

example, transthyretin (TTR), a carrier of thyroid hormones and retinol, is the most abundant protein synthesised and secreted by the choroid plexus (Li, X. and Buxbaum, J.N. (2011) Mol. Neurodegener. 6: 1–17; Richardson, S.J. et al. (2015) Front. Neurosci. 9: 1–8; Johnson, B.A. et al. (2018) Fluids Barriers CNS 15: 22). TTR-mediated delivery of thyroid hormones to the CSF appears to have an important role in regulation of brain development.

The choroid plexus tightly sealed epithelium is also known as the blood-CSF barrier (B-CSF-B). The B-CSF-B, similar to the blood-brain barrier (BBB), prevents toxic substances or signals in the circulation from reaching the brain. However, in contrast to the BBB, the B-CSF-B is formed by a single cell type, the choroid plexus epithelial cells, and it faces the CSF rather than the brain parenchyma directly (5, 6). However, because the CSF has free access to the brain, transport of molecules across the B-CSF-B is an alternative route into the brain.

Little is known about choroid plexus human development. The development of choroid plexus follows a series of stages from a pseudostratified epithelium, to a columnar and then highly folded cuboidal epithelium with microvilli and basally located nuclei at the latest stages. In the dorsal telencephalon, around the 7th week of gestation, the local BMP signalling gradient drives choroid plexus development from the neuroepithelium, which begins to elongate towards the lumen of the ventricle. By establishing local thresholds, the BMP secreted signals gradually specify the choroid plexus epithelium and the adjacent cortical hem, a Wntless-related (WNT) signalling centre from which the hippocampal primordium develops.

Studies toward the patterning of choroid plexus have been carried out *in vitro*. Over the past decade, significant progress in 3D culture systems such as cerebral organoids has allowed fundamental discoveries in human developmental biology (7–12).

However, the induced choroid plexus cells generated so far have not been shown to exhibit the same complex architecture as the *in vivo* choroid plexus, nor have they been shown to be functional in generating CSF.

Accordingly, there remains a significant need in the art for the establishment of functional models of the choroid plexus. The choroid plexus is an important point of entry of drugs to the CNS; however, little is known about the regulation and permeability of the B-CSF-B and how it could be exploited as a tool to improve the availability of certain drugs in the brain. The study of functional models of the choroid plexus may aid understanding in this regard, in particular for assaying candidate drugs for the ability to cross the B-CSF-B and/or BBB.

SUMMARY

The inventors have established a protocol to generate choroid plexus organoids using a combination of signalling molecules.

The organoids (which may be referred to hereinafter as “ChP organoids”) have a selective epithelial barrier that secrete CSF-like fluid in self-contained compartments, much like the CSF of brain ventricles. Characterization of ChP organoids by single cell RNA sequencing reveals the presence of epithelial and stromal ChP populations. Proteomic analysis of the organoid CSF-like fluid reveals human-specific and developmental factors, as well as disease-related biomarkers. Finally, using NMR the inventors show that this *in vitro* barrier

exhibits the same selectivity to small molecules as the *in vivo* counterpart, and that ChP-CSF organoids can predict CNS permeability of novel compounds.

Thus, the organoids provided by the inventors' protocol recapitulate fundamental functions of the choroid plexus, namely secretion and formation of a tight epithelial barrier. The inventors determined that the organoids provided by their protocol reliably and reproducibly develop choroid plexus epithelium with polarised cells that actively secrete a fluid within the organoid with properties closely resembling cerebrospinal fluid (CSF).

Furthermore, the inventors discovered the presence of choroid plexus-specific water channels and transporters localised on the apical brush border of the choroid plexus organoid epithelium using histological and electron microscopy (EM) analysis. These tissues were found to display tight junctions forming the epithelial barrier, and the inventors observed the formation of large fluid-filled cysts protruding from the organoids, the contents of which, analysed by mass spectrometry, highly resemble human embryonic CSF.

The disclosure provides a tool to study, for example, choroid plexus function, CSF production, and the permeability of compounds, peptides and serum-derived proteins into the human brain through the CSF.

Accordingly, in one aspect the disclosure provides a choroid plexus organoid comprising: (a) an epithelium comprising a tight epithelial barrier; and/or (b) one or more cysts surrounded by an epithelium.

In some examples, the choroid plexus organoid comprises an epithelium comprising a tight epithelial barrier. In some examples, the choroid plexus organoid comprises one or more cysts surrounded by an epithelium. In preferred examples, the choroid plexus organoid comprises an epithelium comprising a tight epithelial barrier and one or more cysts surrounded by the epithelium.

