1+) population in assembloids upon culture with MOm, MGLm or cc med. h. TH positive (TH+) neuron population in midbrain organoids (left) and assembloids (right). (n(midbrain organoids)=2, n(assembloids) =5). Error bars are SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using a 2way ANOVA with Dunnett's multiple comparisons test. Abbreviations: BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; TGFβ3, Transforming growth factor beta-3, cAMP, cyclic adenosine monophosphate; ActA, activin A.

[0052] Figure 2. The co-culture medium allows a successful microglia integration and seven other neural cell populations in assembloids. a. Timeline of the co-culture of midbrain organoids with macrophage precursors. B. IBA1 positive (IBA1+) cell percentage in midbrain organoids and assembloids. Assembloids present around 6.4% of IBA1+ cells (n(midbrain organoids)=5, n(assembloids) =15). C. Immunofluorescence staining of midbrain organoids and assembloids with microglia from the line K7 for IBA1, FOXA2 and MAP2 (left panels), and for TH and TUJ1 (right panels). D. UMAP visulaisation of scRNA-seq data - split by microglial presence - shows 8 different defined cell clusters. RGL, radial glia; midNESC, midbrain specific neural epithelial stem cells; PROG, neuronal progenitors; NB, neuroblasts; yDN&CN, young dopaminergic and cholinergic neurons; mDN(A10)&gaN&GlN, mature A10 specific dopaminergic neurons, gabaergic and glutamatergic neurons; mDN(A9)&SN, mature dopaminergic neurons and serotonergic neurons, MGL, microglia. E. Proportions of different cell types in midbrain organoids and assembloids. F. Average expression of cluster defining genes in assembloids. G. Spearman's correlation between different cell types in

assembloids. Error bars are SEM. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 using a Kruskal-Wallis 1way ANOVA with Dunn's multiple comparisons test (for MGLm and cc med vs MOm), and a Mann-Whitney test (for MGLm vs cc med) in a and b, and a Mann-Whitney test in e. LU500789

[0053] Figure 3. Microglia in assembloids have a typical immune cell signature. A. Heatmap showing the average expression of 100 most variable genes between midbrain organoids and assembloids. B. Expression of top 100 microglial marker genes across microglial cells in assembloids. C. Microglia core signature, expression of microglial marker genes as well as genes involved in adhesion, immune response, pathogen recognition and purinergic signaling. D. Gene expression levels of genes related to phagocytic activity. Violin plot shows average expression level. Wilcox one-sample tests * p<0.05, median in red, quantiles in black. Dots represent single cells.

[0054] Figure 4. Microglia in assembloids have phagocytic capacities and release cytokines and chemokines. a. Measured levels of cytokines and chemokines in cell culture media from midbrain organoids and assembloids b. Cytokine (upper graph) and chemokine (bottom graph) levels in midbrain organoids and assembloids (n=9, 3 batches, 3 lines). c. Average expression of cytokines and chemokine genes across cell types. d. Organoid surface area in midbrain organoids and assembloids over time (left graph, n=3). Comparison of the organoid size, measuring the same organoids and assembloids, in four time points during culture (right graph, n=3). e. Cell number (total nuclei count, left panel) and dead cells (pyknotic nuclei) in midbrain organoids and assembloids after 20 days of culture. (n(midbrain organoids)=5, n(assembloids) =15). Error bars are SEM. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 using a Mann-Whitney test in a and b., a Multiple t test with the Holm-Sidak method for d. (left panel), a 2way ANOVA with Tukey's multiple comparisons test for d (right panel) and a Mann-Whitney test in e.

[0055] Figure 5. Microglia affect oxidative stress and trigger immune response in assembloids. A. Differentially expressed genes enrichment analysis in assembloids against midbrain organoids reveals 12 significant network process pathways (FDR<0.05). B. Response to oxidative stress in assembloids compared to midbrain organoids. Box plots show mean expression and standard deviation. Wilcox test, * p< 0.05. Dots represent single cells. C. Immune response of assembloids. The presence of microglia increases antigen presentation and immune response, and decreases autophagy in non-microglia cells. Box plots show mean expression and standard deviation. Wilcox test, * p< 0.05. Dots represent single cells. D. Enrichment analysis of cluster specific DEG p<0.05 between assembloids and midbrain organoids reveals significant FDR<0.05 network processes involved in oxidative stress, immune response as well as synaptic regulation.

[0056] Figure 6. Microglia affect synaptic remodeling in assembloids and lead to mature electrophysiological properties. A. Gene expression of general synaptic markers such as Synaptotagmin (SYT1) and Synaptophysin (SYP), and the dopaminergic neuron circuit formation genes ROBO1 and DCC. B. Expression of genes involved in action potential (CASK, CACNA1A, CACNA1E, CACNA1B) and active zones (HCN1, KCNC3, KCND3) within synapses. Box plots show mean expression and standard deviation. Wilcox test, * p< 0.05. Dots represent single cells. C. Fixation of organoid during recording and post hoc verification of microglia presence. The left half shows an infra-red phase-contrast life image with the fixation pipette. The right half shows the same organoid after immunofluorescence staining for MAP2 and IBA1 (top left). Example traces show voltage response to hyperpolarizing and depolarizing current injections of a neuron inside an assembloid measured by whole-cell patch-clamp. Voltage responses that exhibited action potential (AP) following 50ms after stimulus onset were used for AP analysis (top right). D. Analysis of AP waveform characteristics (bottom left). Voltage thresholds were significantly more depolarized in assembloid neurons (upper right, n =14 neurons in midbrain organoids and n =13 cells in assembloids), although analysis of AP amplitude and half width shows no systematic differences between both groups. Box plots indicate median, 25th, and 75th percentiles and raw data points. Outliers deviating 2.5 standard deviations are marked translucent and were excluded from statistical analysis for normally distributed data. P-values were determined using unpaired t-tests or Mann-Whitney rank test.

[0057] Figure 7 Assembloids show a different metabolic profile compared to midbrain organoids. A. Metabolite levels in the culture media from organoids and assembloids after 48h of culture. The levels of glucose and pyruvic acid were lower in media from assembloids compared to midbrain organoids. B. The levels of the amino acids phenylalanine, tyrosine, methionine, lysine, putrescine, threonine, leucine, isoleucine, valine, asparagine and serine in the media were lower in assembloids, whereas the glutamate levels were higher (n (midbrain organoids)=9, n(assembloids)=27. Each dot represents a sample of pooled medium from 3 organoids. Error bars are SEM. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 using a Mann-Whitney or an unpaired t test.

DETAILED DESCRIPTION OF THE INVENTION

[0058] A major feature of neurological disorders is chronic inflammation (Bradburn et al., 2019; Tu et al., 2019). However, the current midbrain organoid system derived from neuroepithelial stem cells lacks microglia, due to their mesodermal origin. The absence of microglia, with their ability to prune neuronal synapses as well as phagocytose apoptotic cells and debris represents a major disadvantage for the understanding of the physiological

brain. Additionally, neuro-inflammation related disease modeling is not possible in a system that lacks microglia.

[0059] Microglia, the largest population of immune cells in the brain, are tissue-resident macrophages. In the adult brain they represent 5 to 15% of the adult brain cells, depending on the brain region (Thion et al., 2018). Microglia have a unique ontogeny; they are derived from Yolk sac progenitors in a very early embryonic age (Ginhoux et al., 2010; Li and Barres, 2017; Schulz et al., 2012). Microglia have particular functions during brain development. Among others, they establish contacts with neural progenitors to support neurogenesis and proliferation (Choi et al., 2008; Ueno et al., 2013). In the adult brain, they interact with neurons, astrocytes and oligodendrocytes, and their major functions are maintenance of brain homeostasis and immune defense. They also interact with synapses, modulating neuronal activity, and perform synaptic pruning (Tremblay et al., 2011; Wake et al., 2009). They phagocytose apoptotic neurons, induce programmed cell death (Witting et al., 2000), guide sprouting blood vessels, and participate in neuronal maturation (Rymo et al., 2011). Chronic neuro-inflammation is one of the neuropathological characteristics of neurodegenerative disorders, including PD (Shabab et al., 2017).

[0060] With the objective to overcome the deficiencies outlined above, the inventors were able to achieve a stable integration of functional human iPSC-derived microglia in midbrain organoids. This represents a significant advancement of the midbrain model, increasing its complexity and making a step forward from organoids to multi-lineage assembloids (Marton and Pașca, 2020; Pasca, 2019). Moreover, microglia within assembloids are functional. They have phagocytosis ability and are able to release cytokines and chemokines, demonstrating relevant cellular communication abilities. Moreover, the inventors demonstrate that the presence of microglia in assembloids leads to a reduction of cell death and stress in the system. They also participate in synapse remodeling, and lead to increased electrophysiology properties in neurons. Overall, the inventors have established a stable and reproducible way to integrate functional microglia into midbrain organoids, leading to a next generation of brain organoid modelling, with enhanced functionality. The present invention represents a step forward for the understanding and modulation of the complexity of the healthy and diseased human brain, with especial relevance for neuro-inflammatory conditions.

[0061] Accordingly, the present invention relates to a method of generating a midbrain assembloid. In particular, said method comprises the step of contacting a midbrain organoid, which is cultured in a three-dimensional cell culture comprising a matrix, with a co-colture medium comprising macrophage precursor cells, thereby obtaining a midbrain assembloid. Generally, the term “assembloid” refers to a multi-lineage, regionalized organoid, which presents a higher complexity and diversity in terms of cell types and regionalization,

compared to organoids. In this regard, an “organoid” resembles a whole organ, and exhibits an intrinsic potential to self-organise, forming the cellular organisation of an organ. Organoids hold great promise for diagnostic and therapeutic applications. In particular, in the context of the present invention, a “midbrain assembloid” refers to a midbrain organoid comprising microglia, as defined elsewhere herein. Accordingly, a midbrain assembloid of the present invention presents preferably the phenotype of a midbrain and as such, it comprises typical cells/cell types of a midbrain. In particular, the midbrain assembloid of the invention comprises dopaminergic neurons and microglia. Accordingly, a midbrain assembloid of the present invention may comprise a) radial glia, b) midbrain specific neural epithelial stem cells, c) neuronal progenitors, d) neuroblasts, e) young dopaminergic neurons, f) young cholinergic neurons, g) mature dopaminergic neurons, h) GABAergic neurons, i) glutamatergic neurons, j) mature dopaminergic neurons, and/or k) serotonergic neurons (see figure 2 d and e). Furthermore the assembloid as disclosed herein may comprise an asymmetric organization, astrocytes, and/or processes that expand from the midbrain organoid through the matrix. Presence of the cells/cell types in a midbrain assembloid of the present invention can be tested by means and methods known in the art and as described herein in the example section and in the materials and methods section. Likewise, expression of the markers as described herein by said cells/cell types can be tested as is known in the art and as described herein in the example section and in the materials and methods section.

[0062] As disclosed herein, the midbrain assembloid of the present invention is generated by contacting a midbrain organoid, which is cultured in a three-dimensional cell culture comprising a matrix, with a co-culture medium comprising macrophage precursor cells, thereby obtaining a midbrain assembloid. The “midbrain organoid” according to the present invention resembles the midbrain. The midbrain is the region of the brain, where the majority of the neurotransmitter dopamine (DA) is produced. A midbrain organoid of the present invention is preferably produced from a single colony of a neural epithelial stem cells (NESC), preferably a hNESC. Neuroepithelial stem cells (NESCs) when referred to herein can be derived from actual stem cells in several different stages of neural development. Neuroepithelial cells are a class of stem cell and have similar characteristics as stem cells. For example, these cells are able to self-renew. Self-renewal is the ability to go through numerous cell cycles of cell division while maintaining the undifferentiated state. In addition, neuroepithelial stem cell cells have the capacity to differentiate further into multiple types of cells, such as neurons, astrocytes and other glial cells. Thus, these cells are also multipotent. They are restricted to the neural lineage and can differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000). Methods for testing if a cell has the capacity to self-renew and if a cell is multipotent are known to the skilled artesian. Self-renewal may be tested by

passaging the cells over more than 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more passages. Passaging includes splitting of the cells before re-plaiting them as a single cell suspension. Multipotency can be tested by differentiating said cells into different lineages such as astrocytes, oligodendroctyes and neurons.

[0063] Furthermore, a neuroepithelial stem cell can express markers such as PAX6, Notch 1, Nestin, PCNA, Hes5 and Sox1. In particular, the neuroepithelial stem cells used in the methods of the present invention can be mammalian neural plate border stem cells (NPBSC) as described in WO2013104752. Furthermore, the neuroepithelial stem cells used in the methods of the present invention can also be NPBSCs as described in WO2013104752, which are also obtained by the method as described in WO2013104752. These NPBSC can be characterized by the expression of at least three markers selected from the group consisting of FORSE1, MSX1, PHOX2B, PAX3, PAX6, SOX1, SOX2, NESTIN, IRX3, HOXA2, HOXB2, HES5, DACH1, PLZF, LM03, EVI1 and ASCL1. Furthermore, these cells can be characterized by a lack of expression of at least one of the markers OCT4, NANOG, AFP, T, SOX17, EOMES, GSH2, OLIG2, CK8, CK18, NKX2.2, NKX6.1, HOXB8, HOXA5, FOXA2 and VCAM-1.

[0064] The neuroepithelial stem cell can be a mammalian NESC. It is also encompassed by the present invention that the NESC is a human NESC (hNESC). A neuroepithelial stem cell may be obtained by different means and methods known to the skilled artesian. For example, a neuroepithelial stem cell may be derived or obtained from pluripotent cells. NESC of the present invention may be genetically modified or obtained from a patient suffering from a neurological disease, such as PD. Also, NESC may be produced from iPSCs, fibroblasts or PBMCs as described herein.

[0065] As disclosed herein, a midbrain organoid of the present invention may be either an early midbrain organoid or a late midbrain organoid. The early midbrain organoid may be a midbrain organoid, which has been differentiated for 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 days, preferably the early midbrain organoid has been differentiated for 15 days. The late midbrain organoid is a midbrain organoid, which may have been differentiated for at least 25, 30, 35, 40, 50, 60, 70 or more days, preferably the late midbrain organoid has been differentiated for 70 days. In preferred examples, the midbrain organoid of the invention is an early midbrain organoid comprising dopaminergic neurons that have differentiated for 15 days, as defined elsewhere herein. A midbrain organoid as disclosed herein presents preferably the phenotype of a midbrain and as such, it comprises typical cells/cell types of a midbrain. Accordingly, a midbrain organoid as disclosed herein may comprise neural progenitor cells, young neurons, young dopaminergic neurons, mature neurons, mature dopaminergic neurons, an asymmetric organization, oligodendrocytes, oligodendrocyte progenitors, astrocytes, and/or processes that expand from the midbrain

organoid through the matrix. Furthermore, the midbrain organoid may present typical midbrain clustering of dopaminergic neurons and the processes that expand from the midbrain organoid through the matrix. The midbrain organoid of the present invention therefore comprises dopaminergic neurons, however a midbrain organoid according to the invention does not comprise microglia.

[0066] The methods disclosed herein can be carried out in any cell culture, while, however, three-dimensional cell culture is preferred. Culture conditions may vary, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel comprising one or more of the following: a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, gases (O_2, CO_2) and/or regulated physico-chemical environment (pH, osmotic pressure, temperature). Cell culture as described herein refers to the maintenance and growth of cells in a controlled laboratory environment. Such *in vitro* cell culture models are well-known in experimental cell biological research. For example, cells can be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture). Cells can also be grown floating in the culture medium (suspension culture). Furthermore, the cells of the present invention may be cultured under agitating conditions. The methods of the present invention are, however, preferably be carried out in a three-dimensional cell culture. A “three-dimensional cell culture or “3D cell culture” as used herein means that cells are grown in an artificially-created environment in which cells are permitted to grow or interact with its surroundings in all three dimensions. For example, in order to achieve the three-dimensional property of the cell culture, cells are grown or differentiated in matrices or scaffolds. In principle, suitable matrices or scaffolds, which can be used in three dimensional cell cultures are known to the skilled artisan. Such matrices or scaffolds can therefore be any matrix or scaffold. For example, the matrix or scaffold can be an extracellular matrix comprising either natural molecules or synthetic polymers, a biological and synthetic hybrid, metals, ceramic and bioactive glass or carbon nanotubes. Exemplary natural extracellular matrix molecules include collagen, basement membranes such as laminin or fibrin, alginates, chitosan, hyaluronic acid, silk fibroin, cellulose actetate, casein, chitin, fibrinogen, gelatine, elastin or poly-(hydroxyalkanoate). Synthetic extracellular matrix polymers include Hyaluronic acid (HA) modified forms, Poly-ethylen glycol (PEG) modified forms, Self-assembling protein hydrogels, Poly(lactic-co-glycolic acid) (PLGA), Polycaprolactone (PCL), Polyurethane or PGS. Biological and synthetic hybrids can for example include Polycaprolactone-chitosan, PLLA-Hydroxyapatite, Hydroxyapatite-bioglass-ceramic, Poly-(hydroxylalkanoate)-bioglass, Hydroxyapatite-collagen, PCL-gelatin or PCL-collagen. Exemplary metals include Tantalam, Magnesium and its alloys, Titanium and its alloys or Nitinol (nickel and titanium alloys). Examples of Ceramics and bioactive glass matrices/scaffolds include Titanium and tri calcium phosphate,

Hydroxyapatite and Tricalcium phosphate, Bioactive silicate glass (SiO2–Na2O–CaO–P2O5), Hydroxyapatite and bioglass, Calcium phosphate glass or Phosphate glass. Carbon nanotubes can be constructed using graphite ranging from 0.4 to 2 nm. Carbon nanotubes can comprise CNT-polycaprolactone, CNT-ceramic matrix, 45S5 bioglass-CNT, CNT studded with gelatine hydrogel, CNT-TiO2, CNT-laminin, CNT grafted with polyacrylic acid or CNT-TGF-β. The matrix or scaffold can also be a hydrogel such as Matrigel, fibrin gel or alginate gel. Matrigels can be a reconstituted basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Matrigel can be constituted of 60% laminin, 30% type IV collagen and 8% entactin. Optionally growth factors and other molecules can be added to the Matrigel. The Matrigel can also be BD MatrigelTM (obtainable from BD Biosciences).

[0067] Different culture mediums may be used for the generation of the midbrain organoid and the midbrain assembloid of the invention, such as maintenance medium, patterning medium, organoid maturation medium, or co-culture medium. A scheme of the culturing protocol and different mediam and culturing time used is summarized in figure 2a. For example, the midbrain organoid according to the invention may be generated by i) contacting the neural epithelial stem cells with a maintenance medium; ii) contacting the cells of step i) with patterning medium; and iii) contacting the cells of step ii) with organoid maturation medium, thereby obtaining the midbrain organoid. In particular, the NESC (as defined elsewhere herein) are contacted firstly with a maintenance medium and kept in maintenance medium for 1, 2, 3,4 days, preferably, the NESC are cultivated in maintenance medium for 2 days. Generally, a maintenance medium as disclosed herein may comprise (i) a SHH-pathway activator; (ii) canonical WNT-signaling activator; and (iii) an antioxidant. Preferably, the maintenance medium as disclosed herein may be a N2B27 medium (known to the skilled artisan) supplemented with 0.2 mM Ascorbic acid, 3 μM CHIR 99021, 0.5 μM Smoothened Agonist, SAG, 2.5 μM SB-431542 0.1 μM LDN-193189. After preferably 2 days of culture in maintenance medium, the NESC are contacted with patterning medium. Therefore, the maintenance medium is removed and replaced with patterning medium, where the cells are cultured for 2, 4, 6, 8 days, preferably for 6 days. A patterning medium as described herein may comprise (i) a SHH-pathway activator; (ii) canonical WNT-signaling activator; and (iii) an antioxidant, preferably, the patterning medium according to the invention corresponds to the preferred maintenance medium without the TGF-β inhibitors SB-431542 and LDN-193189. Preferably, the patterning medium can be patterning medium 1 or patterning medium 2. Patterning medium 1 (or pre-patterning medium) corresponds to the preferred maintenance medium without the TGF-β inhibitors SB-431542 and LDN-193189. Preferably, the patterning medium 1 as disclosed herein may be a N2B27 medium (known to the skilled artisan) supplemented with 0.2 mM Ascorbic acid, 3 μM CHIR 99021, 0.5 μM Smoothened

Agonist, and SAG. Patterning medium 2, as defined herein, corresponds to the preferred maintenance medium without the TGF-β inhibitors SB-431542 and LDN-193189 and with a lower concentration of CHIR 99021 compared to patterning medium 1. Preferably the patterning medium 2 may be a N2B27 medium (known to the skilled artisan) supplemented with 0.2 mM Ascorbic acid, 0.7 µM CHIR 99021, 0.5 µM Smoothened Agonist, and SAG. Contacting the cells with patterning medium 1 causes dopaminergic neurons differentiation, meaning that dopaminergic neurons start to differentiate from the initial NESC population. Therefore, the first day in which the cells are first contacted with patterning medium 1 corresponds to day 0 of dopaminergic neuron differentiation and, for example, the day six , counting from day 0 as defined herein in which the cells are cultured (in any given medium) corresponds to day 6 of dopaminergic neuron differentiation. Similarly, day 15 counting from day 0 as defined herein in which the cells are cultured (in any given medium) corresponds to day 15 of dopaminergic neuron differentiation.

[0068] After preferably 6 days of culture in patterning medium (preferably 2 days in patterning medium 1, followed by 4 days in patterning medium 2, corresponding to day 6 of dopaminergic neuron differentiation), the cells are contacted with maturation medium (or “organoid maturation medium”). Therefore, the patterning medium is removed and replaced with maturation medium, where the cells are cultured for 3, 6, 9, 12 days, preferably for 9 days. A maturation medium as described herein may comprise an antioxidant, neurotrophins, a TGF pathway ligand, an activator of cAMP-dependent protein kinases and phosphodiesterase inhibitor and a Smad activator. Preferably, the maturation medium according to the invention comprises N2B27 medium, 0.2 mM Ascorbic acid, 10ng/mL Brain Derived Neurotrophic Factor, BDNF (Peprotech, 450-02), 10 ng/mL Glial-Derived Neurotrophic Factor, GDNF (Peprotech, 450-10), 1pg/mL TGF-β3 (Peprotech, 100-36E), 10 µM DAPT (R&D Systems, 2634/10) and 2.5ng/mL Activin A (Thermo Scientific, PHC9564)). In preferred embodiments, the organoid maturation medium according to the invention comprises TGF- β3 and cAMP.

