[0005] Region-specific brain organoids or brain spheroids to not currently contain neuromodulatory systems and there are no human platforms to study, manipulate or investigate neuromodulatory pathways with human patient derived cells.

SUMMARY ~~OF THE INVENTION~~

[0006] Compositions and methods are provided for *in vitro* generation of human raphe nuclei organoids or spheroids (hRNS), which may be generated at least in part from human pluripotent stem (hPS) cells. Such spheroids model the human raphe nuclei and comprise specific sets of cells, e.g. serotonergic neurons, GABAergic neurons, etc that are associated with the raphe nuclei of a human.

[0006a] In one aspect, the present disclosure refers to a method for producing a human raphe nuclei-like spheroid or organoid (hRNS) *in vitro*, the method comprising:

(a) inducing a human pluripotent stem cell in 3D suspension culture to a neural fate to generate a neural spheroid by culturing in a medium comprising an inhibitor of bone morphogenetic protein (BMP) and an inhibitor of transforming growth factor β (TGFβ), and wherein the method comprises supplementing the medium with an inhibitor of GSK-3 for a period of from 2 to 10 days;

(b) differentiating the neural spheroid into a hRNS by culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a sonic hedgehog pathway agonist, and supplementing the neural medium with FGF4; and

(c) maintaining the hRNS in neural medium for at least one week, such that the hRNS comprises serotonergic neurons.

[0007] The hRNS can be functionally integrated with human cerebral cortical spheroids (hCS), which spheroids comprise human cortical neurons, such as glutamatergic neurons, to provide a cortico-raphe nuclei assembloid (hCS-hRNS). Human serotonergic neurons form bidirectional projections between neurons of the hRNS and hCS to generate a neuromodulatory assembloid. This assembloid is comprised of functionally integrated cells, including neurons, which interact in a physiologically relevant manner, *e.g.* forming synapses between classes of neurons so as to provide physiologically relevant, functional neural circuits. Using a combination of viral tracing and live imaging, evidence is provided herein for the formation of an *in vitro* generated human cortico-raphe nuclei neural circuit, which provides useful modeling of cortico-raphe nuclei pathway development and dysfunction.

[0008] In some ~~embodiment~~examples, assembloids are provided in which one or more of the cells have been genetically modified to provide for additional functionality in screening. For example, one or both of cortical neurons and serotonergic neurons can be genetically modified to

express a fluorescent calcium indicator, which indicators are known and used in the art. One or both of cortical neurons and serotonergic neurons can be genetically modified to express a light activated opsin. In some ~~embodiment~~examples, an assembloid comprises serotonergic neurons expressing an opsin, and cortical, e.g. glutaminergic, neurons expressing a fluorescent calcium indicator, where a functional relationship between the neurons is demonstrated by using light to activate the serotonergic neuron, and observing a calcium indicator response from a cortical neuron. 5HT lineage-specific viral tools, such as AAV-based *FEV* minipromoter-driven reporters, are available and hve been shown to work below to specifically probe and study 5HT lineage cells in hRNS and hCS-hRNS.

[0008a] In another aspect, the present disclosure refers to a method for producing a cortico-raphe nuclei assembloid (hCS-hRNS) *in vitro*, the method comprising:

(i) producing a human raphe nuclei spheroid (hRNS) by the method disclosed herein from a first human pluripotent stem cell

(ii) (a) inducing a second human pluripotent stem cell in a second suspension culture to a neural fate to derive a second neural spheroid by culturing in a medium comprising an inhibitor of bone morphogenetic protein (BMP) and an inhibitor of transforming growth factor β (TGFβ), and wherein the method comprises supplementing the medium with an inhibitor of GSK-3 for a period of from 2 to 10 days;

(b) differentiating the second neural spheroid into a cortical spheroid (hCS), and

(iii) culturing the hRNS and hCS under conditions permissive for cell fusion in neural medium, such that the cortico-raphe assembloid comprises human serotonergic neurons with projections between the hRNS and hCS, and neurons from the hCS projecting into hRNS wherein the human neurons form bidirectional projections between the hRNS and hCS.

[0008b] In another aspect, the present disclosure refers to a human raphe nuclei spheroid (hRNS) obtained by any of the method disclosed herein.