In preferred examples, the epithelium is cuboidal epithelium.

In some examples the choroid plexus organoid further comprises (c) stromal cell populations.

In some examples the choroid plexus organoid has a lateral ventricle identity.

In preferred examples, the choroid plexus organoid is LUM+, DCN+, and DLK1+.

In some examples, the choroid plexus organoid is TTR+, MRP1+, Aqp1+ and/or ZO1+. In preferred examples, the choroid plexus organoid is TTR+, MRP1+, Aqp1+ and ZO1+.

In other examples, the choroid plexus organoid is TTR+, MRP1+, MRP4+, Aqp1+ and/or ZO1+. In other examples, the choroid plexus organoid is TTR+, MRP1+, MRP4+, Aqp1+ and ZO1+.

In some examples, the choroid plexus organoid comprises at least 50% TTR+ tissue. In some examples, the choroid plexus organoid comprises at least 60% TTR+ tissue. In some examples, the choroid plexus organoid comprises at least 70% TTR+ tissue. In some

examples, the choroid plexus organoid comprises at least 80% TTR+ tissue. In some examples, the choroid plexus organoid comprises at least 90% TTR+ tissue.

In some examples, the epithelium is TTR+, MRP1+, Aqp1+ and/or ZO1+. In preferred examples, the epithelium is TTR+, MRP1+, Aqp1+ and ZO1+.

5 In some examples, the epithelium is MRP4+.

In some examples, the TTR is enriched on the apical side of the epithelium. In some examples, the Aqp1 is enriched on the apical side of the epithelium. In some examples, the ZO1 is enriched on the apical side of the epithelium.

10 In some examples, the TTR and Aqp1 are enriched on the apical side of the epithelium. In some examples, the TTR, Aqp1 and ZO1 are enriched on the apical side of the epithelium.

In some examples, the MRP1 is enriched on the apical and basal sides of the epithelium.

In some examples, the MRP4 is enriched on the apical and basal sides of the epithelium.

15 Enrichment of a marker in a certain area, for example of a cell, may mean that the marker is present in that area in greater abundance than other areas (e.g. all other areas). The marker may be substantially absent in other areas (e.g. all other areas).

In some examples, the choroid plexus organoid is FOXG1-.

In some examples, the epithelium comprises apical microvilli.

In some examples, the epithelium comprises centro-basally located nuclei.

In some examples, the epithelium is apico-basal polarised.

20 In some examples, the epithelium is polarised, and Aqp1 and ZO1 are expressed on the luminal side of the epithelium.

In some examples, LAT-1 is expressed on the apical side of the epithelium.

25 In some examples, the epithelia are enriched in specific transporters involved in the trafficking of nutrients e.g. SLC23A2 (vitamin C) and/or SLC46A2 (folate). In some examples, the choroid plexus organoid is capable of secreting a liquid comprising one or more proteins selected from the group consisting of transthyretin (TTR), clusterin (CLU), apolipoprotein E (APOE), apolipoprotein A4 (APOA4) and lumican (LUM).

In some examples, the liquid comprises phospholipid transfer protein (PLTP)

In some examples, the liquid comprises alphafetoprotein (AFP).

30 In some examples, the choroid plexus organoid is capable of secreting a cerebrospinal fluid (CSF)-like fluid.

In some examples, the choroid plexus organoid is capable of secreting a cerebrospinal fluid (CSF).

In preferred examples, the cysts comprise a liquid. In preferred examples, the liquid comprises one or more proteins selected from the group consisting of transthyretin (TTR), clusterin (CLU), apolipoprotein E (APOE), apolipoprotein A4 (APOA4) and lumican (LUM). In some examples, the liquid is a CSF.

5 In some examples, the liquid comprises alphafetoprotein (AFP).

In some examples, the liquid comprises developmental-specific CSF proteins e.g. Insulin-like growth factor 2 (IGF2), IGFBP7, and/or Follistatin-like protein 1 (FSTL1).

10 In some examples, the choroid plexus organoid comprises less than 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2% or 1% (v/v) forebrain neuronal tissue. In some examples, the choroid plexus organoid comprises substantially no forebrain neuronal tissue.

In some examples, the choroid plexus organoid comprises less than 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2% or 1% (v/v) FOXG1+ tissue. In some examples, the choroid plexus organoid comprises substantially no FOXG1+ tissue.

In some examples, the choroid plexus organoid comprises extracellular matrix (ECM).

15 In some examples, the choroid plexus organoid have primary cilia, for example, with microvilli apically located, tight junctions between cells, multi-vesicular bodies and extracellular vesicles.