[0069] Contacting the cells with organoid maturation medium, causes the cells in the culture to organize themselves into midbrain organoids, as defined elsewhere herein (and described for example in Nickels et al., 2020; Smits et al., 2019). In particular, according to the present invention the midbrain organoid is cultured in organoid maturation medium until day 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 of dopaminergic neuron differentiation before being contacted with the co-culture medium, preferably the midbrain organoid is cultured in organoid maturation medium until day 15 of dopaminergic neuron differentiation before being contacted with co-culture medium. As used herein, the term “dopaminergic neuron differentiation” refers to a cell culture, which allows the starting population of cells to differentiate into dopaminergic neurons. Day 15 of dopaminergic neuron differentiation corresponds to day 15 counting from

day 0 of dopaminergic neuron differentiation as defined elsewhere herein in which the cells are cultured (in any given medium). Preferably, day 15 of dopaminergic neuron differentiation is reached after to 2 days of culture in patterning medium 1, followed by 4 days of culture in patterning medium 2, followed by 9 days of culture in organoid maturation medium (see also Figure 2A) as defined herein. Presence of dopaminergic neuron differentiation can be determined with techniques known to the skilled artisan, for example, by screening for determined cell-markers characteristic of dopaminergic neurons, as described elsewhere herein. The midbrain organoids according to the invention may be kept under static culture conditions in organoid maturation medium with media changes every third day, preferably until day 15 of dopaminergic neuron differentiation. By “static culture” as used herein it is meant that the cultures are carried in standard non-adherent well-plates (static culture) compatible with unembedded organoid culture protocols (known to the skilled artisan), as opposed to agitating conditions or “shaking cultures” which keeps cells in motion. For example, the cultures according to the methods herein disclosed may be carried out in a 96 well round bottom plates without agitation.

[0070] Organoid maturation medium, as applied in the methods of the present invention may comprise an activator of activin/transforming growth factor- β (TGF- β) signaling pathway. In general, a compound/molecule that can be used as an activator can be any compound/molecule, which can activate the respective pathway or which inhibits a suppressor of the pathway to be activated. Exemplary activators can include suitable binding proteins directed e.g. against suppressors of a certain pathway. An activator may enhance or increase the pathway to be activated by 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 % or more when compared to the activity of the pathway without the addition of the activator. The activin/TGF-□ signaling pathway is known in the art and for example described in Heldin, Miyazono and ten Dijke (1997). In short, Receptor ligands, including, for example, TGFB1, TGFB2, TGFB3, ACTIVIN A, ACTIVIN B, ACTIVIN AB, and/or NODAL, bind to a heterotetrameric receptor complex consisting of two type I receptor kinases, including, for example, TGFBR2, ACVR2A, and/or ACVR2B, and two type II receptor kinases, including, for example, TGFBR1, ACVR1 B, and/or ACVR1C. This binding triggers phosphorylation and activation of a heteromeric complex consisting of an R-smad, including, for example, SMAD2, and/or SMAD3, and a Co-smad, including, for example, SMAD4. Accordingly, the term "activator of the activin/TGF-β signaling pathway" refers to an activator of any one of the above recited molecules that form part of this signaling pathway. Exemplary activators of the activin/TGF-β signaling pathway include TGF-β 1, TGF-β 2, TGF-β 3, activin A, activin B, activin AB or nodal. Thus, the activator of activin/TGF- β signaling pathway in an organoid maturation medium can be TGF-β 3. The activator of the activin/TGF-β signaling pathway such as TGF-β 3 can be utilized in an amount of 0.0001 ng/µl to 0.1 ng/µl such as

e.g. in an amount of 0.001 ng/µl. Organoid maturation medium as applied in the methods of the present invention can further comprise a cAMP analogue. Such cAMP analogs are compounds that have similar physical, chemical, biochemical, or pharmacological properties as the cyclic adenosine monophosphate (cAMP). cAMP is known to the skilled artesian and described in e.g. Fimia GM, Sassone-Corsi P. (2001).

[0071] According to the present invention, after being cultured in organoid maturation medium for preferably 15 days under static conditions, the midbrain organoid as defined herein is contacted with a co-culture medium. Said co-culture medium comprises macrophage precursor cells. The term “macrophage precursor cells” refers to the parent cells in the lineage that gives rise to macrophages. Macrophage precursor cells give also rise to microglia in the brain, after they migrate from the yolk sac into the developing central nervous system where they actively proliferate during development, giving rise to the resident microglial pool (Alliot *et al.*, 1999; Pessac *et al.*, 2001). Said macrophage precursor cells may be produced from induced pluripotent stem cells (iPSCs). A "pluripotent stem cell" when referred to herein relates to a cell type having the capacity for self-renewal, and the potential of differentiation into different cell types. Pluripotent stem cells can differentiate into nearly all cells, i.e. cells derived from any of the three primary germ layers: ectoderm, endoderm, and mesoderm. The term pluripotent stem cells also encompasses stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst. Induced pluripotent stem cells are pluripotent stem cells that are generated from somatic cells (Takahashi and Yamanaka, 2006). "Induced pluripotent stem cells", as used herein, refers to adult somatic cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Thus, induced pluripotent stem cells derived from a non-pluripotent cell. In addition, iPSCs are able to self-renew in vitro and differentiate into all three germ layers. The pluripotency or the potential to differentiate into different cell types of iPSC can tested, e.g., by in vitro differentiation into neural or glia cells or the production of germline chimaeric animals through blastocyst injection. Methods for the generation of human induced pluripotent stem cells are well known to the skilled person. Usually forced expression of Oct3/4, Sox2 and Klf4 (as well as OCT3/4, SOX2 and KLF4) is sufficient to generate an induced pluripotent stem cell out of an adult somatic cell, such as a fibroblast. Further methods for obtaining iPSCs are also known to the skilled artesian and for example described in WO2009115295, WO2009144008 or EP2218778. Thus, the skilled artesian can obtain an iPSC by any method. In principle, induced pluripotent stem cells may be obtained from any adult somatic cell (of a subject). Exemplary somatic cells include peripheral blood Mononuclear Cells (PBMCs) from blood or fibroblasts, such as for example fibroblasts obtained from skin tissue biopsies.

[0072] Therefore, it is envisioned by the present invention that the macrophage precursor cells have been produced or derived or obtained from an induced pluripotent stem cell (iPSC). Different ways how to differentiate iPCSs into macrophage precursor cells are known to the skilled artesian and for example described in van Wilgenburg et al. 2013 and Muffat et al. 2016. In addition, it is envisioned by the present invention that the iPSCs can be produced from somatic cells such as fibroblasts. Furthermore, the iPSC can be a human iPSC (hiPSC).

[0073] Therefore, the co-culture media as applied in the methods of the present invention comprises i) at least two different cytokines, ii) at least two different neurotrophins, iii) a notch pathway inhibitor, iv) an activator of activin/transforming growth factor β (TGF- β) signaling pathway, and v) macrophage precursor cells.

[0074] As defined herein, the co-culture media as applied in the methods of the present invention comprise at least two different cytokines. The term “cytokines” refers to a category of small proteins (~5–20 kDa) important in cell signaling. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors. Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells; a given cytokine may be produced by more than one type of cell. Cytokines are important in health and disease, specifically in host immune responses to infection, inflammation, trauma, sepsis, cancer, and reproduction. Cytokines have been classed as lymphokines, interleukins, and chemokines. Exemplary cytokines include cytokines of the four-α-helix bundle family (including the IL-2 subfamily, the interferon IFN subfamily, and the IL-10 subfamily), the IL-1 family (like IL-1 and IL-18), the cysteine knot family (like TGF-β1, TGF-β2 and TGF-β3), the IL-7 family, IL-34 and GM-CSF. Preferably the cytokines in the co-colture media of the invention do not comprise TGF-β3. Accordingly, the term "at least two different cytokines" may refers to two or more of the recited molecules, excluding TGF-β3. Preferably, the at least two different cytokines are IL-34 and GM-CSF.

[0075] IL-34 and GM-CSF can both independently from each other be employed in a concentration of between about 1 and about 200 ng/µl each, more preferably IL-34 may be employed between about 5 and 200 ng/µl, most preferably at a concentration of about 100 ng/mL and GM-CSF may employed at a concentration between about 1 and 20 ng/mL, most preferably GM-CSF is employed at a concentration of 10 n/mL..

[0076] The co-culture media as applied in the methods of the present invention further comprises at least two different neurotrophins. The term "neurotrophins", as used herein, relates to a family of proteins that regulate the survival, development, and function of neurons. Exemplary neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) as well as GDNF family of ligands and ciliary neurotrophic factor (CNTF). The GDNF family of ligands includes

glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN).

[0077] Accordingly, the term "at least two different neurotrophins" refers to two or more of the recited molecules. Preferably, the at least two different neurotrophins are BDNF and GDNF (Gene Symbols: BDNF and GDNF, respectively). BDNF can e.g. be the human BDNF protein of Uniprot/Swissprot accession no. P23560 (version 1 as of October 31, 1991). GDNF can e.g. be the human GDNF protein of Uniprot/Swissprot accession no. P39905 (version 1 as of January 31, 1995).

[0078] BDNF and GDNF can both independently from each other be employed in a concentration of between about 1 and about 50 ng/µl each, more preferably between about 10 and about 25 ng/µl each, and most preferably the amount is about 10 ng/µl each. BDNF and GDNF may for example be obtained from Peprotech.

[0079] The co-culture media as applied in the methods of the present invention further comprises a notch pathway inhibitor. The “notch pathway inhibitor” as used herein can be any suitable notch pathway inhibitor. In general, a notch pathway inhibitor is an inhibitor of notch pathway. The notch pathway is known to the skilled person. The term "notch signaling pathway” refers to a highly conserved cell signaling system present in most animals (Artavanis-Tsakonas S, Rand MD, Lake RJ (Apr 1999). "Notch signaling: cell fate control and signal integration in development". Science. 284 (5415): 770–6.). Mammals possess four different notch receptors, referred to as NOTCH1, NOTCH2, NOTCH3, and NOTCH4. The notch signaling pathway thus refer to a cascade of cellular events initiated when Notch receptors on the cell surface engage ligands presented *in trans* on opposing cells. Ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to modify gene expression. The Notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling also has a role in the neural function and development (Gaiano N, Fishell G (2002). "The role of notch in promoting glial and neural stem cell fates". Annual Review of Neuroscience. 25 (1): 471–90; Aguirre A, Rubio ME, Gallo V (Sep 2010). "Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal". Nature. 467 (7313): 323–7.). An "inhibitor" of notch pathway as used herein, is defined as any suitable inhibitor capable of a decreasing or inhibting the activity of notch pathway. The inhibitor may be a compound/molecule decreasing or abolishing the activity of the notch pathway. The inhibitor may achieve this effect by decreasing or blocking the transcription of the gene encoding the NOTCH protein receptors and/or decreasing the translation of the mRNA encoding the NOTCH receptors. It can also be that the inhibitor leads to that NOTCH receptors perform their biochemical function with decreased efficiency in the presence of the

inhibitor than in the absence of the inhibitor. Accordingly, the term "inhibitor" also encompasses molecules/compounds that have a directly decreasing effect on the NOTCH pathway but also molecules that are indirectly decreasing, e.g. by interacting for example with molecules that positively regulate the NOTCH pathway, for example DAPT, or RO4929097 which are a γ-secretase inhibitor. The inhibitor can also be an antagonist of the pathway to be inhibited.

[0080] Exemplary notch pathway inhibitors are DAPT, RO4929097, or other notch pathway inhibitors. Preferably, the notch pathway inhibitor in the co-culture medium of the present invention is DAPT.

[0081] The co-culture media as applied in the methods of the present invention further comprises an activator of activin/transforming growth factor- β (TGF- β) signaling pathway, as defined elsewhere herein. Exemplary activators of the activin/TGF-β signaling pathway include TGF-β1, TGF-β2, TGF-β3, activin A, activin B, activin AB or nodal. The activator of the activin/TGF-β signaling pathway such as TGF-β 3 can be utilized in an amount of 0.0001 ng/µl to 0.1 ng/µl such as e.g. in an amount of 0.001 ng/µl. However, the activator of activin/TGF- β signaling pathway comprised in the co-culture medium is not TGF-β 3, preferably the activator of activin/TGF-β signaling pathway comprised in the co-culture medium is activin A.

[0082] In preferred embodiments of the invention the co-culture medium comprises i) IL-34, ii) GM-CSF, iii) BDNF, iv) GDNF, v) DAPT, vi) activin A, and vii) macrophage precursor cells. Additionally, the co-culture medium according to the present invention does not comprise TGF-β3 and cAMP.

[0083] The methods of the present invention envision therefore i) contacting the midbrain organoid as defined elsewhere herein with the co-culture medium as defined elsewhere herein; and ii) culturing the midbrain organoid with the co-culture medium for 20 days, 40 days, 60 days or for 70 days, preferably culturing the midbrain organoid for 20 days wherein the culturing in co-culture medium is performed under agitating conditions, thereby obtaining a midbrain assembloid. In particular, the midbrain organoid is contacted with co-culture medium at preferably day 15 of dopaminergic neuron differentiation, as described elsewhere herein. Preferably, after 5 days of contacting the midbrain organoid with co-culture medium (i. e after five days of addition of macrophage precursor cells), the midbrain assembloid is embedded in an extracellular matrix, as defined elsewhere herein, after which the culturing in co-culture medium was performed under agitating conditions. Agitation" or "agitating" when used herein encompasses any technique that keeps cells in motion, i.e. cells are essentially not allowed to adhere to surfaces. Agitation can be achieved in a number of ways, including shaking, spinning, stirring, moving and/or mixing. Spinning can, for example, be achieved by the use of spinner flasks containing a magnetic paddle or impeller. Alternatively, the methods

of the invention can be carried out in a bioreactor. A number of types of bioreactor are available, including bioreactors in which agitation of the medium is achieved using a paddle or impeller and rotary wall bioreactors. Rotary wall bioreactors can additionally be used to simulate conditions of reduced gravity (microgravity). It is also desirable to monitor and/or control the shear forces experienced by cells in operation of the methods of the present invention. For example, optimal conditions in cultures subjected to agitation require balancing the requirement for even distribution of oxygen and nutrients throughout the culture against the need to avoid cell damage due to excessive shear forces.

[0084] Accordingly, the midbrain assembloids may be embedded into an extracellular matrix, as defined elsewhere herein, allowing three-dimensional cell culture, defined elsewhere herein. Said matrix may be e.g. a droplet of Matrigel. Matrigel droplets are described, for example, in Lancaster and Knoblich (2014a).

[0085] As disclosed herein, the midbrain assembloid of the invention comprise dopaminergic neurons and microglia. In addition to dopaminergic neurons and microglia, the midbrain assembloid according to the invention comprises the following cell types: a) radial glia, b) midbrain specific neural epithelial stem cells, c) neuronal progenitors, d) neuroblasts, e) young dopaminergic neurons, f) young cholinergic neurons, g) mature dopaminergic neurons, h) GABAergic neurons. Importantly, with the methods herein disclosed the inventors were able to generate a midbrain assembloid comprising both dopaminergic neurons and microglia. Presence of dopaminergic neurons in an organoid or in an assembloid according to the invention can be determined by screening for certain markers typical of said cell types, for example, young dopaminergic neurons are characterized by the expression of the markers TUJ1 and tyrosine hydroxylase (TH). Mature dopaminergic neurons are characterized by the expression of the markers MAP2 and TH. Methods of screening for cell markers are known to the skilled artisan and also described herein in the examples and materials and methods section of the present application. Preferably, the dopaminergic neurons according to the invention are characterized by the expression of markers selected from the group consisting of FOXA2, TH, CALB1, ADCYAP1, GIRK2, SLC6A.

[0086] As defined herein, the midbrain assembloid of the invention comprises microglia. Microglia is the largest population of immune cells in the brain, as defined elsewhere herein, and are tissue-resident macrophages and as such they act as the first and main form of active immune defense in the central nervous system (CNS). As such, the microglia represents the pool of immune cells of the brain and is characterized by an immune cell signature. Accordingly, the microglia of the present invention comprised in the midbrain assembloid is characterized by an immune cell signature, meaning that the microglia according to the invention expresses markers (i.e genes and their corresponding gene

products) that are typical of a cell of the immune system. Preferably, said markers are selected from the group consisting of IBA1, PU1, HLA-DMB, CSF1R, IL18, C1QC, TLR2, CLEC7A, MRC1, NAIP, ITGAM, CCL2, P2RX4 (see figure 1a and 2b). Methods of screening for such cell markers are known to the skilled artisan and also described herein in the examples and materials and methods. For example said markers can be identified via immunostaining as described examples 2 and 3 and materials and methods section.

[0087] Furthermore, the immune cell signature of the microglia according to the invention is characterized in that a higher amount of cytokines and chemokines is released in the medium of the midbrain assembloid when compared to a control (see figure 4b). In this regard, the level of secreted cytokines and chemokines may be increased by 10%, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 %,200 % or more when compared to a control as defined herein. Specifically, said control may be a midbrain organoid as defined elsewhere herein, which is not contacted with a co-culture medium as defined elsewhere herein. In particular, said control is an early or late midbrain organoid as defined elsewhere herein, comprising dopaminergic neurons and not comprising microglia and which is not contacted with the co-culture medium defined herein. The medium in which the control is cultured may be the organoid maturation medium as defined elsewhere herein, while the medium of the midbrain assembloid is the co-culture medium defined elsewhere herein. The release of cytokines in the medium may be measured using different techniques known to the skilled artisan, and are described herein in the materials and methods section. Also, specific kits available can be used, like the Human XL Cytokine Discovery Luminex® Performance Assay (RD Systems, #FCTSM18).

[0088] Additionally, the microglia of the present invention is also characterized by the expression of phagocytic cell markers. Microglia have phagocytic abilities as was also observed in 2D monoculture (Fig. 1c). Indeed, the microglia of the invention (comprised in midbrain assembloids of the invention) may express phagocytic gene markers suggesting the presence of a functional phagolysosomal pathway (Figure 3d). Preferably, said phagocytic gene markers are selected from the group consisting of HLA-A, HLA-B, HLA-C, TLR2, TLR4, ATG7, LAMP2, SQSTM1, and CYBB. Methods of screening for such cell markers are known to the skilled artisan and are also described herein in the examples. For example said markers can be identified via immunostaining or RNA sequencing as described in the examples and in the materials and methods section.

[0089] In accordance with the phagocytic activity of the microglia comprised in the midbrain assembloid of the invention – and hence the ability to remove dead cells –, the inventors observed that the midbrain assembloid also displayed a lower cell number compared to a control midbrain organoid as defined elsewhere herein, in accordance with the capacity of the microglia of removing dead cells. Accordingly, the midbrain assembloid of the invention

may display a lower cell number and a smaller size compared to a control midbrain organoid as defined elsewhere herein). The size of a midbrain assembloid of the invention may be decreased by at least about 10%, preferably by at least about 20%, more preferably of at least about 25%, or more when compared to a control as defined herein. Methods of determining and comparing cell numbers of the midbrain assembloid and a control are known to the skilled artisan and described elsewhere herein. For example, cell number might be determined by counting of the cell nuclei and count of dead cells (see for example figure 4e and example 6).

[0090] Additionally, pathway enrichment analysis showed that the presence of microglia in the midbrain assembloid is connected to a downregulation of the expression of genes involved in hypoxia and oxidative stress. Accordingly, the midbrain assembloid of the invention may show a lower expression of genes involved in hypoxia and oxidative stress when compared to a control midbrain organoid as defined elsewhere herein. Preferably, said genes involved in hypoxia and oxidative stress are selected from the group consisting of COX1, SOD1, GPX4, MT-ATP6, and GSTP1. Methods of screening for such cell markers are known to the skilled artisan and also described herein in the examples and materials and methods, additionally, kits to measure ROS (reactive oxygen species) and hypoxia are commercially available and known to the skilled person.

[0091] In terms of gene marker expression, the midbrain assembloid obtained by the methods of the present invention is further characterized by a) higher expression of immune response markers, b) lower expression of synaptic markers, and c) higher expression of action potential markers, when compared to a control as defined herein. Preferably, the immune response markers are selected from the group consisting of the genes HLA-A, HLA-B, STAT2, STAT3, MRC2; the synaptic markers are selected from the group consisting of the genes SYT1, SYP, ROBO1, DDC; the action potential markers are selected from the group consisting of the genes CASK, CACNA1a, CACNA1e, CACNA1B, HCN1, KCNC3, KCND3. In this context, by “higher expression” or “lower expression” of said gene markers refers to the fact that a particular gene is expressed at higher or lower levels (i.e., which are up- or down- regulated) when compared to a control as defined herein. The meaning of “gene (or marker) expression” is well known in the art, and it indicates the information from a gene is used in the synthesis of a functional gene product that enables it to produce end products, protein or non-coding RNA. Therefore, downregulation is the process by which a cell decreases the quantity of a cellular component, such as RNA or protein, in response to an external stimulus. The complementary process that involves increases of such components is called upregulation. In the context of the present invention the higher or lower expression of particular gene markers refers to the up or downregulation of said gene products when compared to a control as defined herein (i.e an early or late midbrain organoid comprising

dopaminergic neurons, not comprising microglia, and which is not contacted with the co-culture medium defined herein). The higher/lower expression of gene markers may refer to an expression which is increased or decreased by at least about 5%, 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 80%, 100% or more when compared to the expression of the same markers by a control as defined herein. Methods of measuring and comparing expression for such cell markers are known to the skilled artisan and are also described herein in the examples

[0092] Furthermore, the midbrain assembloid according to the present invention may show a different metabolism compared to a control as defined herein. Said metabolism may refer in particular to amino-acid metabolism and glucose metabolism. In particular, the midbrain assembloid according to the invention may release a significantly lower amount of certain amino-acids. Said amino acids are preferably selected from the group consisting of Leucine, Isoleucine, Valine, Phenylalanine and Tyrosine. Accordingly, a lower amount of these amino acids is released by the midbrain assembloid in the co-culture medium, when compared to the amount of the same amino acids released by a control as defined elsewhere herein in a culture medium, which may be organoid maturation medium as defined elsewhere herein. In particular, a lower amount of said amino acids is released in the co-culture medium, compared to the amount of the amino acids released in the organoid maturation medium of the control (See figure 7 (this is suppl figure 6 of manuscript) and Example 8). Interestingly, increased plasma levels of these amino acids have been associated with acute hypoxic exposure in rats (Muratsubaki and Yamaki, 2011). Other metabolites whose presence in the culture medium differs between the midbrain assembloid and controls according to the invention include glucose and pyruvic acid. Specifically, the midbrain assembloid according to the present invention may show increased glucose metabolism when compared to a control as defined herein. Said increased glucose metabolism is characterized by a higher glucose and pyruvic acid uptake from the co-culture medium as defined herein when compared to the uptake of glucose and pyruvic acid from the culture medium of a control as defined herein. Different methods of glucose uptake from cells differs throughout tissues depending on two factors; the metabolic needs of the tissue and availability of glucose. The skilled artisan is familiar with these cellular pathways. The release or uptake of metabolites such as amino-acids, glucose and pyruvic acid in a cell culture medium may be measured using different techniques known to the skilled artisan, and are described herein in the examples and materials and methods section. The higher uptake of glucose and pyruvic acid may refer to an uptake which is increased by at least about 5%, preferably by at least about 10% or more when compared to the uptake of the same metabolite by a control as defined herein. Methods of measuring and comparing uptake of such metabolites are known to the skilled artisan and also described herein in the examples and materials and methods.