[0008c] In yet another aspect, the present disclosure refers to a cortico-raphe nuclei assembloid (hCS-hRNS) comprising a raphe nuclei spheroid (hRNS) fused to a cortical spheroid (hCS), wherein the hCS-hRNS comprises human serotonergic neurons projecting between the hRNS and hCS and forming a human neuromodulatory circuit, and wherein the hCS-hRNS is capable of being maintained in suspension culture without adhering to a surface for at least 4 weeks, obtained by any of the method disclosed herein.

[0009] The hRNS spheroid and hCS-hRNS assembloid provide unique opportunities for analysis of the development and function of serotonergic neural circuits between the raphe nuclei and the

[0012] Also provided are methods for determining the activity of a candidate agent neural circuits in the assembloids, the method comprising contacting the candidate agent with the assembloid. The cells present in the assembloid optionally comprise at least one allele encoding a mutation associated with, or potentially associated with, a neurologic or psychiatric disorder, and determining the effect of the agent on morphological, genetic or functional parameters, including without limitation neuron number, neuron function, gene expression profiling, cell death, single cell gene expression (RNA-seq), calcium imaging with pharmacological screens, patch-clamp recordings, modulation of synaptogenesis, and the like.

[0012a] In yet another aspect, the present disclosure refers to a method of determining the effect of a candidate agent on serotonergic modulation on cortical neural circuits, the method comprising: contacting the candidate agent with the cortico-raphe nuclei assembloid (hCS-hRNS) of disclosed herein; and determining the effect of the candidate agent on the ability of serotonergic neurons to modulate function of cortical neural circuits,
optionally wherein cells of the hCS-hRNS comprise at least one allele or genetic event associated with a neurologic or psychiatric disorder, further optionally wherein the neurologic or psychiatric disorder is selected from the group consisting of: schizophrenia, affective disorder and autism spectrum disorder (ASD).

[0013] These and other objects, advantages, and features of the ~~invention~~ disclosure will become apparent to those persons skilled in the art upon reading the details of the subject methods and compositions as more fully described below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0015] **FIG. 1A-1G**. (**A**) Schematic showing hRNS-hCS assembly (**B**) Schematic showing the recipe for deriving human raphe nuclei-like spheroids (hRNS). (**C**) RT-qPCR profiling of genes expressed in the developing hindbrain in human cortical spheroids (hCS) versus hRNS. Different hiPSC lines are represented in different colors. (**D**) Representative images of immunocytochemistry (ICC) showing hindbrain progenitors and (**E**) 5-HT neural lineage cells. (**F**)

5-HT neurotransmitter levels measured by HPLC from hRNS across different *in vitro* stages and hiPSC lines. (**G**) RT-qPCR profiling of different 5-HT receptors (HTRs) in hCS at day 100 of *in vitro* differentiation. HTR subtype is indicated by color.

[0016] **FIG. 2A-2C (A)** UMAP projections of single cell transcriptomic data from 13,708 hRNS cells harvested from 9 spheroids derived from 3 hiPSC lines between day 79 and 82 of *in vitro* differentiation. (**B**) Cluster marker expression showing different neuronal populations in hRNS. (**C**) FEV+ subclustering showing subcluster separated of caudal and rostral identities (top) and heatmap of top 10 differentially expressed genes in each cluster (bottom).

[0017] **FIG. 3A-3C**. (**A**) Assembly of hCS and AAV-Syn1::mCherry-labeled hRNS (Left). 3D reconstruction of a hCS-hRNS assembloid, with hRNS cells labeled in mCherry (Right). (**B**)

That which is claimed is:

1. A method for producing a human raphe nuclei-like spheroid or organoid (hRNS) *in vitro*, the method comprising:

(a) inducing a human pluripotent stem cell in 3D suspension culture to a neural fate to generate a neural spheroid~~;~~

~~(b) differentiating the neural spheroid into a hRNS; and~~

~~(c) maintaining the hRNS in neural medium, such that the hRNS comprises serotonergic neurons.~~

~~2. The method of claim 1, wherein the human pluripotent stem cell is an induced human pluripotent stem cell.~~

~~3. The method of claim 1, wherein inducing the human pluripotent stem cell in suspension culture to the neural fate in step (a) comprises~~ by culturing in a medium comprising an inhibitor of bone morphogenetic protein (BMP) and an inhibitor of transforming growth factor β (TGFβ), and wherein the method comprises supplementing the medium with an inhibitor of GSK-3 for a period of from 2 to 10 days;

(b) differentiating the neural spheroid into a hRNS by culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a sonic hedgehog pathway agonist, and supplementing the neural medium with FGF4; and

(c) maintaining the hRNS in neural medium for at least one week, such that the hRNS comprises serotonergic neurons.