In some examples, the choroid plexus organoid is a human or mouse organoid, preferably a human organoid.

20 In some examples, the choroid plexus organoid is obtainable by the method of the disclosure.

In some examples, the choroid plexus organoid is produced from stem cells, optionally embryonic stem cells or induced pluripotent stem cells.

25 In another aspect, the disclosure provides use of a WNT pathway activator and a bone morphogenetic protein (BMP) signalling pathway activator for producing a choroid plexus organoid, wherein the choroid plexus organoid comprises (a) an epithelium comprising a tight epithelial barrier; and/or (b) one or more cysts surrounded by an epithelium.

30 Although there are previous reports of using Bmp4 and/or CHIR (see (15) and (16)) these earlier publications did not yield ChP organoids having the authentic characteristics demonstrated herein.

In another example, the disclosure provides a method for producing a choroid plexus organoid comprising contacting a population of neuroepithelial cells with a WNT pathway activator and a bone morphogenetic protein (BMP) signalling pathway activator, wherein the choroid plexus organoid comprises: (a) an epithelium comprising a tight epithelial barrier;
35 and/or (b) one or more cysts surrounded by an epithelium.

The method may be performed with or without fibrous microscavolds. The absence of microscavolds may be preferred.

In another aspect, the disclosure provides a method for producing a choroid plexus organoid comprising producing a population of neuroepithelial cells from a population of stem cells, and culturing the population of neuroepithelial cells in the presence of a WNT pathway activator and a bone morphogenetic protein (BMP) signalling pathway activator.

- 5 In preferred examples, the population of neuroepithelial cells is an aggregate of neuroepithelial cells.

In preferred examples, the choroid plexus organoid comprises: (a) an epithelium comprising a tight epithelial barrier; and/or (b) one or more cysts surrounded by an epithelium.

- 10 In some examples, the stem cells are embryonic stem cells. In some examples, the stem cells are induced pluripotent stem cells.

- In some examples, culturing the population of neuroepithelial cells in the presence of a WNT pathway activator and a BMP signalling pathway activator is started less than 18 days e.g. between about 8-12 days after starting culturing the population of stem cells. In some examples, culturing the population of neuroepithelial cells in the presence of a WNT pathway activator and a BMP signalling pathway activator is started between about 9-11 days after starting culturing the population of stem cells. In some examples, culturing the population of neuroepithelial cells in the presence of a WNT pathway activator and a BMP signalling pathway activator is started between about 9.5-10.5 days after starting culturing the population of stem cells. In some examples, culturing the population of neuroepithelial cells in the presence of a WNT pathway activator and a BMP signalling pathway activator is started about 10 days after starting culturing the population of stem cells.
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In another aspect, the disclosure provides a method for producing a choroid plexus organoid comprising the steps:

- 25 (a) producing a population of embryoid bodies by culturing a population of stem cells;
- (b) culturing the population of embryoid bodies under conditions suitable for neural induction;
- (c) embedding the product of step (b) in a three-dimensional matrix, preferably an extracellular matrix (ECM);
- 30 (d) culturing the product of step (c) in the presence of a WNT pathway activator and a bone morphogenetic protein (BMP) signalling pathway activator, preferably wherein step (d) is started between about 8-12 days after the start of step (a).

- 35 In some examples, the stem cells are embryonic stem cells. In some examples, the stem cells are induced pluripotent stem cells.

In some examples, the culturing of step (a) is for about 4-6 days, preferably about 4.5-5.5 days, preferably about 5 days.

In preferred examples, the culturing of step (b) is carried out until neuroepithelia are present. In some examples, the culturing of step (b) is for about 1-3 days, preferably about 1.5-2.5 days, preferably about 2 days.

5 In some examples, the ECM is a polymerised ECM. In some examples, the ECM is a polymerised ECM gel. In some examples, the ECM is Matrigel.

Thus in some examples step (c) is performed using Matrigel, about 5- 9 days, more preferably 6-8 days, most preferably 7 days after starting culture.

10 In some examples, step (c) further comprises culturing the embedded embryoid bodies. In some examples, the culturing of step (c) is for about 2-4 days, preferably about 2.5-3.5 days, preferably about 3 days.

In some examples, step (d) is started between about 9-11 days, preferably about 9.5-10.5 days, preferably about 10 days after the start of step (a).

15 In some examples, the culturing in the presence of a WNT pathway activator and a BMP signalling pathway activator is for about 6-8 days, preferably about 6.5-7.5 days, preferably about 7 days.

In preferred examples, the culturing of step (d) comprises agitation.