[0093] Additionally, the midbrain assembloid according to the present invention is also characterized by an increased neuronal excitability when compared to a control as defined herein. By “lower increased neuronal excitability” is meant that the neurons in the assembloids are characterized by a voltage threshold for the action potential generation which is more negative compared to that of the neurons of a control as defined elsewhere herein. This is an indicator of increased neuronal excitability in mature neurons, and it means that neurons (i.e all the different types of neurons comprised in the midbrain assembloid according to the invention as defined elsewhere herein) in assembloids develop fully mature electrophysiological properties with a lower threshold for action potential generation than in midbrain organoids (Figure 6). Method of measuring action potential are known to the skilled artisan and described herein in the materials and methods section.

[0094] The inventors therefore were able to generate a midbrain assembloid having the characteristics as disclosed herein, obtainable by the methods disclosed herein. Furthermore, said midbrain assembloid can be used for testing the ability of certain compounds of interest to elicit a cellular response. Accordingly, the invention also envisages testing compounds for their ability to elicit a cellular response on said midbrain assembloid. Said compound may be a drug, small molecule, hormone, growth factor, binding protein, nucleic acid molecule, peptide protein, a virus, or (co-cultured) cell. Accordingly, the method for testing a compound of interest for its ability to elicit a cellular response may comprise: (a) contacting the midbrain assembloid as disclosed herein with said compound of interest; and (b) determining whether said compound of interest elicits a cellular response. A cellular response may be for example the frequency or survival of a certain type of cell, for example a dopaminergic neuron. Preferably, said cellular response is promotion or inhibition of a neuro-degenerative disease. In this context, the compound of interest may be tested for its ability to promote (or inhibit) reactive microgliosis, neuro-inflammation or other conditions involved in the development of neuro-degenerative diseases. Said neurodegenerative disease may be for example Parkinson’s disease. Therefore, the methods for testing a compound of interest as described herein may be used to study neuro-inflammation related pathways and, to study new therapeutic targets for compounds that focus on the immune system in the brain. Other diseases that might be investigated using the methods herein disclosed may also include Schizophrenia and Depression.

EXAMPLES OF THE INVENTION

[0095] Example 1 – hiPSC-derived microglia express specific markers and are functional.

[0096] The work presented here is based on the use of quality controlled hiPSC lines (lines: K7, 163, EPI) (Table 1) from three different healthy individuals that express the pluripotency

markers SSEA-4, OCT-4, TRA-1-60, NANOG, TRA-1-81 and SOX2. The Inventors started by deriving macrophage precursors from hiPSCs that were then differentiated into microglia for 10 days, as previously described (Haenseler et al., 2017a). hiPSC-derived microglia in monoculture expressed macrophage-specific markers, including IBA1, PU1 and CD45, and microglia markers, notably TMEM119 and P2RY12, as detected by immunostaining (Fig. 1a). Moreover, PCR confirmed that microglia also expressed the macrophage specific genes IBA1 and CD68, and the microglia-specific genes TMEM119 and P2RY12 (Fig. 1b). After confirming the microglial cell identity, the inventors next assessed their phagocytic capacity. Incubation of microglia with Zymosan particles showed the cells' ability in phagocytosing these particles, detectable within their cell bodies (Fig. 1c). LU500789

[0097] A major challenge in assembloid generation is the compatibility of cell culture conditions for different cell types. In order to assess the microglia compatibility with the organoid culture medium, the inventors first examined the toxicity of each midbrain organoid medium supplement on microglia survival. Macrophage precursors were cultured for 10 days with organoid maturation medium (containing BDNF, GDNF, TGFβ3, db cAMP, DAPT and Activin A), microglia differentiation medium (containing IL-34 and GM-CSF), or microglia medium individually supplemented with each of the neurotrophic factors contained in the organoid medium. After 10 days of exposure to the different media, an MTT viability assay showed a significant decrease in cell viability in the presence of TGFβ3 (69.18% ± 8.371, $p=0.0024$), db cAMP (47.10% ± 8.371, $p<0.0001$) and Activin A (76.01% ± 8.371, $p=0.0292$) compared to microglia medium (Fig. 1d & e). After only 7 days of exposure, an impairment of differentiation and a lower viability were already observed visually in the cells cultured with organoid medium as well as the microglia medium supplemented with TGFβ3 or db cAMP (Fig. 1e). These results indicate that the midbrain organoid medium, containing TGFβ3, db cAMP and Activin A might not be suitable for the cultivation of microglia containing midbrain organoids.

[0098] Example 2 – The co-culture medium allows microglial survival and neuronal differentiation in midbrain organoids

[0099] After testing the organoid medium on microglia, the inventors next assessed the effects of the different media compositions on the neuronal population of the midbrain organoids. Taking into consideration the cytotoxic effect on 2D monoculture microglia, the inventors combined the microglia differentiation medium with the least microglia-toxic neurotrophic factors from the organoid maturation medium. Hence, the inventors supplemented the microglia differentiation medium with BDNF, GDNF, DAPT and Activin A – since they play an important role in neuronal maturation - and refer to this medium combination as the "co-culture medium". In order to study the effects of the co-culture medium on the organoids, the inventors co-cultured organoids with microglia from the three

different cell lines with midbrain organoid medium, microglia differentiation medium or co-culture medium for 20 days (Fig. 1f). As expected, the IBA1 positive microglial population was significantly reduced when organoid medium was used, whereas no difference was observed when cultured with microglia or co-culture medium (Fig. 1g). The major neuronal cell type in midbrain organoids are TH positive dopaminergic neurons. Interestingly, the inventors observed a significant decrease in TH positive neurons in both midbrain organoids and assembloids in the presence of microglia differentiation medium (Fig. 1h).

[00100] The reduction of TH positive cells indicates an impairment of dopaminergic neuron differentiation under microglia medium culture conditions. Notably, in the presence of co-culture medium, the levels of TH in both midbrain organoids and assembloids remained at similar levels as for the organoids cultured with midbrain organoid maturation medium (Fig. 1h). In summary, the inventors here developed a unique co-culture medium that is optimal for both microglial survival and dopaminergic neuron differentiation in human midbrain organoids.

[00101] Example 3 – Assembloids contain IBA1 positive functional microglia and express midbrain specific neuronal markers

[00102] After successfully optimizing the co-culture conditions, midbrain organoids were generated using a pure ventral midbrain-patterned NESC population from the hiPSC line K7 (Fig. 2a) (Nickels et al., 2020; Smits et al., 2019). After 2 days of maintenance, and 15 days of dopaminergic neuron differentiation induction, the organoids were co-cultured with macrophage precursors derived from the hiPSC lines K7, 163 and EPI. Thus, midbrain organoids from one individual will separately contain microglia from three different individuals. During the whole study, the inventors did not observe any major line specific differences. During the co- culture, the macrophage precursors first aggregated, forming smaller round colonies that attached to the surface of the organoid and then incorporated within that same structure After 20 days of co-culture, the inventors validated the incorporation of microglia into the organoids by IBA1 immunostaining. the inventors then assessed the percentage of IBA1 positive cells in assembloids using a computer-assisted image analysis pipeline for marker identification and quantification. On average, 6.4% of the assembloid cells were IBA1 positive (Fig 2b). The morphology of the incorporated microglial cells varied between round and partially ramified. the inventors confirmed the presence of a neuronal population by a Beta-tubulin III (TUJ1) staining, and assessed further neuronal differentiation by MAP2 staining. In addition, FOXA2 positive midbrain specific dopaminergic neuron precursors and TH positive differentiated dopaminergic neurons were observed in assembloids (Fig. 2c) After culturing the assembloids for 70 days, the inventors further confirm the presence of a GFAP positive astrocytic population in the assembloids. Moreover, the system was compatible with the embedding into the extracellular matrix Geltrex™. the

inventors embedded the assembloids after 5 days of co-culture, and kept them until day 20. the inventors observed uniform distribution of the microglia into the assembloids' core as well as into the extracellular matrix containing the neurite projections.

[00103] Example 4 – Single nuclear RNA-sequencing reveals eight different cell populations within midbrain-microglia assembloids.

[00104] In order to further characterize the microglia within assembloids the inventors performed single nuclear RNA-sequencing (snRNA-Seq). Separation of clusters in cells coming from midbrain organoids and assembloids clearly showed that the microglia cluster is specific for assembloids and not present in midbrain organoids (Fig. 2d). Cell types were identified based on marker gene expression (Table 5). Quality controlled clustering of single cells showed eight different cell types: radial glia (RGL), midbrain specific neural epithelial stem cells (midNESC), neuronal progenitors (PROG), neuroblasts (NB), young dopaminergic and cholinergic neurons yDN&CN, mature dopaminergic neurons, GABAergic and glutamatergic neurons (mDN(A10)&GABA&GLUT), mature dopaminergic neurons and serotonergic neurons (mDN(A9)&SN) as well as microglia (MGL). Proportions of the different clusters are represented in a pie chart (Fig.2e). midNESC were the most prominent cell type by representing 35% of total cells. More than 60% of the organoid and assembloid cells showed neuronal identity (corresponding to the groups NB, yDN&CN, mDN(A10)&GlN&Gan, mDN(A9)&SN). Less than 5% were represented by glial cells (astrocytes, oligodendrocytes and microglia) at this stage of differentiation. Progenitors were closest to neuroblasts and young neurons, whereas radial glial cells were very similar to midNESCs. The different mature neuronal groups clustered together. Clustering of the marker genes showed the relation between different cell clusters (Fig. 2f). As expected, MGL cells clustered apart from all other cell types. A distinct genetic signature of the microglia cells was further validated by a Spearman correlation analysis (Fig. 2g).

[00105] Example 5 – Microglia in assembloids show a typical immune cell signature

[00106] Next, the inventors aimed at identifying gene signatures unique to assembloids' microglia in comparison to the other cell types. Firstly, the inventors used the expression of the 100 most variable genes obtained from the snRNA-Seq analysis to cluster the different cell populations observed in the assembloids(Fig. 3a). Indeed most variability between cells clusters came from the microglia, which had a completely different genetic signature (Fig. 3a). Then, the inventors identified the top 100 markers defining microglia identity (Fig. 3b). From those, several canonical marker genes were chosen in order to characterize the main functions of microglia (Fig. 3c). General microglial marker genes such as *IBA1* and *PU1* were significantly and specifically expressed in microglia. In addition, the microglia core signature revealed genes involved in antigen presentation (*HLA-DMB*), cytokine and complement signaling (*CSF1R, IL18, C1QC*) as well as pathogen and self-recognition such as toll-like

receptor signaling (*TLR2*), C-type lectins (*CLEC7A*) and mannose and nod-like receptors (*MRC1, NAIP*). Moreover, genes involved in microglial adhesion (*ITGAM*) and motility through chemokine signaling (*CCL2*), as well as purinergic signaling (*P2RX4*) were significantly expressed in microglia. Furthermore, several chemokines and cytokines were expressed predominantly in microglia (Fig. 3d). LU500789

[00107] In order to address microglia functionality, the inventors then measured chemokine and cytokine release in midbrain organoids and assembloids. For that, medium from midbrain organoids and assembloid media was used. The medium collected was in contact with the organoids or assembloids (20 days after the microglia addition) for 3 days. As expected, in a hierarchical clustering analysis, midbrain organoids clustered in a different group than the microglia containing assembloids (Fig. 4a). For a total of 18 analyzed cytokines (IL-7, IL-12p17, IL-3, TNFα, IL-1α, IL-1ß, IL-6, IFNα, IL-10 and IFNγ) and chemokines (CXCL8, CCL2, CXCL2, CCL3, CXCL1, CCL4, CX3CL1 and MIP-3ß) the inventors observed a significant increase in the medium from assembloids compared to midbrain organoids, suggesting that immune functionality was acquired through the microglia incorporation (Fig 4b). Interestingly, although the RNA expression levels for IL-6, IL-7 and CX3CL1 were higher in non-microglia cells (Fig. 4c), the levels of released cytokines were higher in the assembloids medium (Fig. 4b). Hence, potentially the released cytokines are not necessarily produced in the microglia, but their secretion is stimulated by the presence of microglia.

[00108] Example 6 – Microglia in assembloids are functional and improve overall cell survival

[00109] As the inventors previously demonstrated, microglia have the ability to phagocytose Zymosan particles in 2D monoculture (Fig. 1c). Therefore, the inventors assessed if microglia in assembloids express genes involved in phagocytosis. Indeed, in assembloids the inventors observed a higher expression of phagocytic genes suggesting the presence of a functional phagolysosomal pathway (Fig. 3d). Genes included antigen recognition (*HLA-A,B,C*), receptor (*TLR2,4*) and internal signaling (*ATG7*), as well as autophagic vesicle formation (*LAMP2*, *SQSTM1* (*p62*)) and lysosomal degradation/oxidation (*CYBB*). These results indicate these microglia should have phagocytic capacity and hence the ability to remove dead cells. Interestingly and in agreement with this hypothesis, when the inventors measured the organoid size throughout the culture period the inventors observed a decrease of the assembloid size over time (Fig. 4d). Using our computer-assisted image analysis pipeline for cell type segmentation, the inventors quantified the DNA dye Hoechst signal. Midbrain organoids and assembloids were stained using Hoechst, and levels of total cells, live cells and necrotic/late apoptotic cells were evaluated. the inventors observed that the total amount of cells was lower in assembloids compared to midbrain organoids, correlating

with the size measurements. Particularly, the amount of dead cells was significantly lower in the assembloids (Fig. 4e), suggesting that microglia eliminate dead cells in the assembloids. LU500789

[00110] Example 7 – Microglia decrease oxidative stress and trigger immune responses in assembloids

[00111] After establishing the successful integration of microglia into midbrain organoids and showing important aspects of microglia functionality, the inventors investigated the potential influence of microglia on the neural cells in midbrain organoids. the inventors performed differential gene expression analysis over the neural cells in assembloids and midbrain organoids, which revealed 423 significantly different genes ($p<0.05$). The top 100 differentially expressed genes (DEGs) across all cells are represented in a heat-map and clustered by cell type. the inventors assigned DEGs to each cell type and represented their overlap in Venn diagrams ($p<0.05$). The left Venn diagram shows an overlap of all three neuronal clusters and the right Venn diagram overlaps NB, RGL, PROG and midNESCs. Most DEG were cell type specific, although some cell types shared DEGs, which was especially the case between both mature neuronal clusters.

[00112] Next, a pathway enrichment analysis was performed MetaCore using the DEG across all cell types in midbrain organoids and assembloids (Fig. 5a). The analysis revealed 12 significantly enriched biological pathways. Besides ribosomal and cytoskeletal genes, genes for oxidative stress, the immune response as well as neurogenesis and axonal guidance were significantly different. Interestingly, the inventors found 10 enriched genes involved in hypoxia and oxidative stress. Among those, six genes showed a clear downregulation of this pathway in the presence of microglial cells; the expression of mitochondrial cytochrome oxidase 1 (complex IV) (COX1), peroxiredoxin1 (PRDX1), superoxide dismutase 1 (SOD1), glutathione peroxidase 4 (GPX4), ATPase (complex V) (MT-ATP6), as well as glutathione S-transferase 1 (GSTP1) was significantly lower in assembloids (Fig. 5b).

[00113] Next, the inventors assessed the genes involved in the immune response and in the antigen presentation processes (Fig. 5c). Indeed, non-microglial cells expressed genes from the MHCII such as HLA- *C* and *B*. Moreover, the *STAT2* and *STAT3* genes were upregulated in the presence of microglia. The MRC2 (ENDO180) receptor involved in collagen internalization and remodeling was upregulated, while genes involved in cytokine mediated phagocytosis (*ELMO1*) and autophagy (MAP1LC3A (LC3), SQSTM1 (p62)) were downregulated (Fig. 5c). An enrichment analysis of the DEGs for each cell cluster showed differences in genes involved in immune response, inflammation, phagocytosis and response to hypoxia and oxidative stress, among others.

[00114] Microglia are important to main brain homeostasis. However, neuro-inflammation might occur when this homeostasis is compromised. Therefore, the inventors assessed the

expression of genes involved in pyroptosis – inflammation related cell death through inflammasome activation – including CASP1, NLRP3, PYCARD (data not shown) PPIA. Neuro-inflammation related genes were unchanged or decreased in assembloids, suggesting that presence of microglia in organoids does not lead to neuro-inflammation in physiological conditions. LU500789

[00115] Example 8 – Microglia mediate synaptic remodeling in assembloids

[00116] Enrichment analysis of the DEGs for each cell cluster showed differences in genes involved in synaptic vesicle exocytosis, synaptic contact, synaptogenesis and axonal guidance in assembloids (Fig. 5d). To further investigate the microglia effects on synaptic pathways, the inventors performed an extensive analysis of genes involved in synaptic processes. In assembloids, general synaptic markers such as *Synaptotagmin* (*SYT1*) and *Synaptophysin* (*SYP*), as well as the dopaminergic neuron circuit formation genes *ROBO1* and *DCC* were significantly downregulated across cell types (Fig. 6a). Other important genes involved in synaptic vesicle exocytosis – such as *VMAT2* and *SNAP25* - were differentially expressed. Moreover, the inventors assessed axonal guidance and growth molecules, such as semaphorins (*SEMA3C*, *DPYSL2*), plexins, ephrins (*EPHA5*), neuropilins, neurofilaments and actin cytoskeleton (*NEFM, ACTB*). These genes were all differentially expressed, depending on the cell type, indicating that axonal remodeling is influenced by the presence of microglia. Furthermore, the inventors examined some cell specific genes in mature neurons involved in action potential (*CASK, CACNA1A, CACNA1E, CACNA1B*) and active zones (*HCN1, KCNC3, KCND3*) within synapses and observed that those genes are upregulated in assembloids (Fig. 6b). Together these results suggest a role of microglia in synaptic remodeling and maturation within midbrain organoids.

[00117] In order to investigate the functional impact of microglia in midbrain organoids the inventors performed electrophysiological measurements of passive and active membrane properties as well as firing behavior. the inventors performed patch-clamp experiments of visually identified neurons in the intact organoids from 20 to 35 days after microglia addition (Fig 6c). Neurons in midbrain organoids and assembloids exhibited similar resting membrane potentials and input resistances and reliably fired repetitive action potentials in response to somatic current injections (n midbrain organoids =14; n assembloids = 13 cells. Depolarizing steps in voltage-clamp configuration triggered strong inward currents in all tested neurons, indicative of fast voltage-activated sodium currents. The amplitude of these currents was not different between both groups. Firing characteristics and action potential waveforms (Fig. 6d) varied considerably, which was expected from neurons at different degrees of maturation. Importantly, the voltage threshold for the action potential generation was more negative in the group of assembloid neurons (-39.86 (midbrain organoids) against -36.35 (assembloids) ± 3.281, p=0.0480, Fig 6d), which is a common and strong indicator of increased neuronal

excitability in mature neurons. In sum, neurons in assembloids develop fully mature electrophysiological properties with a lower threshold for action potential generation than in midbrain organoids. LU500789

[00118] In order to support these findings and investigate further the differences between midbrain organoids and assembloids, the inventors performed a non-polar exo-metabolomic analysis from culture supernatants 20 days after microglia addition. A total number of 14 metabolites were significantly different. Among them, the inventors observed a higher uptake of glucose and pyruvic acid from assembloids (Fig. 7a). Regarding amino-acid metabolism, the inventors observed a lower secretion of phenylalanine, tyrosine, methionine, lysisne, putrescine, threonine, leucine, isoleucine and valine by the assembloids compared to midbrain organoids. Furthermore, the levels of uptaken asparagine and serine from the medium were higher in assembloids. The secretion of glutamine was higher assembloids (Fig. 7b).

MATERIALS AND METHODS

[00119] Table 1

Name	Simplified identifier	Identifier	Patient	Gender	Age of sampling	Source
K7	200	2.0.0.10.1.0	K7.1 WT/C4 WT	Female	81	Reinhardt et al., 2013
EPI	201	2.0.0.15.0.0	A13777	Female	Cord Blood	GIBCO/A13777
163	304	2.0.0.79.0.0	163	Male	66	SYSMED

[00120] Table 1 related to experimental procedures. iPS cell lines used in this study. Macrophage precursors were derived from iPSCs as described in the Experimental procedures section. Human neural precursors were derived from human iPSCs from the simplified identifier 200. Human midbrain-specific organoids were generated with neural precursors as described in the Experimental procedures section.

[00121] Table2

Antibody	Species	Source	Ref.-No.	Dilution
IBA1	Goat	Abcam	ab5076	1:250

PU1	Rabbit	Cell signalling	2258S	1:250
CD11b	Rat	Abcam	ab8878	1:1000
CD45	Mouse	Biolegend	304002	1:1000
TMEM119	Rabbit	Sigma	HPA051870	1:250
P2RY12	Rabbit	Sigma	HPA014518	1:250
FOXA2	Mouse	Santa Cruz	sc-101060	1:250
LMX1A	Rabbit	Abcam	ab139726	1:100
PAX6	Rabbit	Biolegend	901301	1:300
SOX2	Goat	R&D systems	AF2018	1:100
SOX2	Rabbit	Abcam	ab97959	1:100
Nestin	Mouse	Millipore	MAB5326	1:100
Sox1	Goat	R&D systems	AF3369	1:100
TH	Rabbit	Abcam	ab112	1:1000
TUJ1	Chicken	Millipore	AB9354	1:1000
MAP2	Chicken	Abcam	ab5392	1:1000
MAP2	Mouse	Millipore	MAB3418	1:200
GFAP	Chicken	Millipore	AB5541	1:1000
SSEA-4	Mouse	Millipore	MAB4304	1:50
Oct-4	Rabbit	Abcam	ab19857	1:400
TRA-1-60	Mouse	Millipore	MAB4360	1:50
Nanog	Rabbit	Millipore	AB5731	1:200
TRA-1-81	Mouse	Millipore	MAB4381	1:50

[00122] Table 2 related to experimental procedures. Antibodies used in this study.

[00123] Table 3

Primer	**Sequence (5’ to 3’)**	**Region (Purpos**	**SEQ ID NO:**

		e)	
h-RPL37A-F	GTGGTTCCTGCATGAAGACAGTG	RT-PCR	1
h-RPL37A-R	TTCTGATGGCGGACTTTACCG	RT-PCR	2
2241_AIF1_F	AGACGTTCAGCTACCCTGACTT	RT-PCR	3
2242_AIF1_R	GGCCTGTTGGCTTTTCCTTTTCTC	RT-PCR	4
2243_CD68_F	CTTCTCTCATTCCCCTATGGACA	RT-PCR	5
2244_CD68_R	GAAGGACACATTGTACTCCACC	RT-PCR	6
2289_P2RY12_F	AAGAGCACTCAAGACTTTAC	RT-PCR	7
2290_P2RY12_R	GGGTTTGAATGTATCCAGTAAG	RT-PCR	8
2291_TMEM119_F	AGTCCTGTACGCCAAGGAAC	RT-PCR	9
2292_TMEM119_R	GCAGCAACAGAAGGATGAGG	RT-PCR	10

Table 3 related to experimental procedures. Primers used in this study.