2. A method for producing a cortico-raphe nuclei assembloid (hCS-hRNS) *in vitro*, the method comprising:

(i) producing a human raphe nuclei spheroid (hRNS) by the method of claim 1 from a first human pluripotent stem cell

(ii) (a) inducing a second human pluripotent stem cell in a second suspension culture to a neural fate to derive a second neural spheroid by culturing in a medium comprising an inhibitor of bone morphogenetic protein (BMP) and an inhibitor of transforming growth factor β (TGFβ), and wherein the method comprises supplementing the medium with an inhibitor of GSK-3 for a period of from 2 to 10 days;

(b) differentiating the second neural spheroid into a cortical spheroid (hCS), and

(iii) culturing the hRNS and hCS under conditions permissive for cell fusion in neural medium, such that the cortico-raphe assembloid comprises human serotonergic neurons with projections between the hRNS and hCS, and neurons from the hCS projecting into hRNS wherein the human neurons form bidirectional projections between the hRNS and hCS.

43. The method of claim 31 or claim 2, wherein the inhibitor of BMP is selected from the group of dorsomorphin, LDN-193189, and LY364947, and the inhibitor of TGFβ is SB-431542.

5. The method of claim 3 or claim 4, wherein and the inhibitor of GSK-3 is CHIR99021.

6. The method of claim 3, wherein culturing in the medium comprising the inhibitor of BMP and inhibitor of TGFβ is for a period of from 2 to 10 days.

7. The method of claim 6, optionally wherein the medium is supplemented with the inhibitor of GSK-3 after a period of 1 to 2 days such that the medium comprises the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3, optionally wherein culturing in the medium comprising the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3 is for a period of from 4 to 8 days.

84. The method of any of claims 1-3claim 7, wherein in step (a) culturing in the medium comprising the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3 comprises:

(1) culturing in a medium comprising the inhibitor of BMP, the inhibitor of TGFβ, and inhibitor of GSK-3 for a period of 2 to 5 days, wherein the inhibitor of TGFβ is present at a concentration of between 5 μM to 20 μM; and subsequently

(2) culturing in a medium comprising the inhibitor of BMP, the inhibitor of TGFβ, and inhibitor of GSK-3 for a period of 2 to 5 days, wherein the inhibitor of TGFβ is present at a concentration of between 1 μM to 5 μM.

9. The method of claim 1, wherein the suspension culture of step (a) is feeder layer free.

~~10. The method of claim 1, wherein differentiating the neural spheroid into the hRNS of step (b) comprises culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a sonic hedgehog pathway agonist, and wherein the method comprises supplementing the neural medium with FGF4.~~

~~11. The method of claim 10, wherein the inhibitor of GSK-3 is CHIR99021.~~

~~12. The method of claim 10 or claim 11, wherein the sonic hedgehog (SHH) pathway agonist is smoothened agonist (SAG).~~

~~13~~5. The method of any of claims ~~10~~1-4, wherein in step (b) the neural spheroid is cultured in neural medium comprising the inhibitor of GSK-3 and a SHH pathway agonist for a period of 1 to 3 weeks, and wherein the neural medium is supplemented with FGF4 after a period of 2 to 10 days.

~~14~~6. The method of any of claims 1-5, wherein step (b) further comprises supplementing the neural medium with at least one of the compounds selected from the group consisting of: brain-derived neurotrophic factor (BDNF), NT3, L-Ascorbic Acid 2-phosphate Trisodium Salt (AA), N6, 2'-O-Dibutyryladenosine 3', 5' -cyclic monophosphate sodium salt (cAMP), cis-4, 7, 10, 13, 16, 19- Docosahexaenoic acid (DHA), and DAPT,

optionally wherein the neural medium is supplemented with the at least one compound after a period of 1 to 3 weeks.