In some examples, agitation is not applied during contact with the WNT pathway activator and the BMP signalling pathway activator. In some examples, the agitation is started at about day 15.

20 In some examples, the method comprises feeding the culture every 3-4 days with culture medium starting at day 15, optionally wherein ECM is added to the culture medium starting at day 30. Preferably, the ECM is soluble ECM – for example dissolved Matrigel.

In preferred examples, the method is an in vitro method.

25 In some examples, the WNT pathway activator is selected from the group consisting of CHIR99021, Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8a, Wnt8b, Wnt10a, Wnt10b, BML-284, 6-bromoindirubin-3'-oxime (BIO), WAY-316606, IQ1, QS11, SB-216763, LY2090314, DCA, 2-amino-4-[3,4-(methylenedioxy)benzyl-amino]-6-(3-methoxyphenyl)pyrimidine and lithium.

In preferred examples, the WNT pathway activator is CHIR99021.

30 In some examples, the BMP signalling pathway activator is selected from the group consisting of Bmp4, Bmp2, Bmp3, Bmp5, Bmp6, Bmp7, Bmp8a, Bmp8b, Bmp10, Bmp11, Bmp15, isoliquiritigenin, 4'-hydroxychalcone, apigenin, diosmetin, and a ventromorphins (e.g. SJ000291942, SJ000063181 and/or SJ00037178).

In preferred examples, the BMP signalling pathway activator is Bmp4.

35 In preferred examples, the WNT pathway activator is CHIR99021 and the BMP signalling pathway activator is Bmp4.

In some examples, the method or use does not comprise contacting the cells with a WNT pathway inhibitor.

In some examples, the method or use does not comprise exposing the cells to high oxygen partial pressure, for example an oxygen partial pressure greater than that of air (20%).

5 In some examples, the method or use comprises culturing only under an oxygen partial pressure of less than or equal to 20%.

As explained above, as part of its selective barrier function, the ChP epithelium is also highly selective to small molecules, including therapeutics. The present disclosure has particular utility in investigating and screening CNS-active drugs.

10 New drugs too often progress to clinical trial before failing for lack of efficacy, inability to cross in to the CNS, or limited translatability between animal models (52).

The present ChP organoids have utility in, for example, preclinical testing and modelling *in vivo* drug permeability into the CNS which may have benefits compared to the current standard in preclinical assays of CNS permeability (e.g. canine MDCK or Caco-2 cells, or
15 artificial membrane assays that are based on phospholipid mixtures which lack transporters and carrier proteins (53)).

In another aspect, the disclosure provides use of the choroid plexus organoid of the disclosure for drug discovery screening or assaying drug toxicity. The drug may be a CNS drug e.g. a small molecule drug e.g. for a neurodegenerative disease such as Alzheimer's
20 Disease

In some examples, the use is for testing the ability of a candidate agent to cross and/or selectively cross the blood-cerebrospinal fluid (CSF) barrier. In another aspect, the disclosure provides a method for testing the ability of a candidate agent to cross the blood-cerebrospinal fluid (CSF) barrier, comprising contacting the choroid plexus organoid of the
25 disclosure with the candidate agent and determining if the candidate agent crosses the epithelium.

DESCRIPTION OF THE DRAWINGS

Figure 1. Generation of choroid plexus organoids from human PSCs. (A) Protocol timeline showing different images from H9 organoid stages of growth. BMP4 and Wnt activator CHIR are added at day 10 of the protocol to promote dorsalisation. Scale bar: 1000µm. (B) Comparison of H1 treated, choroid plexus (ChP) organoid to untreated (UT), forebrain organoid at day 55. Scale bar: 1 mm. (C) H&E stained sections of H1 forebrain and choroid plexus organoids at day 40. Scale bar: 500µm. (D) Histological sections of E18.5 mouse embryonic brain (H&E stained), human fetal ChP at 15 post-conception weeks (Nissl stained, taken from Allen Brain Map <http://portal.brain-map.org/>), H1 human ChP organoid at day 40 (H&E stained). Scale bar: 500µm, 50µm for magnification. (E), (F) Representative confocal images of IMR90-4 iPSC derived forebrain and ChP organoids stained for TTR (grey, anti-sheep Alexafluor 647), ZO1 (green, anti-mouse Alexafluor 488) and Sox2 (magenta, anti-rabbit Alexafluor 568) at day 28 (E) and day 39 (F). Nuclei in blue are stained for DAPI. Scale bar: 100µm. (G) Quantification of percentage of TTR positive area over total organoid area in n=4 independent H9 batches (3-4 organoids per batch collected at d30, d40, d48).
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