[00124] Table 4

Name	Identifier
Cell 1	MglK7_CATCAGAAGGCCATAG.1
Cell 2	MglK7_AGGGATGGTGTCCTCT.1
Cell 3	MglK7_CTAAGACGTCTAGCGC.1
Cell 4	MglK7_CCGTGGACATCCTTGC.1
Cell 5	MglK7_AGTTGGTCAGCCTTTC.1
Cell 6	Mgl163_ACGCCAGTCATTGCCC.1
Cell 7	Mgl163_CATATTCTCCAATGGT.1
Cell 8	MglK7_CGCTGGAGTCCAGTTA.1
Cell 9	MglK7_AAGCCGCAGCTTCGCG.1
Cell 10	MglK7_CTCCTAGTCCTGCTTG.1
Cell 11	MglK7_CAAGGCCGTAGGCATG.1
Cell 12	MglK7_ACAGCCGCAGCTGCAC.1
Cell 13	MglK7_CAACCAACAGGAATGC.1
Cell 14	MglK7_ACGGGCTGTACAAGTA.1
Cell 15	MglK7_GTCTTCGCAATCCGAT.1
Cell 16	Mgl163_GCACATACATCGGACC.1
Cell 17	Mgl163_AGATCTGCAAACGTGG.1
Cell 18	Mgl163_ACGTCAAAGTTTAGGA.1

Cell 19	MglK7_GGATGTTGTCGCCATG.1
Cell 20	MglK7_TGTTCCGCAAGAGGCT.1
Cell 21	MglK7_GACTACAAGTAGGCCA.1
Cell 22	MglK7_TTAGGACAGGTTACCT.1
Cell 23	MglK7_GCTGGGTAGCGATATA.1
Cell 24	MglK7_GCTTGAAAGATACACA.1
Cell 25	MglK7_AAAGTAGGTGACTACT.1
Cell 26	MglK7_TATCTCACAGGTTTCA.1

[00125] Table 4 related to results. Cell identifiers for the top 100 microglia marker genes.

[00126] Table 5

Stem cells	DA	VTA	Maturity	CN	GAN	GLN	SN
SOX2 PAX6 HES5 ASCL1 SOX1 PAX3 DACH 1 LMO3 NR2F1 PLAGL 1 LIX1 HOXA 2 FOXA2 SLC1A 3 MSI1 VIM NES SHH	NR4A2 PBX1 GRIA3 TH EN1 TMCC3 NTM DDC CAMK2N 1 ALDH1A 1 APP PDZRN4 PCDH10 ERBB4 SLC10A 4 BEX5 NPY1R GPC2 HTRA2 HTRA3 HTRA4 PTX3 OTX2 EN2 SOX6 FOXA2 LMX1A LMX1B KCNJ6 CALB1 SLC6A3	ALDH1A 1 TRHR CD24 SLC18A 2 FGF1 NRIP3 MPP6 NTS CCK SOX6 GRIN2C SNCG IGF1 ADCYAP 1 GRP LPL CALB1 SLC32A 1 VIP TACR3 DCC OTX2 SATB1	SPRYD7 TUBB2A SKP1 GNAI1 MRAS ATP1A3 MAGED1 ARL2 MCFD2 MORF4L 2 NRSN2 NAP1L3 NGRN OLFM1 DKK3 CCDC13 6 COX6C HSP90AB 1 CALM2 ATP1B1 UQCRB COX4I1 PSMB5 FXYD7 RTN1 SEC62 COX7C CNTN1 FAIM2 SLC48A1 RAB3B	CHAT SLC18A 3 ACHE	GAD1 GAD2 GABARAP GABARAP L1 GABARAP L2 ABAT	SLC1A1 SLC1A2 SLC1A3 SLC17A 6 SLC17A 7 GLS GLS2 GRIN1 GRIN2A GRIN2B GRIN2C GRIN2D GRIN3A GRIN3B GRINA GRIA1 GRIA2 GRIA3 GRIA4 HTRA1	SLC6A4 SLC18A2 TPH1 TPH2 FEV HTR1D HTR1E HTR1F HTR2A HTR2A−A S1 HTR2B HTR2C HTR3A HTR3B HTR3D HTR4 HTR5A HTR5A−A S1

	SHH NKX6−1		NPTXR PDGFA NDUFC2 BEX2 UCHL1				

[00127] Table 5 related to results. Genes used for defining a cell cluster. DA = Dopaminergic; VTA = ventral tegmental area; CN = Cholinergic; GAN = Gabaergic; GLN = Glutamatergic; SN = Serotonergic.

[00128] STAR Methods

[00129] 2D cell culture

[00130] Generation of iPSCs was performed as described in (Reinhardt et al., 2013). iPSCs (Table 1) where cultured in Matrigel® (Corning, 354277) coated 6-well plates (Thermo Scientific, 140675), using Essential 8 Basal medium (Thermo Scientific, A1517001) supplemented with ROCK Inhibitor (Y-27632, Millipore, SCM075) for the first 24 hours after plating. The medium was exchanged on a daily basis. Confluence iPSCs (~70-90%) slipt using Accutase® (Sigma, A6964) and plated at around 300,000 cells per well. Neural progenitor cells needed to generated organoids were derived from iPSCs and maintained in culture as described previously by us (Nickels et al., 2020; Smits et al., 2019). iPSCs were also used to generate Macrophage precursors(van Wilgenburg et al., 2013) and further differentiate them into microglia as described previously(Haenseler et al., 2017b).

[00131] 3D cell culture

[00132] Midbrain organoid generation and culture

[00133] Midbrain organoids generation is described in (Nickels et al., 2020; Smits et al., 2019). Shortly, 6,000 cells per well were seeded in an Ultra-Low Attachment 96-well Well plate (Merck, CLS3474) and kept under maintenance conditions (N2B27 medium supplemented with 0.2 mM Ascorbic acid, 3 µM CHIR 99021, 0.5 µM Smoothened Agonist, SAG, 2.5 µM SB-431542, 0.1 µM LDN-193189) for 2 days. After that, the inventors started the pre-patterning (day 0 of dopaminergic differentiation) by removing SB and LDN from the medium. Two days after, CHIR concentration was reduced to 0.7 µM. On day 6 of dopaminergic differentiation, the medium was changed into maturation medium (N2B27 plus 0.2 mM Ascorbic acid, 10ng/mL Brain Derived Neurotrophic Factor, BDNF (Peprotech, 450-02), 10 ng/mL Glial-Derived Neurotrophic Factor, GDNF (Peprotech, 450-10), 1pg/mL TGF-β3 (Peprotech, 100-36E), 10 µM DAPT (R&D Systems, 2634/10) and 2.5ng/mL Activin A (Thermo Scientific, PHC9564)). Organoids were kept under static culture conditions with media changes every third day until day 15 of dopaminergic differentiation.

[00134] Co-culture of midbrain organoids with macrophage precursors

[00135] At day 15 of dopaminergic differentiation, organoids medium was fully . After that, replaced by co-culture medium (Advanced DMEM/F12, 1x N2 supplement, 1x GlutaMAX™, 50 µM 2-mercaptoethanol, 100 U/mL Penicillin-Streptomycin, 10 ng/mL BDNF, 10 ng/mL GDNF, 10 µM DAPT and 2.5 ng/mL Activin A) containing 186,000 freshly harvested macrophage precursor cells per organoid. After, the plate was centrifuged at 100 xg for 3 minutes to promote the attachment of the cells to the surface of the organoids. Medium was changed every 2-3 days until 20 or 70 days of co-culture (35 and 85 days of dopaminergic differentiation, respectively).

[00136] For the embedding in an extracellular matrix, assembloids were transferred into a 30 µl of Geltrex™ (Invitrogen, A1413302) droplet five days after the addition of macrophage precursors. Geltrex droplets polymerization was done for 25 minutes at 37C. Afterwards, organoids were transfer to a 24-well plate, and cultured with co-culture medium in shaking conditions (80 rpm).

[00137] Phagocytosis assay

[00138] For immunofluorescence staining macrophage precursors were harvested and 30,000 cells per well were plated in 96-glass bottom well plates (IBL Baustoff, 220.230.042) and differentiated into microglia. At day 10 2x Zymosan A (S. cerevisiae) BioParticles™ (Thermo Fisher, Z23373) per cell were added (60,000 particles / well). Then, cells were incubated for 30 minutes at 37C, washed with 1x PBS and fixed with 4 % formaldehyde. For live imaging, 100,000 cells per well were seeded in 8-well Nunc™ Lab-Tek™ Chamber Slides (Thermo Fisher, 177402PK) and immediately imaged.

[00139] Viability assay (MTT)

[00140] 50K macrophage precursors were plated per well in a 96-well plate. After 10 days of microglia differentiation induction, 10ul of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, M2128) at 5 mg/ml) was added to each well. Cells were incubated for 4h. Then, the medium was aspirated and 100ul of DMSO were added to each well, pipetting vigorously in order to detach and disrupt the cells. Absorbance was measure at 570nm. Results were compared to midbrain organoid medium (MOm).

[00141] Immunofluorescence staining in 2D

[00142] Cells cultured in glass coverslips (150K cells/well) or 96-well imaging plates were fixed for 15 min with 4 % formaldehyde (Sigma, 100496) and washed 3x with PBS. Permeabilization was done by using 0.3% Triton X-100 in 1x PBS for 15 minutes. The cells were washed 3x with PBS and blocked the cells with 3% BSA (Carl Roth, 80764) in PBS at room temperature. , Cells were then incubated in a wet chamber, overnight (16h) at 4°C with the primary antibodies (diluted in 3% BSA + 0.3% Triton X-100 in 1xPBS). Cells were rinsed 3x with PBS and further incubated with secondary antibodies diluted in 3% BSA + 0.3%

Triton X-100 in 1x PBS for 1 hour at room temperature. After 3 more PBS washes, the plates were directly imaged and the coverslips were mounted in a glass slide with Fluoromount-G® (Southern Biotech, Cat. No. 0100-01). The antibodies used are listed in Table 2. LU500789

[00143] Immunofluorescence staining in 3D

[00144] Midbrain organoids and assembloids were fixed with 4 % Formaldehyde overnight at 4 °C and washed 3x with PBS for 15 min. Organoids were embedded in 4 % low-melting point agarose (Biozym, Cat. No. 840100) in PBS. 70 µm sections sections were obtained using a vibratome(Leica VT1000 S). The sections were blocked with 0.5 % Triton X-100, 0.1 % sodium azide, 0.1 % sodium citrate, 2 % BSA and 5 % donkey serum in PBS for 90 min at room temperature. Primary antibodies were diluted in 0.1 % Triton X-100, 0.1 % sodium azide, 0.1 % sodium citrate, 2 % BSA and 5 % donkey serum and were incubated for 48 h at 4 °C in a shaker. After incubation with the primary antibodies (Table 2), sections were washed 3x with PBS and subsequently incubated with secondary antibodies in 0.05 % Tween-20 in PBS for 2 h at RT and washed with 0.05 % Tween-20 in PBS. Sections were mounted in Fluoromount-G mounting medium on a glass slide.

[00145] Imaging

[00146] Qualitative images were acquired with a confocal laser-scanning microscope (Zeiss LSM 710). For quantitative image analysis, the Operetta CLS High-Content Analysis System (Perkin Elmer) was used to automatically acquire 25 planes per organoid section. For live imaging, the Cell Observer SD and the CSU-X1 Spinning Disc Unit (ZEISS) were used. Images were modified with the ZEN blue Software.

[00147] Image analysis

[00148] Immunofluorescence 3D images of midbrain organoids and assembloids were analyzed in Matlab (Version 2017b, Mathworks) following (Monzel et al., 2017; Smits et al., 2019). The in-house developed image analysis algorithms facilitate the segmentation of Nuclei, neurons and microglia, obtaining as a result the positive pixel surface for a selected marker. To estimate the Iba1 positive cell number, the inventors first used the *regionprops* Matlab function to obtain the Iba1 and nuclei area from more than 300 cells from 8 different assembloid sections. After obtaining an average number, the inventors divided the pixel mask per plane by the area number per cell. To obtain the percentage of Iba1 positive cells per assembloid, the Iba1 positive cell number was divided by the live nuclei number.

[00149] RT-PCR

[00150] Between one and three million microglia cells were used per RNA extraction. the inventors used the RNeasy Mini Kit (Qiagen) as well as DNase I Amplification Grade (Sigma-Aldrich) to isolate RNA. After conducting reverse395 transcription by following the protocol of the High Capacity RNA to DNA Kit (Thermo Fisher Scientific), RT–PCRs were performed

using GreenTaq polymerase and 50ng of cDNA per reaction. An initial denaturing step, 5 min at 95 °C, 40 cycles of denaturation for 30 s at 95 °C, annealing for 45 s at 55 °C (for Iba-1, CD68, and TMEM119) or 61 °C (for RPL37A and P2RY12), extension for 30 s at 72 °C and a final extension for 5 min at 72 °C. The used primers are listed in Table 3.

[00151] Cytokine and chemokine release assay

[00152] Cytokine and chemokine measurements were performed using the Human XL Cytokine Discovery Luminex® Performance Assay (RD Systems, #FCTSM18). the inventors collected supernatants from three midbrain organoid and assembloid batches and three biological replicates (microglia lines K7, 163 and EPI). When values were too low to be detected, they appeared as 'out of range'. In order to consider them statistically, they were assigned the lowest measured value of the standard curve for that metabolite. The statistics were run with three batches and three biological conditions (Midbrain organoids vs assembloids K7, 163 and EPI).

[00153] Metabolomics

[00154] For the extracellular metabolomics analysis, the inventors used snap frozen media from 20 days of co-culture old midbrain organoids or assembloids after 48h of culture. the inventors also incubated co-culture media, not in contact with organoids, as a control. From the measured results, the control (basal medium) was substracted in order to discriminate secreted (positive numbers) against uptaken (negative numbers) metabolites.

[00155] Electrophysiology

[00156] Passive and active electrophysiological properties of cells in assembloids (with microglia from line 163) and midbrain organoids were characterized by whole-cell patch-clamp recordings in voltage and current clamp.

[00157] Metabolomics

[00158] Polar metabolite extraction, derivatization, and GC-MS measurement

[00159] Extracellular metabolites from media samples were extracted using a methanolic extraction fluid (5:1, methanol/water mixture, v/v). The water fraction contained two internal standards Pentanedioic acid-D6 (c = 10 µg/mL; C/D/N Isotopes Inc.) and [UL-13C5]-Ribitol (c = 20 µg/mL; Omicron Biochemicals). 40 µL of medium was added to 240 µL ice-cold extraction fluid. After adding 100 µl ice-cold chloroform, the mixture was shaken for 5 min at 4 °C. For phase separation, 100 µl chloroform and 100 µl water were added and vortexed for 1 min. Then, the mixture was centrifuged at 21,000 xg for 5 min at 4 °C. 250 µL of the polar (upper) phase was transferred to GC glass vial with micro insert (5-250 µL) and evaporated to dry under vacuum at -4 °C.

[00160] Metabolite derivatization was performed by using a multi-purpose sample preparation robot (Gerstel). Dried medium extracts were dissolved in 30 µl pyridine, containing 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich), for 120 min at 45 °C

under shaking. After adding 30 µl N-methyl-N-trimethylsilyl-trifluoroacetamide (Macherey-Nagel), samples were incubated for 30 min at 45 °C under continuous shaking. LU500789

[00161] GC-MS analysis was performed by using an Agilent 7890A GC coupled to an Agilent 5975C inert XL Mass Selective Detector (Agilent Technologies). A sample volume of 1 µl was injected into a Split/Splitless inlet, operating in split mode (10:1) at 270 °C. The gas chromatograph was equipped with a 30 m (I.D. 0.25 mm, film 0.25 µm) DB-5ms capillary column (Agilent J&W GC Column) with 5 m guard column in front of the analytical column. Helium was used as carrier gas with a constant flow rate of 1.2 ml/min. The GC oven temperature was held at 90 °C for 1 min and increased to 220 °C at 10 °C/min. Then, the temperature was increased to 280 °C at 20 °C/min followed by 5 min post run time at 325 °C. The total run time was 22 min. The transfer line temperature was set to 280 °C. The MSD was operating under electron ionization at 70 eV. The MS source was held at 230 °C and the quadrupole at 150 °C. Mass spectra were acquired in full scan mode (m/z 70 to 700).

[00162] Data normalization and data processing

[00163] All GC-MS chromatograms were processed using MetaboliteDetector, v3.220190704 (REF). Compounds were annotated by retention time and mass spectrum using an in-house mass spectral library. The internal standards were added at the same concentration to every medium sample to correct for uncontrolled sample losses and analyte degradation during metabolite extraction. The data set was normalized by using the response ratio of the integrated peak area_analyte and the integrated peak area_internal standard. The results correspond to triplicates from three co-cultured batches and three biological replicates (midbrain organoids against assembloids with microglia from lines K7, 163 and EPI).

[00164] Electrophysiology

[00165] Each organoid was transferred from the incubator to a submerged type recording chamber with constant perfusion of carbogen-buffered artificial cerebrospinal fluid (ACSF) at 32°C. The ACSF contained (in mM): 124 NaCl, 3 KCl, 1.8 MgSO4, 1.6 CaCl2, 10 glucose, 1.25 NaH2PO4, 26 NaH2CO3 with an osmolarity of 295 mOsm/l. The organoid was fixated between a large diameter pipette and a custom made platinum harp. Cells were visualized using phase-contrast on an upright BX51 microscope (Olympus, Hamburg, Germany) with a 60x water-immersion objective. Recording electrodes were pulled using borosilicate glass on a Flaming/Brown P-97 Puller (Sutter Instrument, Novato, CA, USA) to yield a resistance of 3–6 MΩ. The electrode solution contained (in mM): 126 potassium gluconate, 4 KCl, 10 HEPES, 0.3 EGTA, 4 MgATP, 0.3 Na_2GTP, and 10 phosphocreatine adjusted to pH 7.2 using KOH and to 288 mOsm/l by adding sucrose. Recordings were obtained in voltage and current-clamp mode with an ELC-03XS amplifier (NPI electronic, Tamm, Germany). Signals were low-pass filtered at 3 kHz and digitized with 20 kHz using a Micros 1401MKII AC-

converter (CED, Cambridge, UK). Data were collected using the Signal 4.10 software (CED). LU500789
Voltages were not corrected for the calculated liquid-junction potential of +14.5 mV. Test pulses of -50 pA and 100 ms were applied regularly to control for changes in series resistance.

[00166] Putative neurons were visually identified by their size and shape. After obtaining the whole-cell configuration, the resting membrane potential was determined in current clamp and the cell subsequently stabilized at -70 mV by continuous current injection. To assess active and passive membrane properties, hyper- and depolarizing current steps were injected at increments of 10 pA and of 500 ms length starting from -50 pA. Passive parameters were assessed by the smallest negative current step that yielded a constant plateau potential, which varied due to the heterogeneity of input resistances. To measure maximum firing rates, cells were depolarized by voltage steps of increasing amplitudes until APs started to fail due to sodium channel inactivation (up to +500 pA depending on input resistance). Action potential waveforms characteristics were analyzed for the first action potential that fired 50 ms after the onset of current injections. The voltage threshold was defined as the potential at which the rising slope exceeded 5 mV/ms and amplitudes were measured from threshold to peak. In voltage-clamp mode, cells were held at -70 mV and positive voltage steps of 300 ms duration and 10 mV increments were applied every 5 s. Maximal voltage was +30 mV. The power of voltage-gated cation channels (predominantly sodium) was quantified at -30mV and used for statistical comparison. Data analysis was performed using Stimfit (Guzman, 2014) and custom-written Phyton routines. Values were tested for Gaussian distribution by D'Agostino-Pearson omnibus normality test. Unpaired t-tests were used to assess statistical significance in normally distributed data and Mann-Whitney tests for non-normally distributed data (indicated as p in Figures). Outliers deviating 2.5 standard deviations were excluded from statistical analysis but indicated in the Figures.

[00167] The presence of microglia in the recorded assembloids was confirmed using immunohistochemical staining and confocal imaging. Organoids were fixed in 4% formaldehyde and stained for nuclei (Fluoroshield with DAPI, Sigma-Aldrich), neuronal markers markers (MAP2, Sigma-Aldrich Chemie GmbH), and microglia (anti-Iba1, WAKO Chemicals). Imaging was carried out on an A1 Nikon confocal microscope at the Nikon Imaging Center Heidelberg, Germany.

[00168] Statistical analyses

[00169] First, Gaussian distribution was evaluated by performing D'Agostino & Pearson omnibus normality test. According to this distribution, either a 1way ANOVA or a Kruskal-Wallis test with a Dunnett's test for multiple comparisons were used to evaluate statistical significance. For the pooled results (organoids against assembloids), gaussian distribution was also tested. Depending on the outcome, an unpaired t-test or Mann-Whitney test was

used to assess the difference between groups. Outliers deviating 2.5 standard deviations were excluded from statistical analysis but indicated in the Figures. Cells that showed a resting membrane potential above -40 mV and action potentials shorter than 50 mV and wider than 3 ms in half-width were excluded from analysis, assuming that these cells were either not fully matured neurons or recording conditions were poor. The presence of microglia in the recorded organoids was confirmed using immunohistochemical staining and confocal imaging. For the image analysis, a 2way ANOVA, Tukey's multiple comparisons test was performed to evaluate statistical significance. Data is presented as mean ± SEM. All analyses were performed with three different biological replicates (assembloids microglia from three different cell lines). LU500789

[00170] Single-nuclei RNA sequencing (snRNAseq)

[00171] Five snap-frozen organoids or assembloids per condition were used to perform snRNAseq. Whole frozen organoids and assembloids (with microglia from lines K7, 163 and EPI) were dissociated for generating single-nuclei gene-expression libraries.

[00172] Data generation

[00173] Whole frozen organoids and assembloids (with microglia from lines K7, 163 and EPI) were dissociated for generating single-nuclei gene-expression libraries. The following steps were performed on ice using chilled and freshly prepared buffers. In brief, organoids and assembloids were gently dissociated using 500 mL of 0.1X Lysis Buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% Tween-20, 0.1% Nonidet P40 Substitute, 0.01% digitonin, 1% BSA, nuclease-free water) by pipetting 10X with a wide-pore 1000 µL pipette, followed by a 10 min incubation period on ice. This was repeated for 2 cycles. To reduce batch effects and increase the number of nuclei per experiment, material from 4 different organoids or assembloids were pooled for each group. Cells were filtered through a 40-µM strainer using 300 µL at a time, using a new 40-µM strainer. The same procedure was done with 20-µM (Sysmex, AN777717) and 10-µM (Sysmex, AP275603) strainers, followed by centrifugation for 5 min at 500 xg, at 4°C. Nuclei were then stained using Hoechst 33342 (1:2000) for 5 min at room temperature and counted on the Countess II.