~~15~~7. The method of any of claims 1-6, wherein differentiating the neural spheroid into the hRNS of step (b) comprises:

(1) culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a sonic hedgehog pathway agonist for a period of 2 to 10 days;

(2) supplementing the neural medium with FGF4; and

(3) culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3, sonic hedgehog pathway agonist and FGF4 for a period of 1 to 3 weeks.

~~16~~8. The method of claim ~~15~~7, further comprising following step (3):

(4) culturing the neural spheroid in suspension culture for a period of 2 to 10 days in neural medium comprising FGF4 and at least one of the compounds selected from the group consisting of: brain-derived neurotrophic factor (BDNF), NT3, L-Ascorbic Acid 2-phosphate Trisodium Salt (AA), N6, 2'-O-Dibutyryladenosine 3', 5' -cyclic monophosphate sodium salt (cAMP), cis-4, 7, 10, 13, 16, 19- Docosahexaenoic acid (DHA), and DAPT; and

(5) culturing the neural spheroid in suspension culture for at least 1 week in neural medium comprising the at least one compound in the absence of FGF4.

~~17. The method of claim 1, wherein maintaining the hRNS in step (c) is carried out in neural medium in the absence of growth factors.~~

~~18. The method of claim 1, wherein maintaining the hRNS in step (c) is for at least 1 week.~~

~~19~~. The method of any of claims 1-8, wherein cells of the hRNS comprise at least one allele associated with a neurologic or psychiatric disorder~~.~~.

~~20. The method of claim 17,~~ wherein the neurologic or psychiatric disorder is selected from the group consisting of: schizophrenia, affective disorder and autism spectrum disorder (ASD), optionally wherein the affective disorder is major depressive disorder, bipolar disorder or an anxiety disorder.

~~21. A human raphe nuclei spheroid (hRNS) comprising human serotonergic neurons, wherein the hRNS is capable of being maintained in suspension culture without adhering to a surface for at least 1 month.~~

~~22. A human raphe nuclei spheroid (hRNS) obtained by the method of claim 1.~~

~~23. A method for producing a cortico-raphe nuclei assembloid (hCS-hRNS) *in vitro*, the method comprising:~~

~~(i) (a) inducing a first human pluripotent stem cell in suspension culture to a neural fate to provide a first neural spheroid;~~

(b) differentiating the first neural spheroid into a human raphe nuclei spheroid (hRNS),

(ii) (a) inducing a human pluripotent stem cell in a second suspension culture to a neural fate to derive a second neural spheroid;

(b) differentiating the second neural spheroid into a cortical spheroid (hCS), and

(iii) culturing the hRNS and hCS under conditions permissive for cell fusion in neural medium, such that the cortico-raphe assembloid comprises human serotonergic neurons with projections between the hRNS and hCS, and neurons from the hCS projecting into hRNS.

24. The method of claim 23, wherein human neurons form bidirectional projections between the hRNS and hCS.

25. The method of claim 23, wherein the first and/or second human pluripotent stem cell is an induced human pluripotent stem cell.

26. The method of claim 23, wherein inducing the first human pluripotent stem cell in suspension culture to the neural fate in step (i)(a) comprises culturing in a medium comprising an inhibitor of bone morphogenetic protein (BMP) and an inhibitor of transforming growth factor β (TGFβ), and wherein the method comprises supplementing the medium with an inhibitor of GSK-3.

27. The method of claim 23, wherein the inhibitor of BMP is dorsomorphin and the inhibitor of TGFβ is SB-431542.

28. The method of claim 23 - 26, wherein the inhibitor of GSK-3 is CHIR99021.

29. The method of claim 23, wherein culturing in the medium comprising the inhibitor of BMP and inhibitor of TGFβ is for a period of from 2 to 10 days.

30. The method of claim 29, wherein the medium is supplemented with the inhibitor of GSK-3 after a period of 1 to 2 days such that the medium comprises the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3, optionally wherein culturing in the medium comprising the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3 is for a period of from 4 to 8 days.