[00174] Using Diluted Nuclei Buffer (10x Genomics, 2000153) each sample was adjusted to a concentration of 4000 nuclei/µL. We generated one library for each sample, aiming for 6000 nuclei. Single-nuclei experiments were performed using the 10x Genomics Next GEM Single Cell 5′ Library Kit v1.1 (1000168) to encapsulate nuclei and amplify cDNA, to generate sequencing libraries. Each library was barcoded using i7 barcodes provided by 10x Genomics. cDNA and sequencing library quality and quantity were determined using Agilent's High Sensitivity DNA Assay (5067-4626) and KAPA (KK4824). Final libraries were pooled, loaded on two lanes of the Illumina's HiSeq X and sequenced in 150PE mode.

[00175] Count matrix generation

[00176] Following steps are performed on default settings if not otherwise specified. Single-nucleus libraries were demultiplexed based on their i7 index sequences and for each library mapping to the human genome was performed using the Cell Ranger 4.0.0 software and human reference GRCh38 3.0.0, both provided by 10x Genomics. Next, count matrix files for each sample were generated using cellranger count. LU500789

[00177] Data pre-processing

[00178] Count matrixes for the assembloids with K7 and 163 microglia, as well as the midbrain organoid control were uploaded and Seurat Objects were created. Data was preprocessed as decribed by https://satijalab.org/seurat/v3.2/pbmc3k_tutorial.html. An independent data quality control was performed of all three objects, by checking levels of ribosomal genes, and by removing cells with a high mitochondrial gene fraction. Moreover, cells containing less than 100 or more than 5000 (100 < RNA n Feature < 5000) genes were removed from the analysis, being considered empty droplets or doublets, respectively. After quality assessment all three objects were combined, log normalized, scaled, and a linear dimensional reduction was performed (PCA). Dimensionality was assessed (20) and cells were clustered with a resolution of 0.5. A non-linear dimensional reduction using “umap” was performed, and cluster markers were assessed. Cluster names were defined by their characteristic marker gene expression.

[00179] Cell type identification

[00180] RGLs expressed *SLC1A3 (Glast*). MidNESCs expressed midbrain markers *SHH*, *LMX1A* and *FOXA2* and stem cell markers *SOX2* and *MSI1* (*Musashi*). PROG highly expressed *VIM (Vimentin*) and lower levels of stem cell markers. NB were positive for young neuronal markers *DCX* (*doublecortin*) and *STMN1* (*sthaminin*) without expressing mature neuronal markers. YDN&CN still expressed *DCX* and *STMN1* but also some mature neuronal markers for synapses such as *SYP (synaptophysin*) and subtype specification markers such as *TH* (dopaminergic neurons) and *SLC18A3* (cholinergic neurons). Mature neurons expressed low levels of *STMN1* and *DCX* but high levels of *SYP*. Subtype specification revealed that mDN(A10)&GaN&GlN expressed high levels of genes that define neurons from the ventral tegmental area (VTA). Besides, cells expressing dopaminergic marker *TH* also expressed the marker for A10 DN *CALB1*, as well as *ADCYAP1*. Moreover, the glutamatergic *SLC18A1* and GABAergic *GAD1* transporters were highly expressed in these neurons. Last mDN(A9)&SN were qualified by high amounts of *TH* and KCNJ6 (*Girk2)* as well as DN defining synaptic markers *ROBO1*. Moreover, also serotonergic transporters *SLC*18A2 were expressed in that cluster. Microglial cells expressed *IBA1.* Cell type proportions were calculated by counting the cells of each cluster. A spearman correlation and a heatmap clustering (pheatmap) were performed on the average expression of clusters defining genes. The top 100 genes defining the microglial cluster are shown in a heatmap.

Moreover, the average gene expression across all microglial cells was exported, and representative genes (Galatro et al. 2017). are presented in a violin plot. Significances were calculated based on a one sample Wilcoxon test p<0.05 *. The same was done for genes involved in the phagocytic pathway. Cyto and chemokines were represented in a heatmap. LU500789

[00181] Differential expressed gene and enrichment analysis

[00182] The most variable features were identified and a heatmap clustering (pheatmap) was performed on the average expression of the top 100 most variable genes. DEG were identified between assembloids and midbrain organoids. This was done for all the cell types together and each cell type independently. Data of the DEG lists is represented in heatmaps (top 100), or underwent metacore enrichment analysis. Metacore analysis was based on the following arbitrary threshold p<0.05, adj p<0.5. Moreover, Venn diagrams were formed using nVenn http://degradome.uniovi.es/cgi-bin/nVenn/nvenn.cgi. Genes involved in the enriched pathways are shown separately in box plots. Statistics were performed using a Wilcoxon test p<0.05*.

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* * *

[00236] Unless otherwise stated, the following terms used in this document, including the description and claims, have the definitions given below.

[00237] Those skilled in the art will recognize, or be able to ascertain, using not more than routine experimentation, many equivalents to the specific embodiments of the invention

described herein. Such equivalents are intended to be encompassed by the present invention.

[00238] It is to be noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a reagent" includes one or more of such different reagents and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[00239] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[00240] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

[00241] The term "about" or "approximately" as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. It includes, however, also the concrete number, e.g., about 20 includes 20.

[00242] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or “including” or sometimes when used herein with the term “having”.

[00243] When used herein “consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[00244] In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms.

[00245] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[00246] All publications cited throughout the text of this specification (including all patents, patent applications, scientific publications, manufacturer’s specifications, instructions, etc.) are hereby incorporated by reference in their entirety. Nothing herein is to be construed as

an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. LU500789

Claims

1. A method of generating a midbrain assembloid, comprising:
contacting a midbrain organoid, which is cultured in a three-dimensional cell culture comprising a matrix, with a co-culture medium comprising macrophage precursor cells, thereby obtaining a midbrain assembloid.

2. The method of claim 1, wherein the midbrain organoid is produced from neural epithelial stem cells (NESC).

3. The method of claim 1, wherein the midbrain organoid has been produced by:
i) contacting the neural epithelial stem cells with a maintenance medium;
ii) contacting the cells of step i) with patterning medium; and
iii) contacting the cells of step ii) with organoid maturation medium,
thereby obtaining the midbrain organoid.

4. The method of claim 3, wherein:
a) the cells are kept in maintenance medium for 1, 2, 3, 4 days, preferably for 2 days;
b) the cells are kept in patterning medium for 2, 4, 6, 8 days, preferably for 6 days;
c) the cells are kept in organoid maturation medium for 3, 6, 9, 12 days, preferably for 9 days.

5. The method of any one of the preceding claims, wherein the midbrain organoid is cultured in organoid maturation medium until day 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 of dopaminergic neuron differentiation before being contacted with the co-culture medium, preferably the midbrain organoid is cultured in organoid maturation medium until day 15 of dopaminergic neuron differentiation before being contacted with co-culture medium.

6. The method of claim 5, wherein the midbrain organoid is kept under static culturing conditions until day 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 of dopaminergic differentiation, preferably the midbrain organoid is kept under static culturing conditions until day 15 of dopaminergic differentiation.

7. The method of any one of the preceding claims, wherein the midbrain organoid is an early or late midbrain organoid.

8. The method of any one of the preceding claims, wherein the midbrain organoid comprises dopaminergic neurons.

9. The method of any one of the preceding claims, wherein the midbrain organoid does not comprise microglia.

10. The method of any one of the preceding claims, wherein the organoid maturation medium comprises TGF- β3 and cAMP.

11. The method of any one of the preceding claims, wherein the macrophage precursor cells have been produced from induced pluripotent stem cells (iPSC).

12. The method of any one of the preceding claims, wherein the co-culture medium comprises:
i) at least two different cytokines,
ii) at least two different neurotrophins,
iii) a notch pathway inhibitor,
iv) an activator of activin/transforming growth factor β (TGF- β) signaling pathway, and
v) macrophage precursor cells.

13. The method of claim 12, wherein the at least two cytokines are selected from the group consisting of IL-34, GM-CSF, IL-1, IL-2, IL-7, IL-10, IL-18, wherein preferably the at least two cytokines are IL-34 and GM-CSF.

14. The method of claim 12, wherein the at least two different neurotrophins are selected from the group consisting of BDNF, GDNF, NGF, NT-3, NT-4, CNTF, preferably the at least two neurotrophins are GDNF and BDNF.

15. The method of claim 12, wherein the notch pathway inhibitor is selected from the group consisting of DAPT, RO4929097, preferably the notch pathway inhibitor is DAPT.

16. The method of claim 12, wherein the activator of activin/transforming growth factor β (TGF- β) signaling pathway is selected from the group consisting of TGFβ1, TGFβ2, activin A, activin B, activin AB and nodal, preferably the activator of activin/TGF-β signaling pathway is activin A.

17. The method of claim 12, wherein the co-culture medium comprises:
i) IL-34,
ii) GM-CSF,
iii) BDNF,
iv) GDNF,
v) DAPT,
vi) activin A, and
vii) macrophage precursor cells.

18. The method of any one of claims 12-17, wherein the co-culture medium does not comprise TGF-β3 and cAMP.

19. The method of any one of the preceding claims, comprising:
i) contacting the midbrain organoid as defined in any one of claims 7-9 with the co-culture medium as defined in any one of claims 12-18; and
ii) culturing the midbrain organoid with the co-culture medium for 20 days, 40 days, 60 days or for 70 days, preferably culturing the midbrain organoid for 20 days wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain assembloid.

20. The method of claim 19, wherein the midbrain assembloid is embedded in an extracellular matrix five days after the addition of macrophage precursors.

21. The method of any one of the preceding claims, wherein the midbrain assembloid comprises dopaminergic neurons and microglia.

22. The method of claim 21, wherein the midbrain assembloid further comprises
a) radial glia,
b) midbrain specific neural epithelial stem cells,
c) neuronal progenitors,
d) neuroblasts,
e) young dopaminergic neurons,
f) young cholinergic neurons,
g) mature dopaminergic neurons,
h) GABAergic neurons,
i) glutamatergic neurons,

j) mature dopaminergic neurons, and/or
k) serotonergic neurons.

23. The method of claim 21, wherein the dopaminergic neurons are characterized by the expression of the markers selected from the group consisting of FOXA2 and TH, CALB1, ADCYAP1, GIRK2 and SLC6A3

24. The method of claim 21, wherein the microglia is characterized by an immune cell signature.

25. The method of claim 21, wherein the immune cell signature is characterized in that the microglia expresses the markers selected from the group consisting of IBA1, PU1, HLA-DMB, CSF1R, IL18, C1QC, TLR2, CLEC7A, MRC1, NAIP, ITGAM, CCl2, P2RX4.

26. The method of claim 25, wherein the immune cell signature is further characterized in that a higher amount of cytokines and chemokines is released in the medium of the midbrain assembloid when compared to a control.

27. The method of claim 26, wherein the control is a midbrain organoid as defined in any one of claims 7-9 which is not contacted with a co-culture medium as defined in any one of claims 12-18.

28. The method of claim 19, wherein the medium of the midbrain organoid is organoid maturation medium as defined in claim 10.

29. The method of claim 21, wherein the microglia expresses phagocytic gene markers.

30. The method of claim 29, wherein the phagocytic gene markers are selected from the group consisting of HLA-A, HLA-B, HLA-C, TLR2, TLR4, ATG7, LAMP2, SQSTM1, and CYBB.

31. The method of any one of claims 21-30, wherein the midbrain assembloid has a lower cell number compared to a control as defined in claim 27.

32. The method of claim 31, wherein the midbrain assembloid has a smaller size compared to a control as defined in claim 27.

33. The method of any one of claim 21-32, wherein the assembloid shows lower expression of genes involved in hypoxia and oxidative stress compared to a control as defined in claim 27.

34. The method of claim 33, wherein the genes involved in hypoxia and oxidative stress are selected from the group consisting of COX1, SOD1, GPX4, MT-ATP6, GSTP1.

35. The method of any one of claims 21-34, wherein higher amount of leucine, isoleucine, valine, phenylalanine and tyrosine is released in the medium of the midbrain assembloid when compared to a control as defined in claim 27.

36. The method of any one of claims 21-35, wherein the midbrain assembloid is further characterized by
a) higher expression of immune response markers,
b) lower expression of synaptic markers, and
c) higher expression of action potential markers,
when compared to a control as defined in claim 27.

37. The method of claim 36, wherein the immune response markers are selected from the group consisting of: HLA-A, HLA-B, STAT2, STAT3, MRC2; the synaptic markers are selected from the group consisting of SYT1, SYP, ROBO1, DDC; the action potential markers are selected from the group consisting of CASK, CACNA1a, CACNA1e, CACNA1B, HCN1, KCNC3, KCND3.

38. The method of claim 36 or37 wherein the midbrain assembloid shows increased glucose metabolism when compared to a control as defined in claim 27.

39. The method of claim 38, wherein the increased glucose metabolism is characterized by a higher glucose and pyruvic acid uptake from the co-culture medium as defined in any one of claims 12-18 when compared to a control as defined in claim 27.

40. The method of any one of claim 36-39, wherein the midbrain assembloid shows an increased neuronal excitability when compared to a control as defined in claim 27.

41. The method of claim 40, wherein the increased neuronal excitability is characterized by a lower threshold of action potential generation when compared to that of a control as defined in claim 27. LU500789

42. A midbrain assembloid obtainable by the method of claims 1-41.

43. The midbrain assembloid of claim 42, wherein said midbrain assembloid is characterized by the features referred to in any one of claims 21-42.

44. A method of testing a compound of interest for its ability to elicit a cellular response comprising
i) contacting the midbrain assembloid of claim 42 with said compound of interest; and
ii) determining whether said compound elicits a cellular response.

45. The method of claim 44, wherein said cellular response is promotion or inhibition of neuro-degenerative disease.

46. The method of claim 45, wherein said neurodegenerative disease is selected from the group consisting of Parkinson’s disease.

47. The method of claim 44, wherein said compound it’s a drug, small molecule, peptide, protein, virus.

Ansprüche

1. Verfahren zur Herstellung eines Mittelhirnassembloids, umfassend:
Inkontaktbringen eines Mittelhirnorganoids, das in einer dreidimensionalen Zellkultur, die eine Matrix umfasst, kultiviert wird, mit einem Co-Kulturmedium, das Makrophagenvorläuferzellen umfasst, wodurch ein Mittelhirnassembloid erhalten wird.

2. Verfahren nach Anspruch 1, wobei das Mittelhirnorganoid aus neuralen epithelialen Stammzellen (NESC) hergestellt wird.

3. Verfahren nach Anspruch 1, wobei das Mittelhirnorganoid hergestellt wurde durch:
i) Inkontaktbringen der neuralen epithelialen Stammzellen mit einem Erhaltungsmedium;
ii) Inkontaktbringen der Zellen aus Schritt i) mit einem Musterungsmedium; und
iii) Inkontaktbringen der Zellen aus Schritt ii) mit einem Organoid-Reifungsmedium, wodurch das Mittelhirnorganoid erhalten wird.

4. Verfahren nach Anspruch 3, wobei:
a) die Zellen für 1, 2, 3, 4 Tage, vorzugsweise für 2 Tage, in einem Erhaltungsmedium gehalten werden;
b) die Zellen für 2, 4, 6, 8 Tage, vorzugsweise für 6 Tage, in einem Musterungsmedium gehalten werden;
c) die Zellen für 3, 6, 9, 12 Tage, vorzugsweise 9 Tage, in einem Organoid-Reifungsmedium gehalten werden.

5. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Mittelhirnorganoid in Organoid-Reifungsmedium bis zum Tag 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 der dopaminergen Neuronendifferenzierung kultiviert wird, bevor es mit dem Co-Kulturmedium in Kontakt gebracht wird, vorzugsweise wird das Mittelhirnorganoid in Organoid-Reifungsmedium bis zum Tag 15 der dopaminergen Neuronendifferenzierung kultiviert, bevor es mit dem Co-Kulturmedium in Kontakt gebracht wird.

6. Verfahren nach Anspruch 5, wobei das Mittelhirnorganoid unter statischen Kulturbedingungen bis zum Tag 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 der dopaminergen Differenzierung gehalten wird, vorzugsweise wird das Mittelhirnorganoid unter

statischen Kulturbedingungen bis zum Tag 15 der dopaminergen Differenzierung gehalten.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Mittelhirnorganoid ein frühes oder spätes Mittelhirnorganoid ist.

8. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Mittelhirnorganoid dopaminerge Neuronen umfasst.

9. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Mittelhirnorganoid keine Mikroglia enthält.

10. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Organoid-Reifungsmedium TGF-β3 und cAMP enthält.

11. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Makrophagen-Vorläuferzellen aus induzierten pluripotenten Stammzellen (iPSC) hergestellt wurden.

12. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Co-Kulturmedium umfasst:
i) mindestens zwei verschiedene Zytokine,
ii) mindestens zwei verschiedene Neurotrophine,
iii) einen Inhibitor des Notch-Wegs
iv) einen Aktivator des Aktivin/Transforming Growth Factor β (TGF- β)-Signalwegs, und
v) Makrophagen-Vorläuferzellen.

13. Verfahren nach Anspruch 12, wobei die mindestens zwei Zytokine ausgewählt sind aus der Gruppe bestehend aus IL-34, GM-CSF, IL-1, IL-2, IL-7, IL-10, IL-18, wobei die mindestens zwei Zytokine vorzugsweise IL-34 und GM-CSF sind.

14. Verfahren nach Anspruch 12, wobei die mindestens zwei verschiedenen Neurotrophine ausgewählt sind aus der Gruppe bestehend aus BDNF, GDNF, NGF, NT-3, NT-4, CNTF, wobei die mindestens zwei Neurotrophine vorzugsweise GDNF und BDNF sind.

15. Verfahren nach Anspruch 12, wobei der Inhibitor des Notch-Wegs ausgewählt ist aus der Gruppe bestehend aus DAPT, RO4929097, wobei der Inhibitor des Notch-Wegs vorzugsweise DAPT ist.

16. Verfahren nach Anspruch 12, wobei der Aktivator des Aktivin/Transforming Growth Factor β (TGF-β)-Signalwegs ausgewählt ist aus der Gruppe bestehend aus TGF1-β, TGF2-β, Aktivin A, Aktivin B, Aktivin AB und Nodal, wobei der Aktivator des Aktivin/TGF- β-Signalwegs vorzugsweise Aktivin A ist.

17. Verfahren nach Anspruch 12, wobei das Co-Kulturmedium umfasst:
i) IL-34,
ii) GM-CSF,
iii) BDNF,
iv) GDNF,
v) DAPT,
vi) Aktivin A, und
vii) Makrophagen-Vorläuferzellen.

18. Verfahren nach einem der Ansprüche 12-17, wobei das Co-Kulturmedium kein TGF-β3 und cAMP enthält.

19. Verfahren nach einem der vorhergehenden Ansprüche, umfassend:
i) Inkontaktbringen des Mittelhirnorganoids gemäß einem der Ansprüche 7-9 mit dem Co-Kulturmedium gemäß einem der Ansprüche 12-18; und
ii) Kultivieren des Mittelhirnorganoids mit dem Co-Kulturmedium für 20 Tage, 40 Tage, 60 Tage oder 70 Tage, vorzugsweise Kultivieren des Mittelhirnorganoids für 20 Tage, wobei die Kultivierung unter schüttelnden Bedingungen durchgeführt wird, wodurch ein Mittelhirnassembloid erhalten wird.

20. Verfahren nach Anspruch 19, wobei das Mittelhirnassembloid fünf Tage nach der Zugabe von Makrophagen-Vorläufern in eine extrazelluläre Matrix eingebettet wird.

21. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Mittelhirnassembloid dopaminerge Neuronen und Mikroglia umfasst.

22. Verfahren nach Anspruch 21, wobei das Mittelhirnassembloid ferner umfasst:
a) radiale Glia,

b) Mittelhirn-spezifische neurale epitheliale Stammzellen,
c) neuronale Vorläufer,
d) Neuroblasten,
e) junge dopaminerge Neuronen,
f) junge cholinerge Neuronen,
g) reife dopaminerge Neuronen,
h) GABAerge Neuronen,
i) glutamaterge Neuronen,
j) reife dopaminerge Neuronen und/oder
k) serotonerge Neuronen.

23. Verfahren nach Anspruch 21, wobei die dopaminergen Neuronen durch die Expression von Markern ausgewählt aus der Gruppe bestehend aus FOXA2 und TH, CALB1, ADCYAP1, GIRK2 und SLC6A3 gekennzeichnet sind.

24. Verfahren nach Anspruch 21, wobei die Mikroglia durch eine Immunzellsignatur gekennzeichnet ist.

25. Verfahren nach Anspruch 21, wobei die Immunzellsignatur dadurch gekennzeichnet ist, dass die Mikroglia die Marker exprimiert, die ausgewählt sind aus der Gruppe bestehend aus IBA1, PU1, HLA-DMB, CSF1R, IL18, C1QC, TLR2, CLEC7A, MRC1, NAIP, ITGAM, CCl2, P2RX4.

26. Verfahren nach Anspruch 25, wobei die Immunzellsignatur ferner dadurch gekennzeichnet ist, dass eine höhere Menge an Zytokinen und Chemokinen im Medium des Mittelhirn-Assembloids im Vergleich zu einer Kontrolle freigesetzt wird.

27. Verfahren nach Anspruch 26, wobei die Kontrolle ein Mittelhirnorganoid gemäß einem der Ansprüche 7-9 ist, das nicht mit einem Co-Kulturmedium gemäß einem der Ansprüche 12-18 in Kontakt gebracht wird.

28. Verfahren nach Anspruch 19, wobei das Medium des Mittelhirnorganoids ein Organoid-Reifungsmedium gemäß Anspruch 10 ist.

29. Verfahren nach Anspruch 21, wobei die Mikroglia phagozytische Genmarker exprimiert.

30. Verfahren nach Anspruch 29, wobei die phagozytischen Genmarker ausgewählt sind aus der Gruppe bestehend aus HLA-A, HLA-B, HLA-C, TLR2, TLR4, ATG7, LAMP2, SQSTM1 und CYBB.

31. Verfahren nach einem der Ansprüche 21-30, wobei das Mittelhirnassembloid eine geringere Zellzahl aufweist im Vergleich zu einer Kontrolle gemäß Anspruch 27.

32. Verfahren nach Anspruch 31, wobei das Mittelhirnassembloid eine geringere Größe aufweist im Vergleich zu einer Kontrolle wie in Anspruch 27 definiert.

33. Verfahren nach einem der Ansprüche 21-32, wobei das Mittelhirnassembloid eine geringere Expression von Genen zeigt, die an Hypoxie und oxidativem Stress beteiligt sind, im Vergleich zu einer Kontrolle wie in Anspruch 27 definiert.

34. Verfahren nach Anspruch 33, wobei die an Hypoxie und oxidativem Stress beteiligten Gene ausgewählt sind aus der Gruppe bestehend aus COX1, SOD1, GPX4, MT-ATP6, GSTP1.

35. Verfahren nach einem der Ansprüche 21 bis 34, wobei eine höhere Menge an Leucin, Isoleucin, Valin, Phenylalanin und Tyrosin im Medium des Mittelhirnassembloids freigesetzt wird, als bei einer Kontrolle wie in Anspruch 27 definiert.

36. Verfahren nach einem der Ansprüche 21-35, wobei das Mittelhirnassembloid ferner gekennzeichnet ist durch:
a) höhere Expression von Immunantwortmarkern,
b) geringere Expression von synaptischen Markern, und
c) eine höhere Expression von Aktionspotentialmarkern,
im Vergleich zu einer Kontrolle wie in Anspruch 27 definiert.

37. Verfahren nach Anspruch 36, wobei die Immunreaktionsmarker ausgewählt sind aus der Gruppe bestehend aus: HLA-A, HLA-B, STAT2, STAT3, MRC2; die synaptischen Marker sind ausgewählt aus der Gruppe bestehend aus SYT1, SYP, ROBO1, DDC; die Aktionspotentialmarker sind ausgewählt aus der Gruppe bestehend aus CASK, CACNA1a, CACNA1e, CACNA1B, HCN1, KCNC3, KCND3.

38. Verfahren nach Anspruch 36 oder 37, wobei das Mittelhirnassembloid einen erhöhten Glukosestoffwechsel aufweist im Vergleich zu einer Kontrolle wie in Anspruch 27 definiert.

39. Verfahren nach Anspruch 38, wobei der erhöhte Glukosestoffwechsel durch eine höhere Glukose- und Aufnahme von Pyruvat aus dem Co-Kulturmedium gemäß einem der Ansprüche 12 bis 18 gekennzeichnet ist im Vergleich zu einer Kontrolle gemäß Anspruch 27.

40. Verfahren nach einem der Ansprüche 36 bis 39, wobei das Mittelhirn-Assembloid eine erhöhte neuronale Erregbarkeit aufweist im Vergleich zu einer Kontrolle gemäß Anspruch 27.

41. Verfahren nach Anspruch 40, wobei die erhöhte neuronale Erregbarkeit durch einen niedrigeren Schwellenwert für die Erzeugung von Aktionspotentialen gekennzeichnet ist, im Vergleich zu einer Kontrolle gemäß Anspruch 27.

42. Mittelhirn-Assembloid, erhältlich nach dem Verfahren der Ansprüche 1-41.

43. Mittelhirn-Assembloid nach Anspruch 42, wobei das Mittelhirn-Assembloid durch die in einem der Ansprüche 21-42 genannten Merkmale gekennzeichnet ist.

44. Verfahren zum Testen einer Verbindung von Interesse auf ihre Fähigkeit, eine zelluläre Reaktion auszulösen, umfassend:
i) Inkontaktbringen des Mittelhirn-Assembloids nach Anspruch 42 mit der Verbindung von Interesse; und
ii) Bestimmen, ob die Verbindung eine zelluläre Reaktion hervorruft.

45. Verfahren nach Anspruch 44, wobei die zelluläre Reaktion die Förderung oder Hemmung einer neurodegenerativen Erkrankung ist.

46. Verfahren nach Anspruch 45, wobei die neurodegenerative Erkrankung vorzugsweise Parkinson-Krankheit ist.

47. Verfahren nach Anspruch 44, wobei es sich bei der Verbindung um ein Medikament, ein kleines Molekül, ein Peptid, ein Protein oder ein Virus handelt.

Figure 1

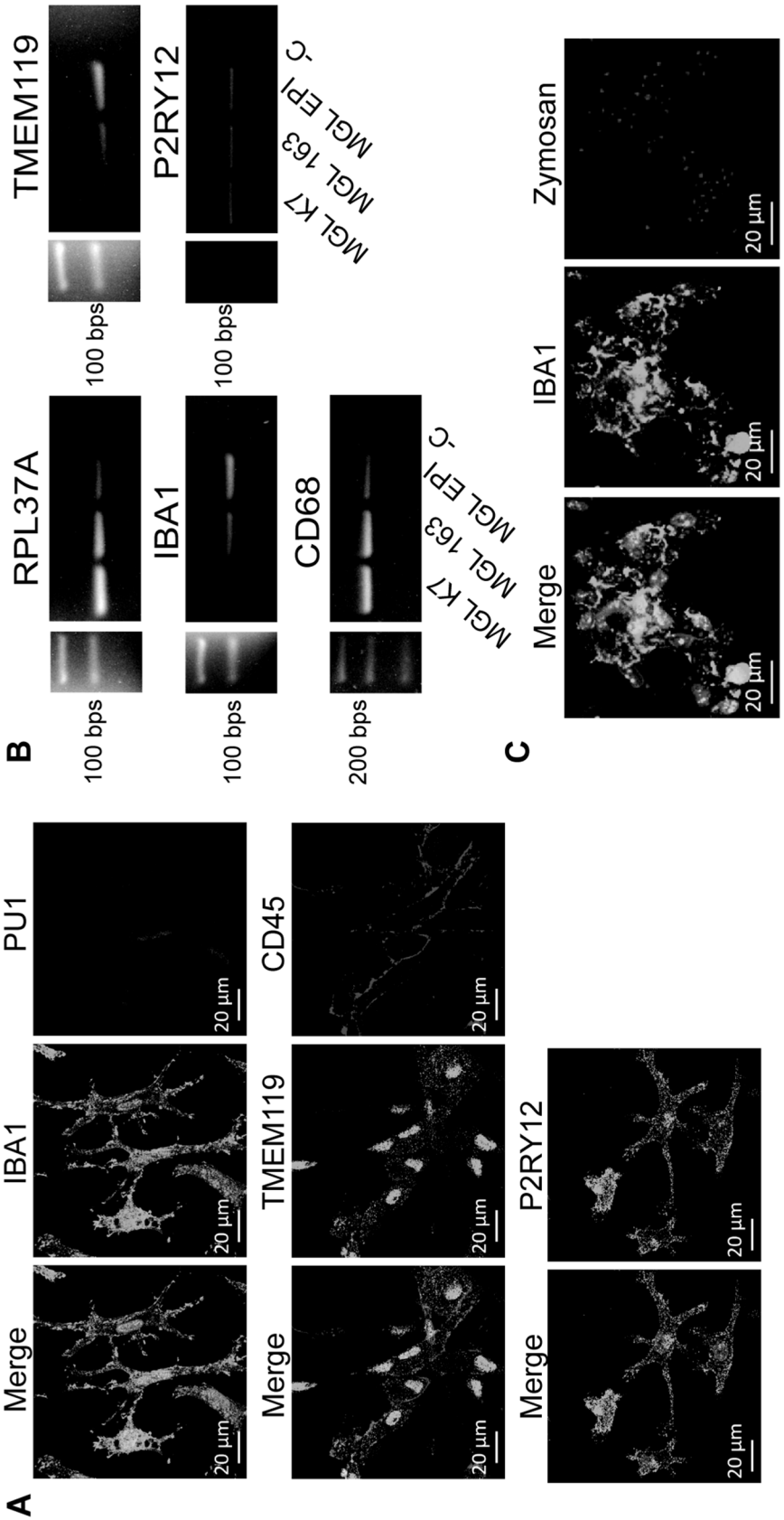

A
Merge
IBA1
PU1
20 µm
Merge
TMEM119
CD45
20 µm
Merge
P2RY12
20 µm
B
RPL37A
IBA1
CD68
100 bps
100 bps
200 bps
TMEM119
P2RY12
100 bps
100 bps
MGL K7
MGL 163
MGL EPI
C
C
Merge
IBA1
Zymosan
20 µm

Figure 1 continued

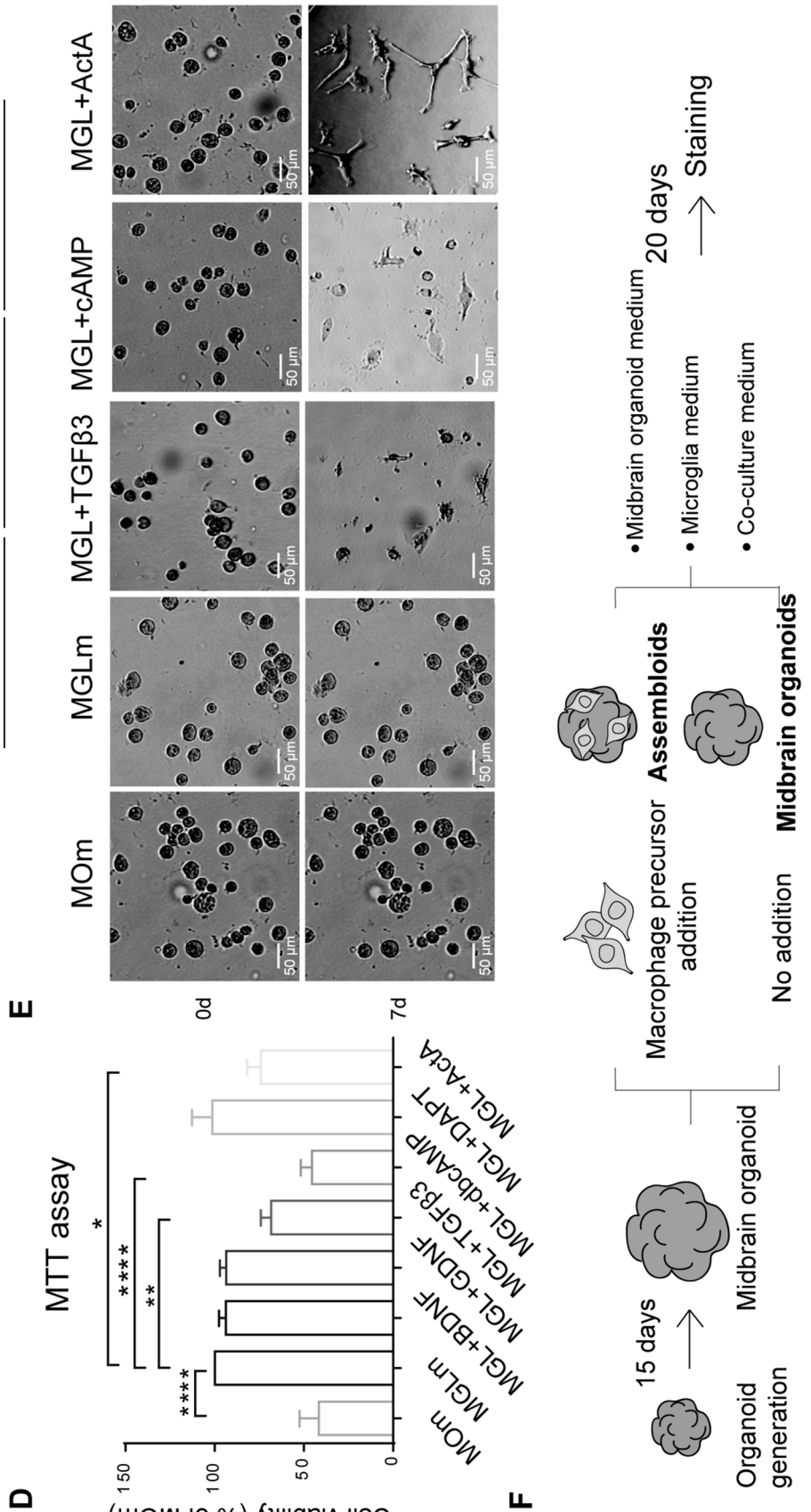


Figure 1 continued

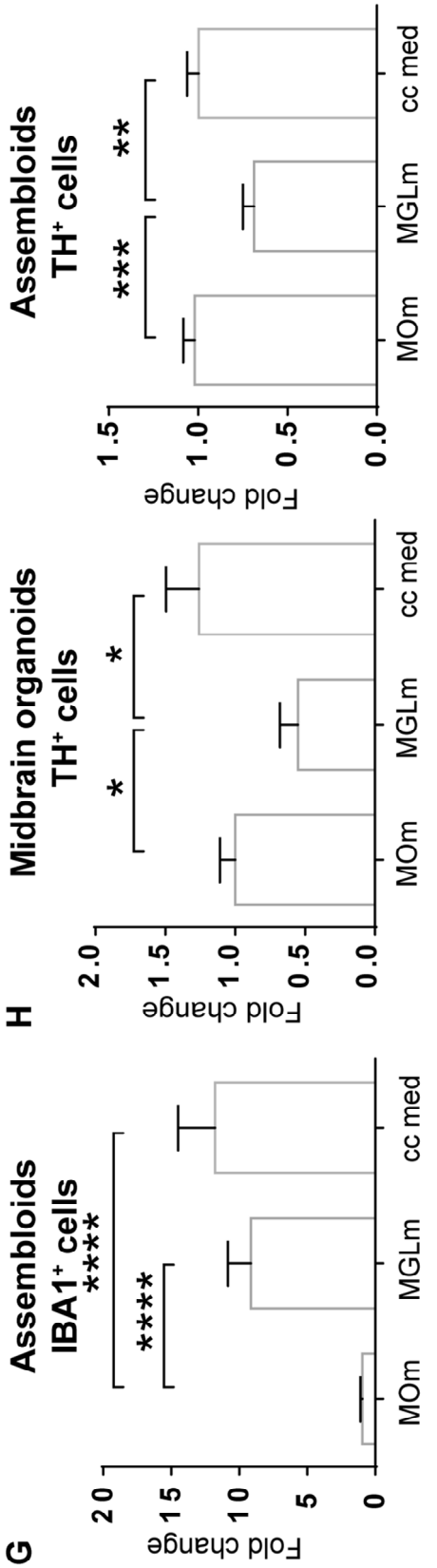


Figure 2

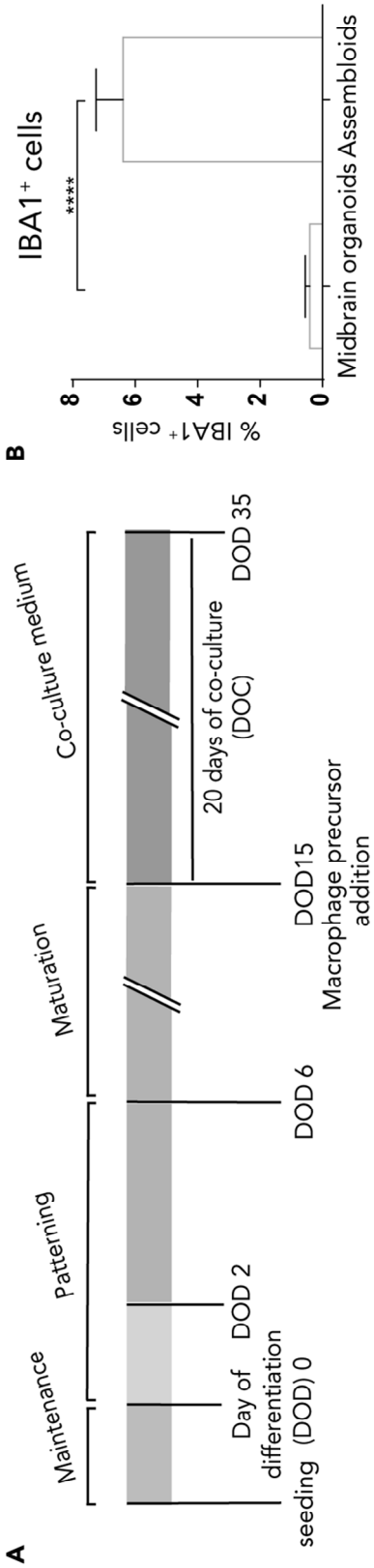

A
Maintenance
Patterning
Maturation
Co-culture medium
Day of differentiation seeding (DOD) 0
DOD 2
DOD 6
DOD15
Macrophage precursor addition
20 days of co-culture (DOC)
DOD 35
B
IBA1+ cells
****
% IBA1+ cells
0
2
4
6
8
Midbrain organoids
Assembloids

Figure 2 continued

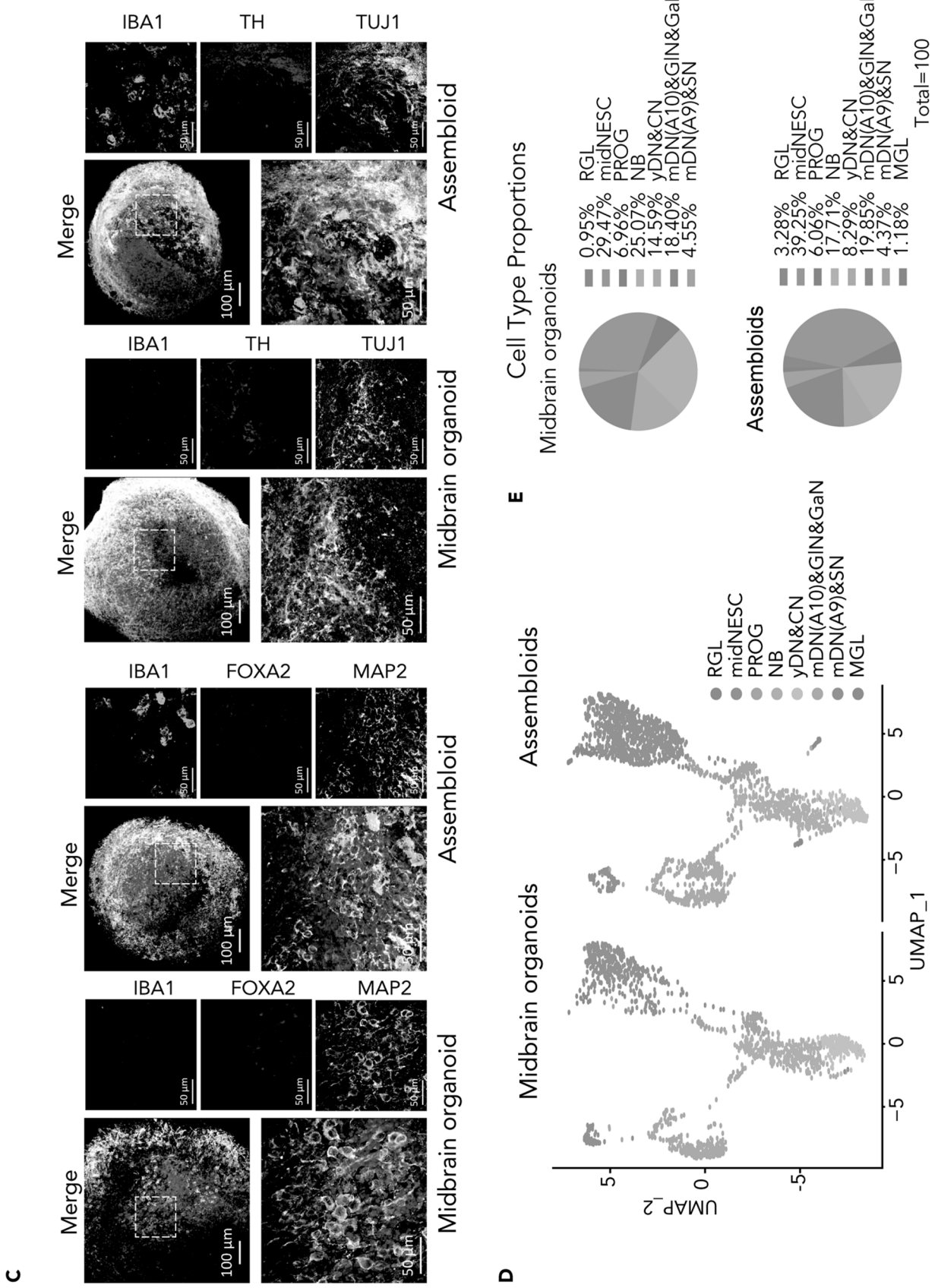


Figure 2 continued

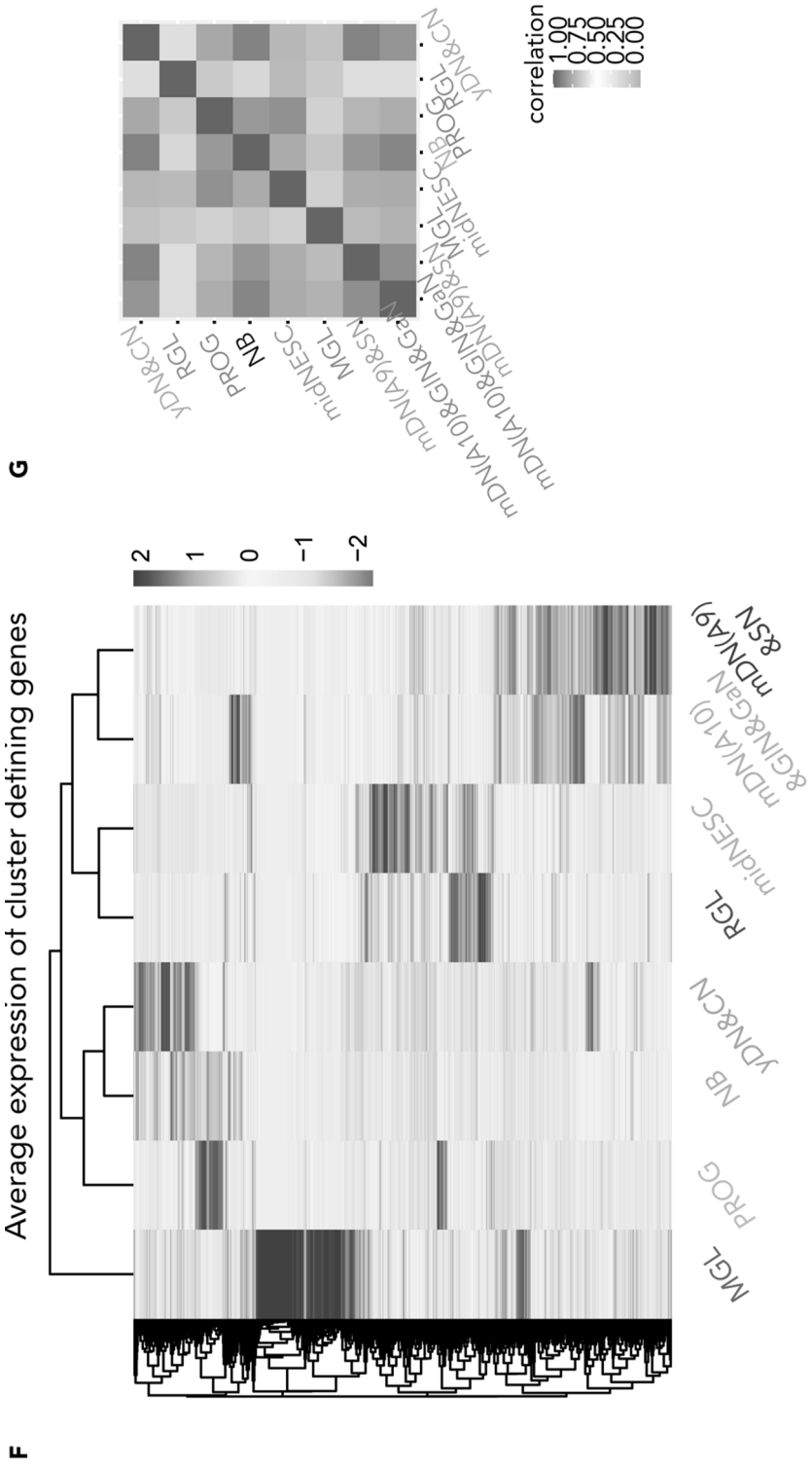


Figure 3

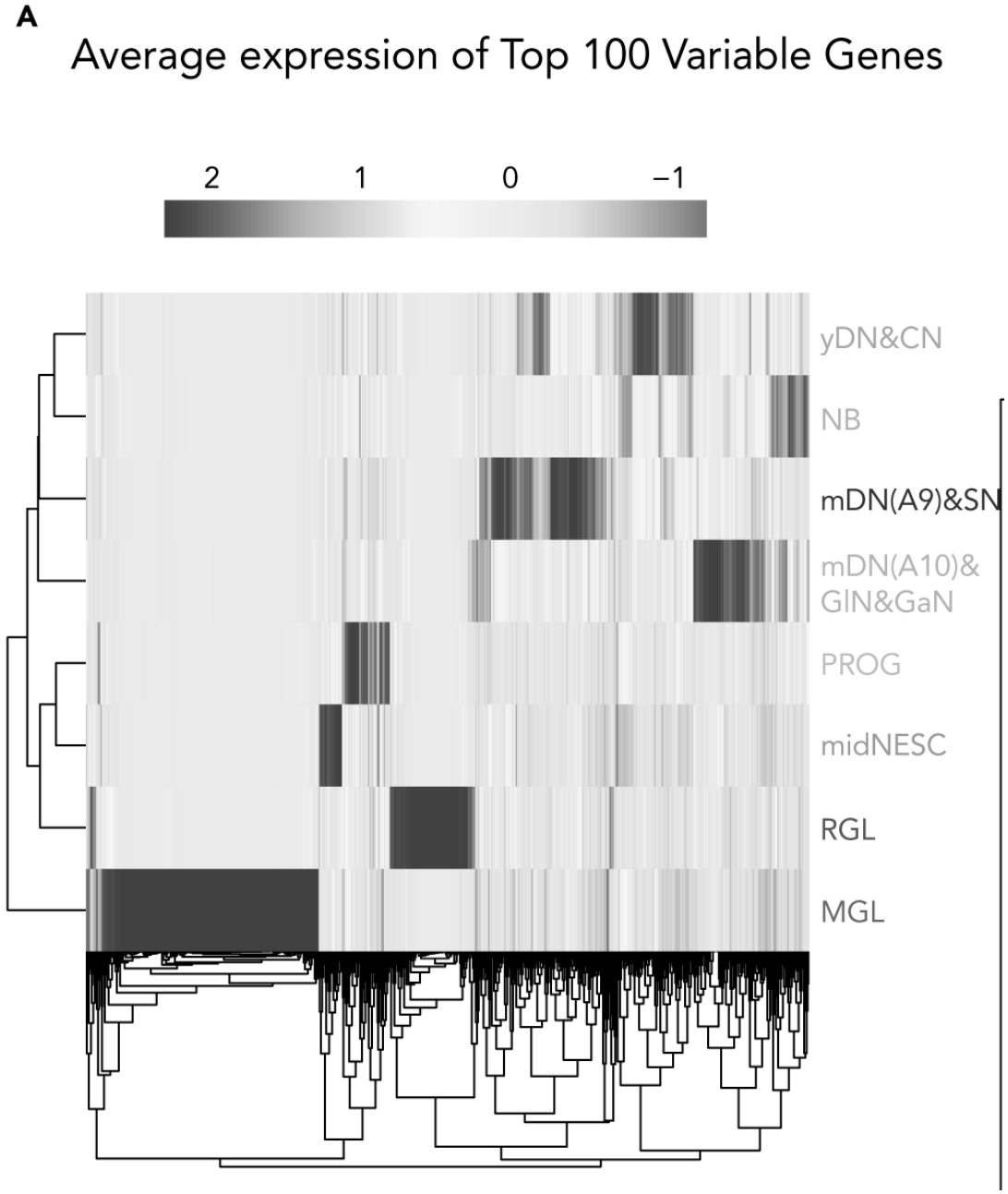

A
Average expression of Top 100 Variable Genes
2
1
0
−1
yDN&CN
NB
mDN(A9)&SN
mDN(A10)&
GlN&GaN
PROG
midNESC
RGL
MGL

Figure 3 continued

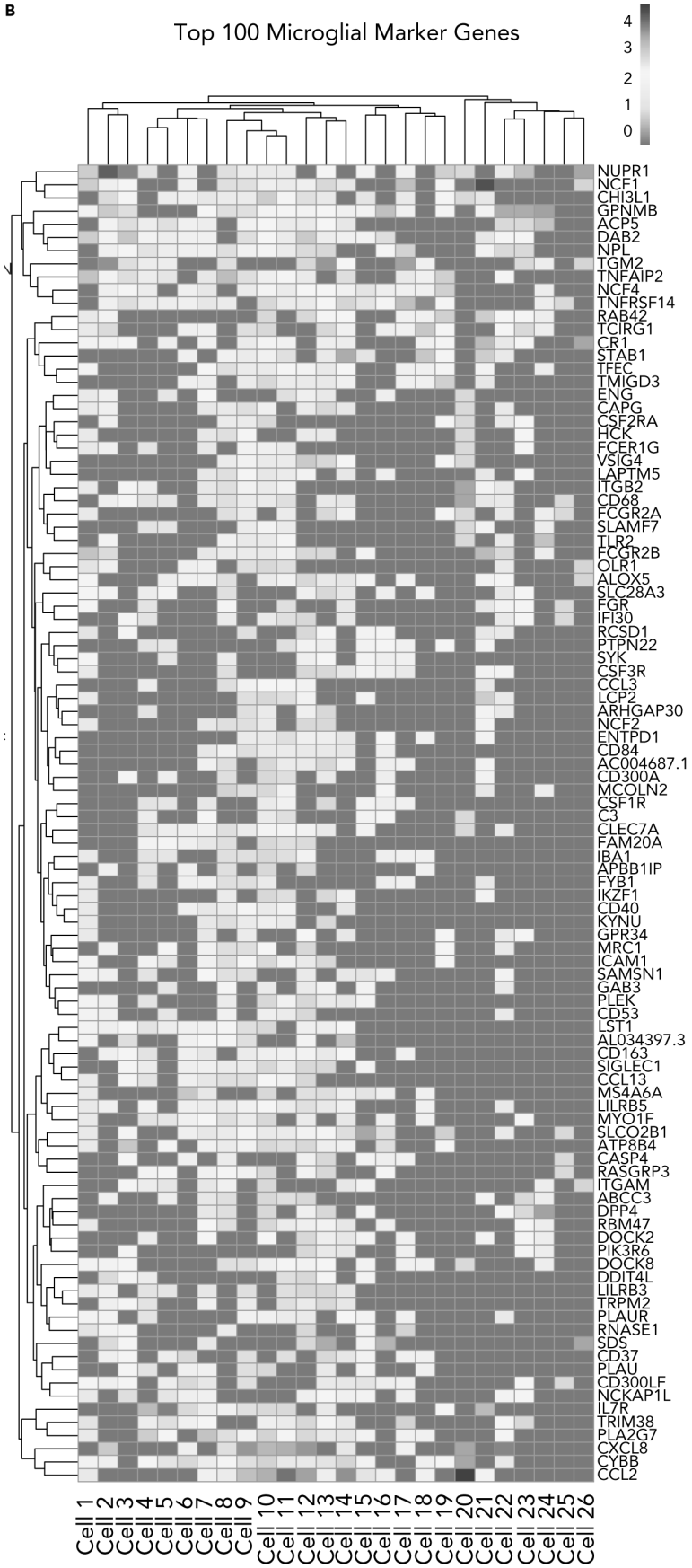


Figure 3 continued

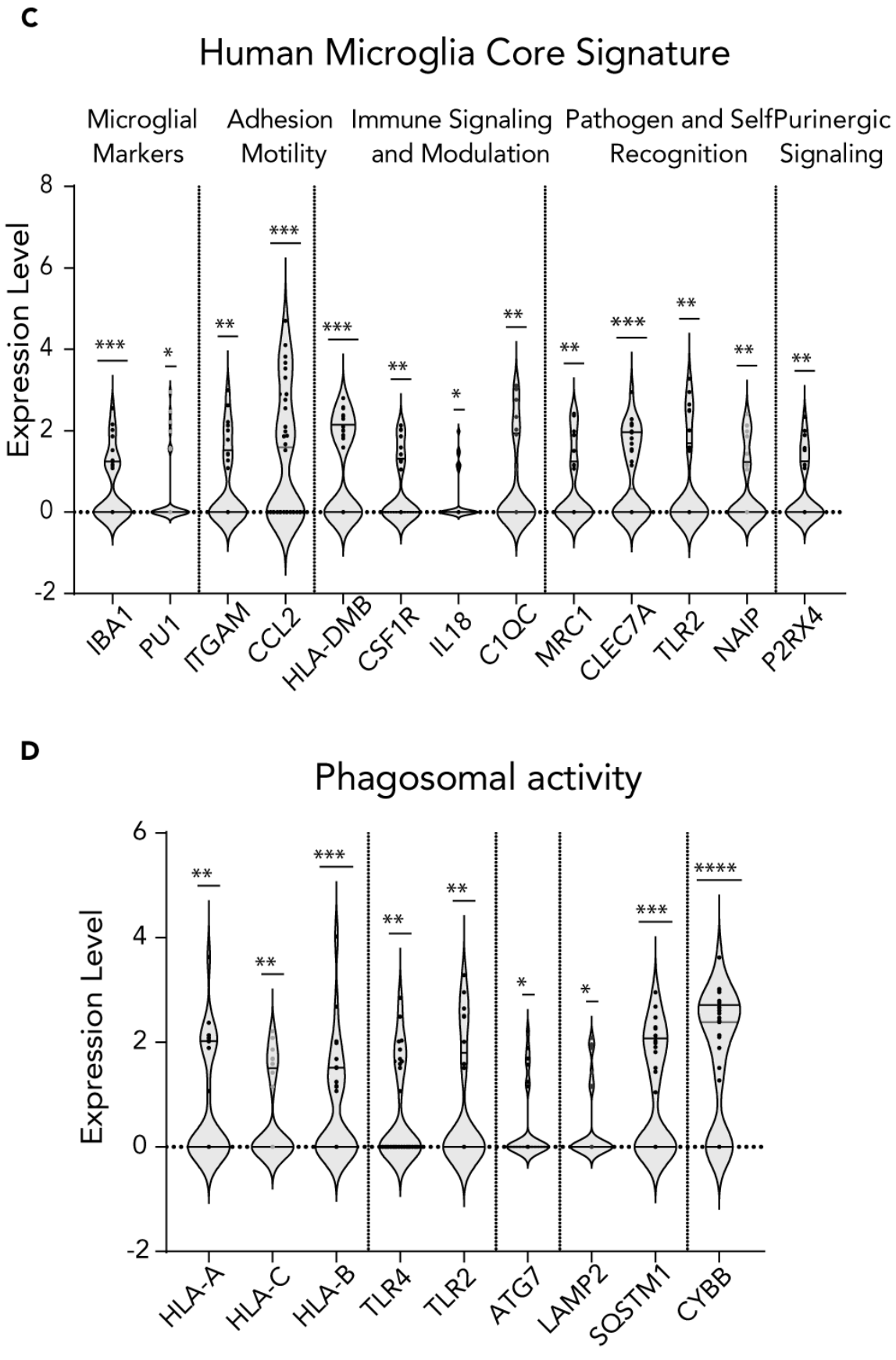


Figure 4

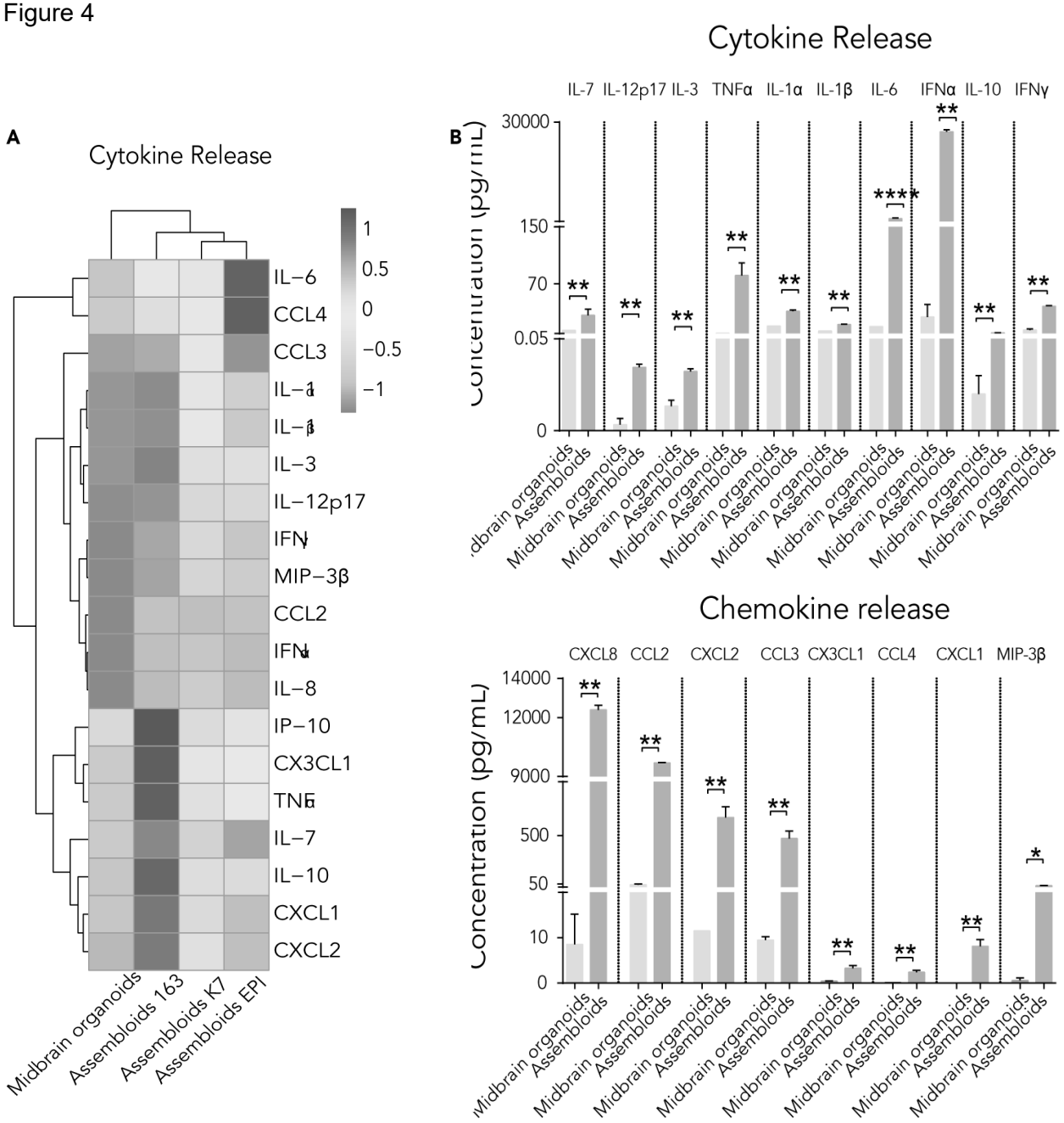

A
Cytokine Release
IL−6
CCL4
CCL3
IL−1α
IL−1β
IL−3
IL−12p17
IFNγ
MIP−3β
CCL2
IFNα
IL−8
IP−10
CX3CL1
TNFα
IL−7
IL−10
CXCL1
CXCL2
1
0.5
0
−0.5
−1
Midbrain organoids
Assembloids 163
Assembloids K7
Assembloids EPI
B
Cytokine Release
IL-7 IL-12p17 IL-3 TNFα IL-1α IL-1β IL-6 IFNα IL-10 IFNγ
30000
150
70
0.05
0
Midbrain organoids
Assembloids
Chemokine release
CXCL8 CCL2 CXCL2 CCL3 CX3CL1 CCL4 CXCL1 MIP-3β
Concentration (pg/mL)
14000
12000
9000
500
50
10
0

Figure 4 continued

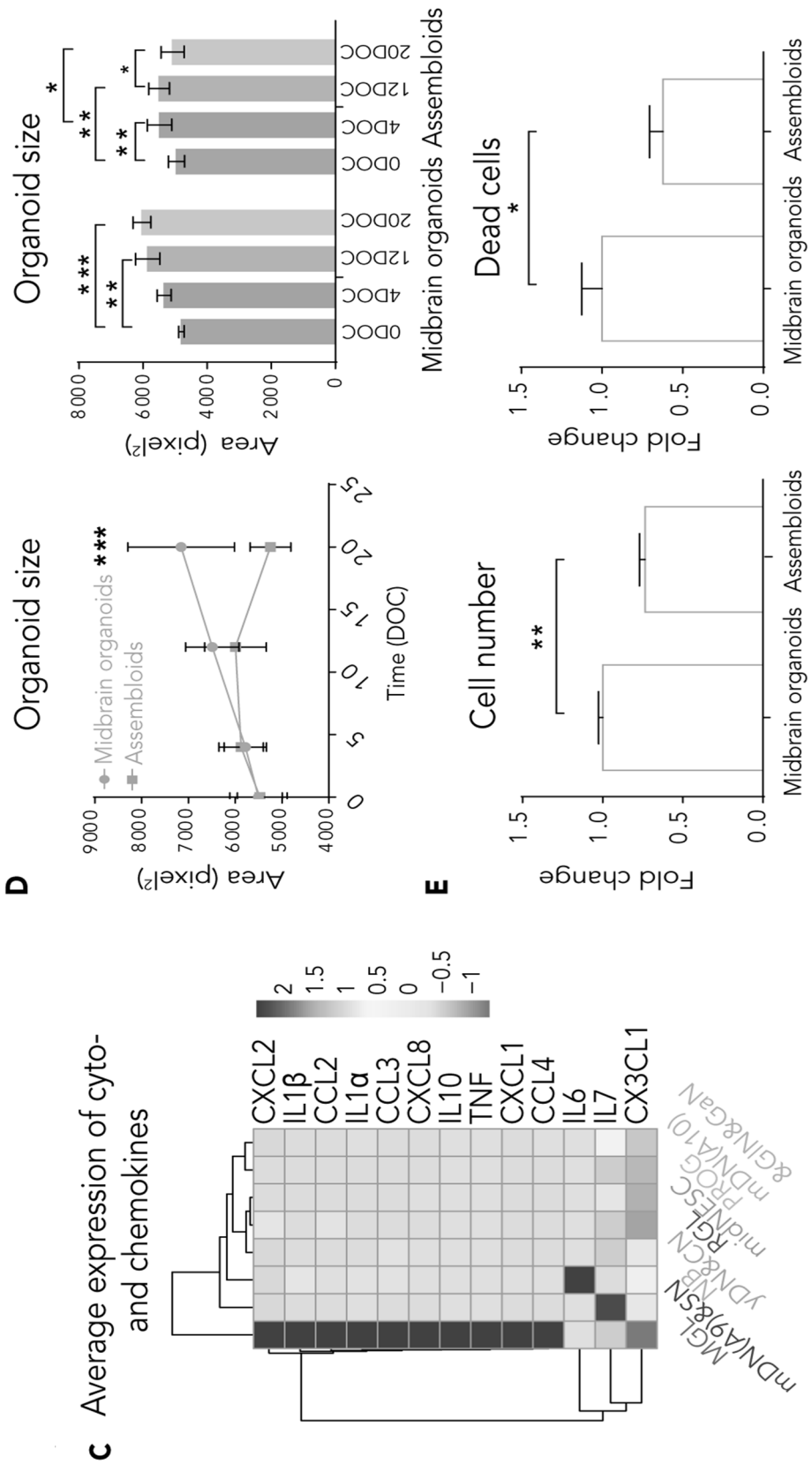


Figure 5

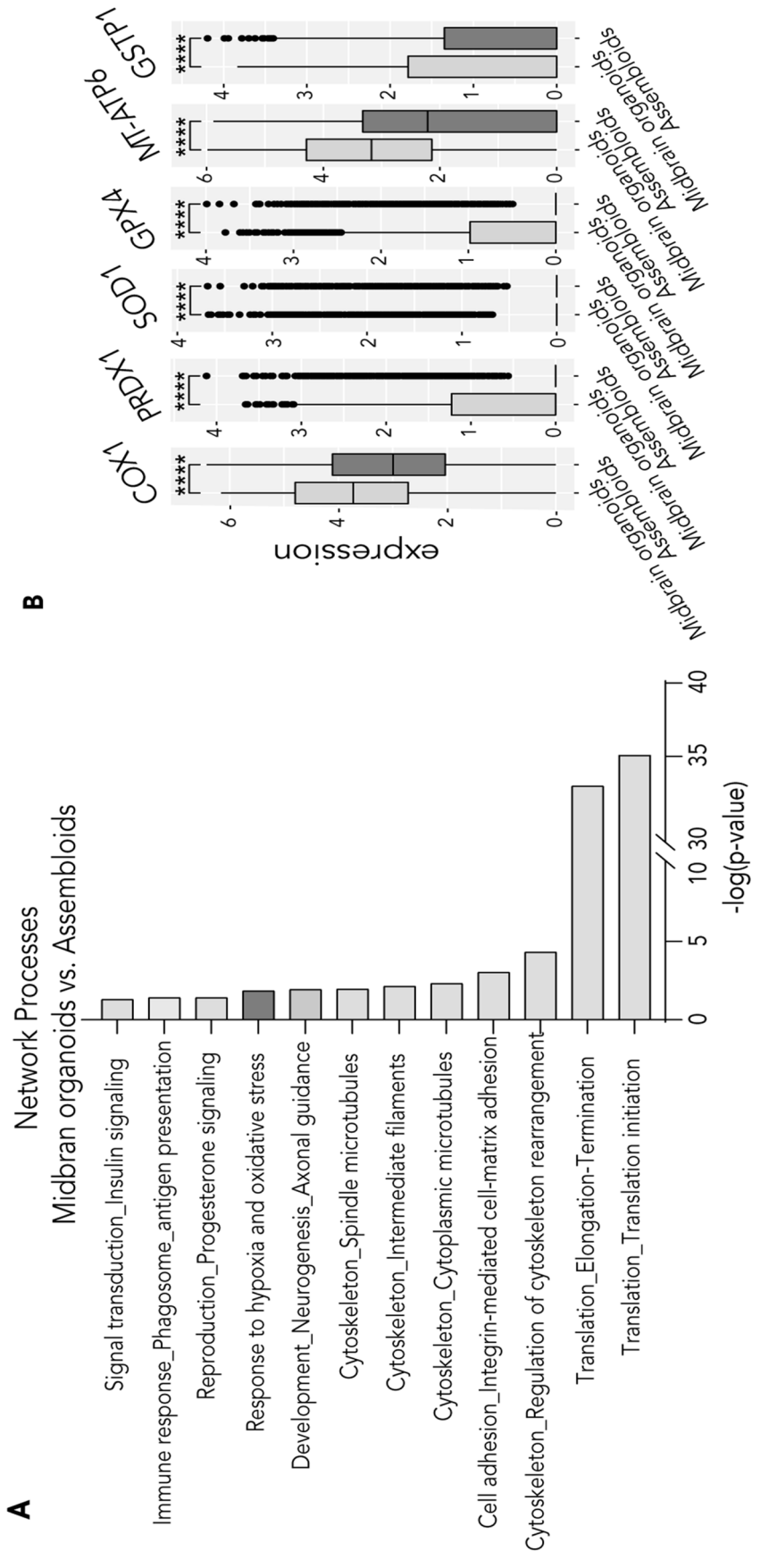
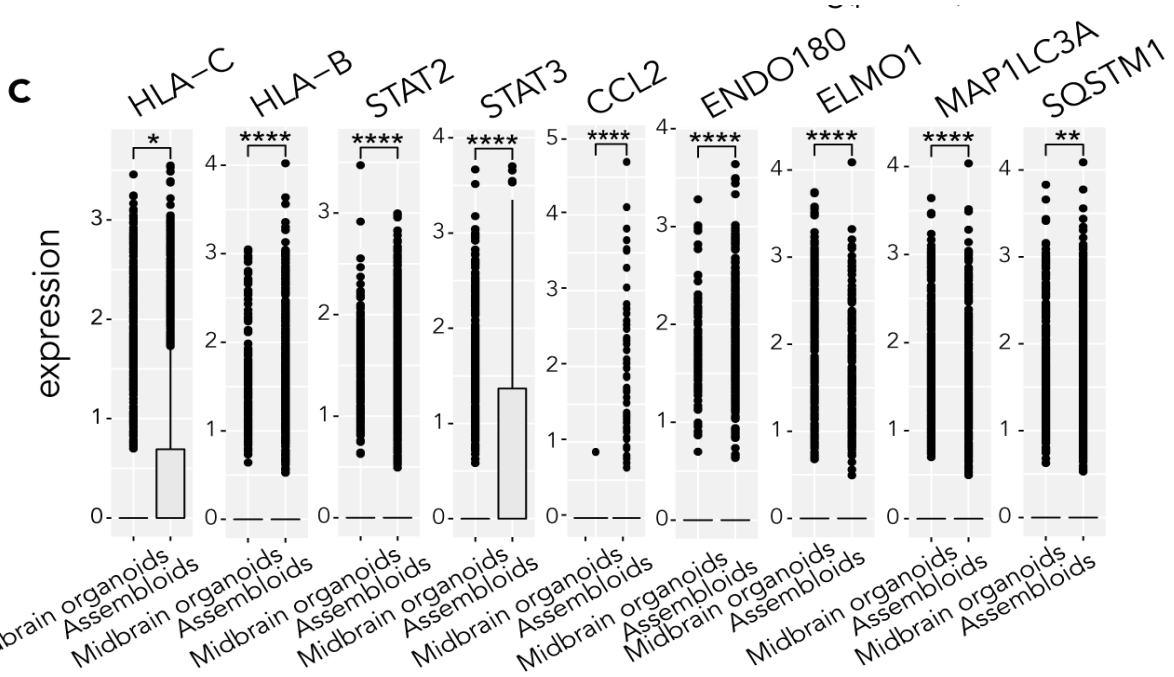

A
Network Processes
Midbran organoids vs. Assembloids
Signal transduction_Insulin signaling
Immune response_Phagosome_antigen presentation
Reproduction_Progesterone signaling
Response to hypoxia and oxidative stress
Development_Neurogenesis_Axonal guidance
Cytoskeleton_Spindle microtubules
Cytoskeleton_Intermediate filaments
Cytoskeleton_Cytoplasmic microtubules
Cell adhesion_Integrin-mediated cell-matrix adhesion
Cytoskeleton_Regulation of cytoskeleton rearrangement
Translation_Elongation-Termination
Translation_Translation initiation
0
5
10
30
35
40
-log(p-value)
B
COX1
PRDX1
SOD1
GPX4
MT-ATP6
GSTP1
expression
****
Midbrain organoids
Midbrain Assembloids

Figure 5 continued



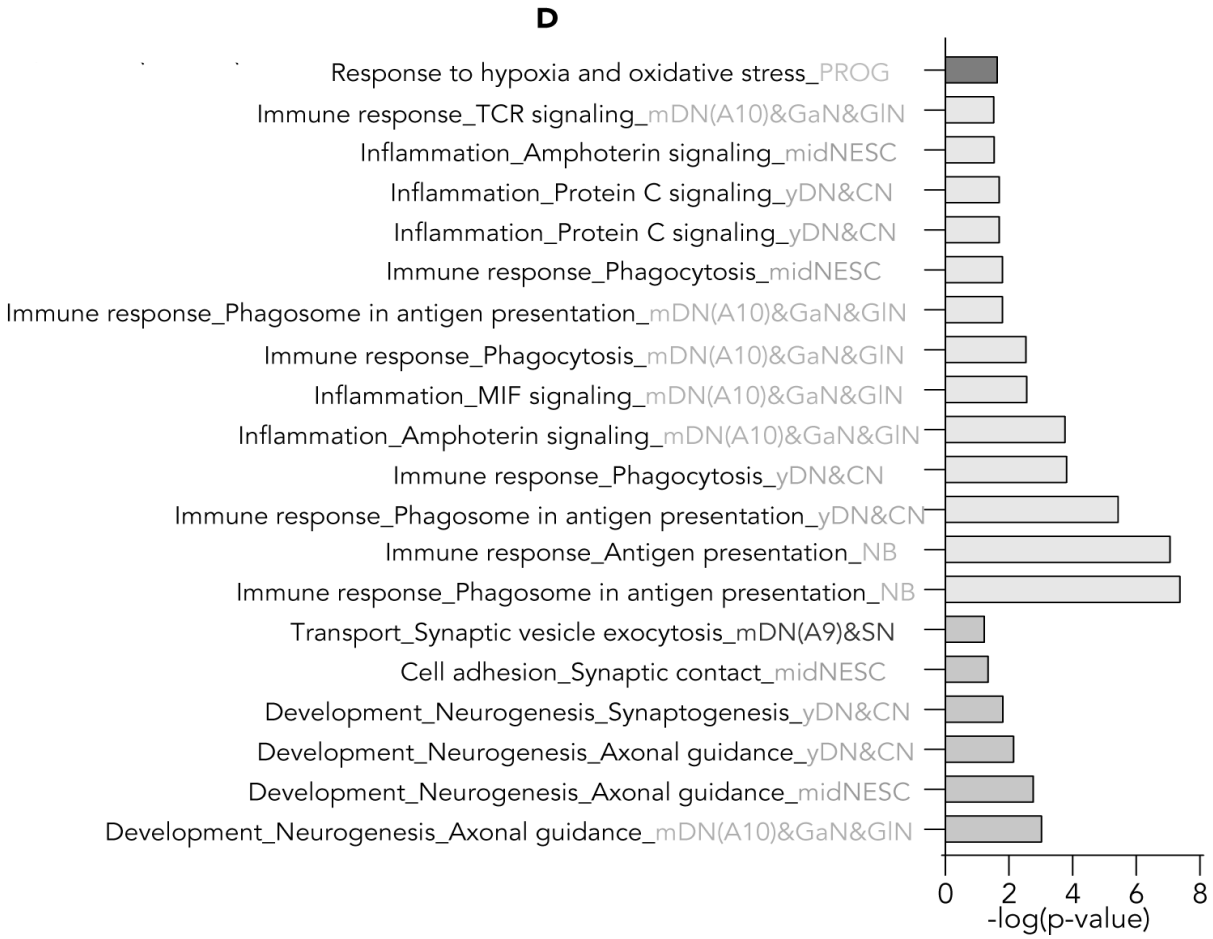


Figure 6

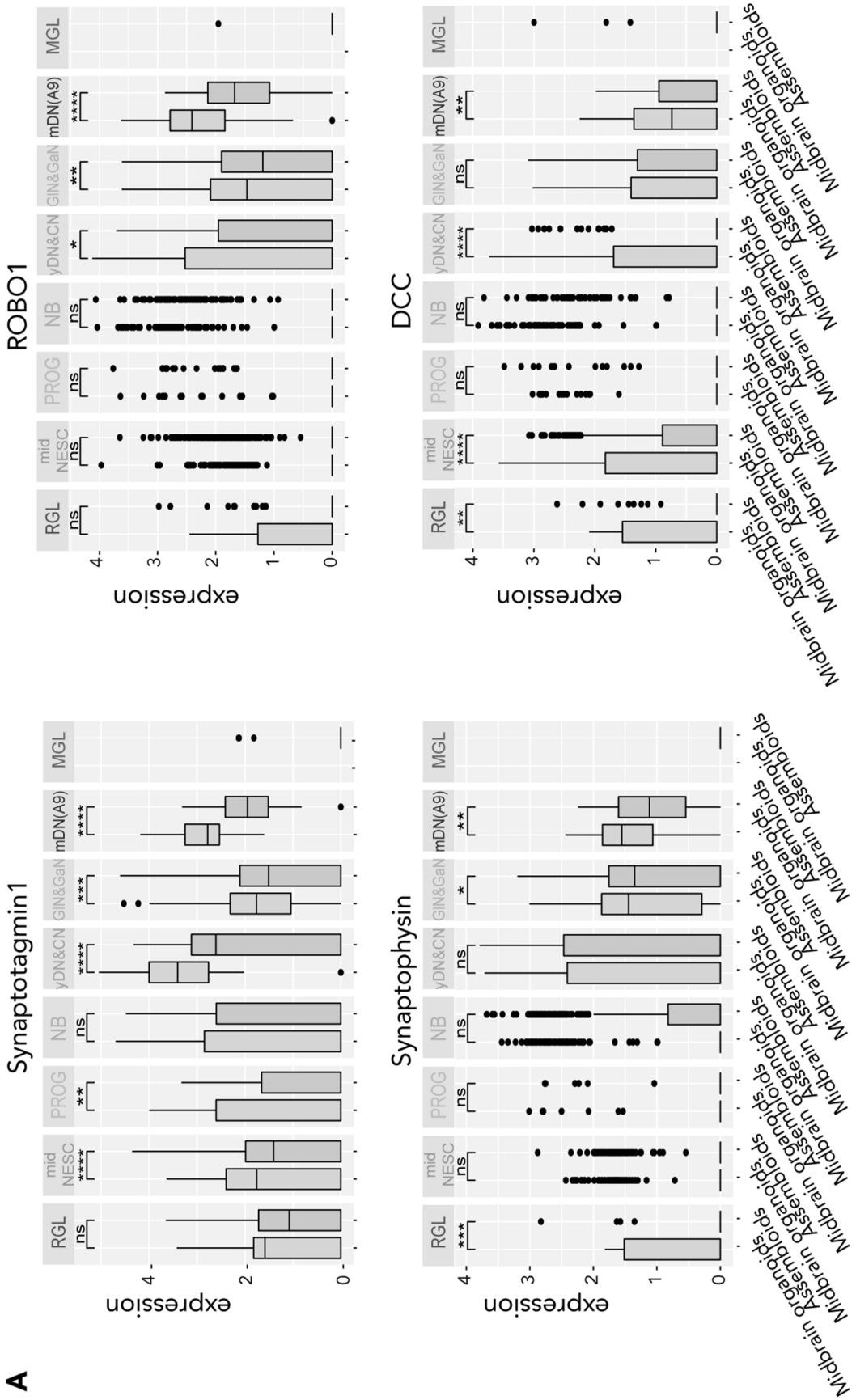

A
Synaptotagmin1
Synaptophysin
ROBO1
DCC
expression
RGL
mid NESC
PROG
NB
yDN&CN
GIN&GaN
mDN(A9)
MGL
ns
*
**
***
****
Midbrain Assembloids
Midbrain organoids
Midbrain Assembloids:organoids

Figure 6 continued

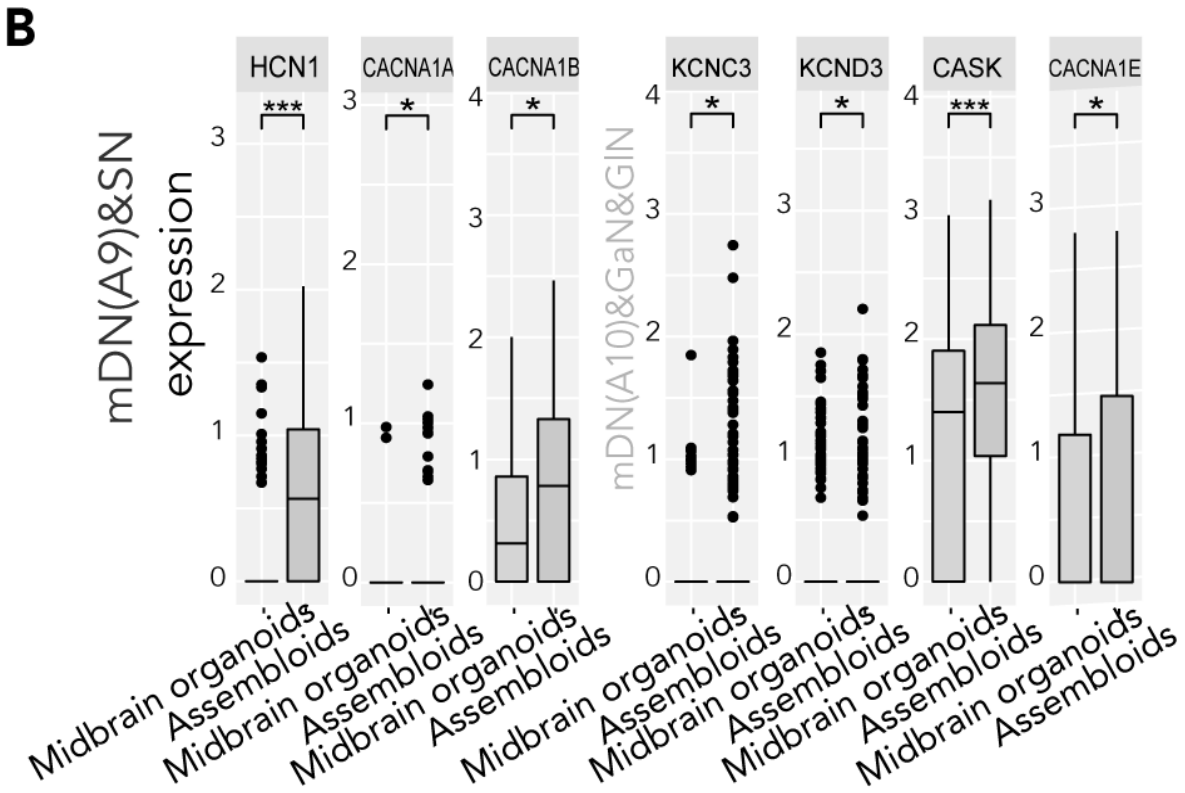


Figure 6 continued

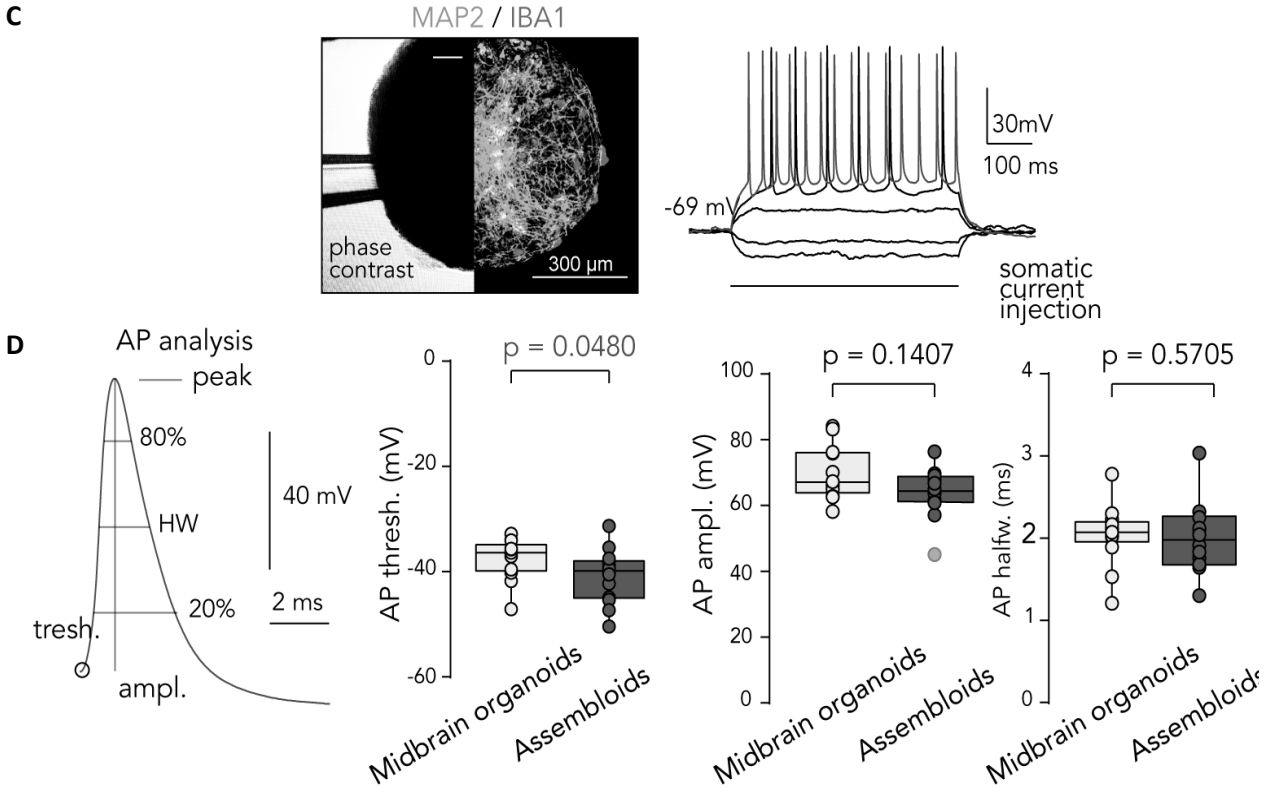
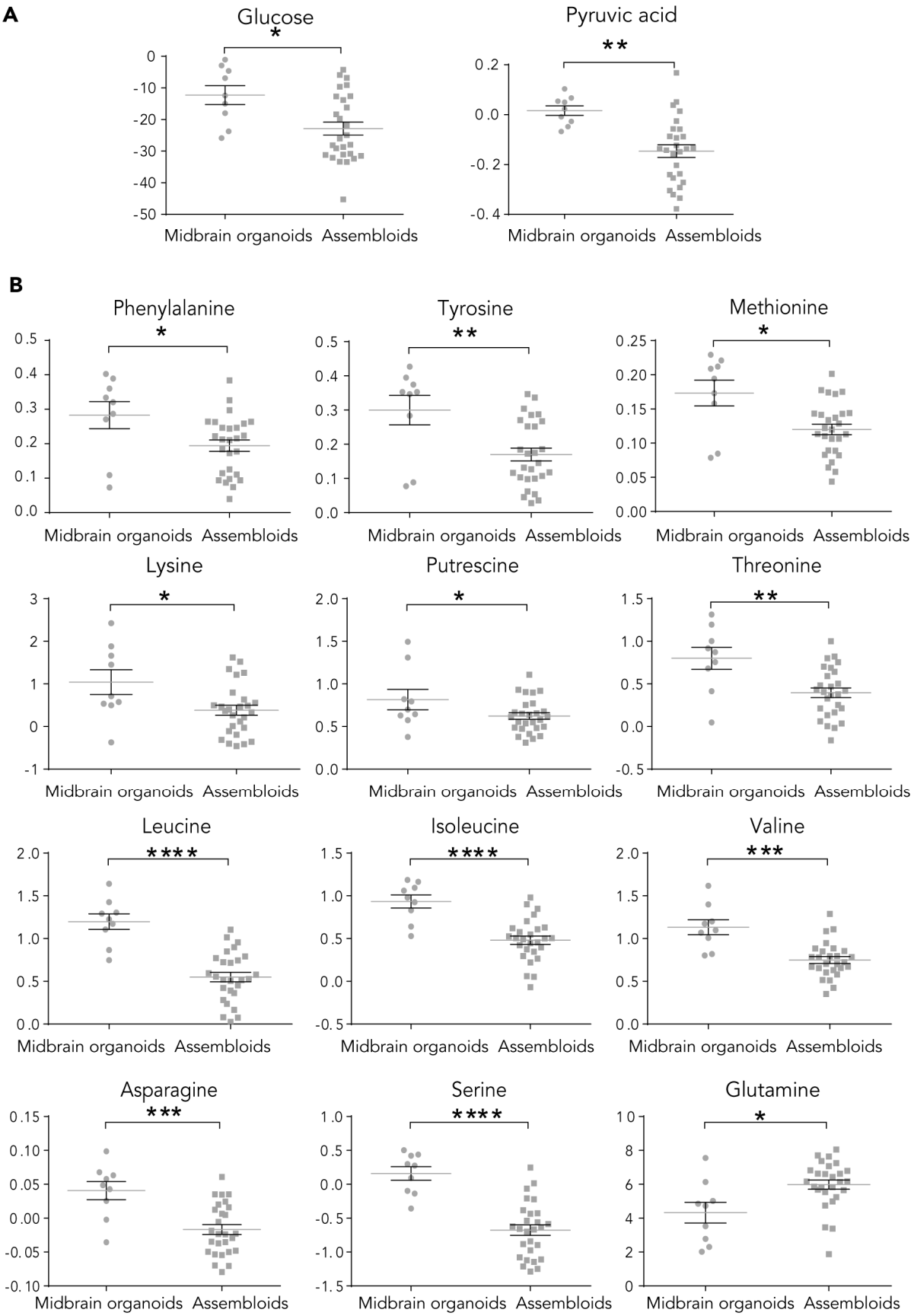


Figure 7


A
Glucose
*
0
-10
-20
-30
-40
-50
Midbrain organoids
Assembloids
Pyruvic acid
**
0.2
0.0
-0.2
-0.4
Midbrain organoids
Assembloids
B
Phenylalanine
*
0.5
0.4
0.3
0.2
0.1
0.0
Midbrain organoids
Assembloids
Tyrosine
**
0.5
0.4
0.3
0.2
0.1
0.0
Midbrain organoids
Assembloids
Methionine
*
0.25
0.20
0.15
0.10
0.05
0.00
Midbrain organoids
Assembloids
Lysine
*
3
2
1
0
-1
Midbrain organoids
Assembloids
Putrescine
*
2.0
1.5
1.0
0.5
0.0
Midbrain organoids
Assembloids
Threonine
**
1.5
1.0
0.5
0.0
-0.5
Midbrain organoids
Assembloids
Leucine
****
2.0
1.5
1.0
0.5
0.0
Midbrain organoids
Assembloids
Isoleucine
****
1.5
1.0
0.5
0.0
-0.5
Midbrain organoids
Assembloids
Valine
***
2.0
1.5
1.0
0.5
0.0
Midbrain organoids
Assembloids
Asparagine
***
0.15
0.10
0.05
0.00
-0.05
-0.10
Midbrain organoids
Assembloids
Serine
****
1.0
0.5
0.0
-0.5
-1.0
-1.5
Midbrain organoids
Assembloids
Glutamine
*
10
8
6
4
2
0
Midbrain organoids
Assembloids