31. The method of claim 30, wherein culturing in the medium comprising the inhibitor of BMP, inhibitor of TGFβ and inhibitor of GSK-3 comprises:

(1) culturing in a medium comprising the inhibitor of BMP, the inhibitor of TGFβ, and inhibitor of GSK-3 for a period of 2 to 5 days, wherein the inhibitor of TGFβ is present at a concentration of between 5 µM to 20 µM; and subsequently

(2) culturing in a medium comprising the inhibitor of BMP, the inhibitor of TGFβ, and inhibitor of GSK-3 for a period of 2 to 5 days, wherein the inhibitor of TGFβ is present at a concentration of between 1 µM to 5 µM.

32. The method of claim 23, wherein differentiating the first neural spheroid into the hRNS of step (i)(b) comprises culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a SHH pathway agonist, and wherein the method comprises supplementing the neural medium with FGF4.

33. The method of claim 32, wherein the inhibitor of GSK-3 is CHIR99021.

34. The method of claim 32-33, wherein the SHH pathway agonist is smoothened agonist (SAG).

35. The method of claim 32, wherein the neural spheroid is cultured in neural medium comprising the inhibitor of GSK-3 and a sonic hedgehog pathway agonist for a period of 1 to 3 weeks, and wherein the neural medium is supplemented with FGF4 after a period of 2 to 10 days.

36. The method of claim 23, wherein step (i)(b) further comprises supplementing the neural medium with at least one of the compounds selected from the group consisting of: brain-derived neurotrophic factor (BDNF), NT-3, L-Ascorbic Acid 2-phosphate Trisodium Salt (AA), N6, 2'-O-Dibutyryladenosine 3', 5' -cyclic monophosphate sodium salt (cAMP), cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid (DHA), and DAPT,

optionally wherein the neural medium is supplemented with the at least one compound after a period of 1 to 3 weeks.

37. The method of claim 23, wherein differentiating the first neural spheroid into the hRNS of step (i)(b) comprises:

(1) culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3 and a sonic hedgehog pathway agonist for a period of 2 to 10 days;
(2) supplementing the neural medium with FGF4; and
(3) culturing the neural spheroid in suspension culture in neural medium comprising an inhibitor of GSK-3, sonic hedgehog pathway agonist and FGF4 for a period of 1 to 3 weeks.

38. The method of claim 37, further comprising following step (3):
(4) culturing the neural spheroid in suspension culture for a period of 2 to 10 days in neural medium comprising FGF4 and at least one of the compounds selected from the group consisting of: brain-derived neurotrophic factor (BDNF), NT3, L-Ascorbic Acid 2-phosphate Trisodium Salt (AA), N6, 2'-O-Dibutyryladenosine 3', 5' -cyclic monophosphate sodium salt (cAMP), cis-4, 7, 10, 13, 16, 19- Docosahexaenoic acid (DHA), and DAPT; and
(5) culturing the neural spheroid in suspension culture for at least 1 week in neural medium comprising the at least one compound in the absence of FGF4.

3910. The method of claim 23, wherein step (iii) comprises culturing the hRNS and hCS are cultured under conditions permissive for cell fusion for at least 3 days, optionally in direct physical contact.

40. The method of claim 23, wherein step (iii) comprises culturing the hRNS and hCS in direct physical contact to each other.

41. The method of claim 23, wherein the suspension culture of step (i)(a) and/or step (ii)(a) is feeder layer free culture condition.
11. The method of any of claims 1-10, wherein one or more of the suspension cultures are feeder layer free culture conditions.

4212. The method of any of claims 231-11, wherein cells of the human raphe nuclei-like spheroid or organoid (hRNS), or cortico-raphe nuclei assembloid comprise at least one allele or genetic event associated with a neurologic or psychiatric disorder.

43. The method of claim 23, wherein either the first or second human pluripotent stem cell comprises at least one allele or genetic event associated with a neurologic or psychiatric disorder.

~~44. The method of claim 42-43,~~ wherein the neurologic or psychiatric disorder is selected from the group consisting of: schizophrenia, affective disorder and autism spectrum disorder (ASD), optionally wherein the affective disorder is major depressive disorder, bipolar disorder or an anxiety disorder.

13. A human raphe nuclei spheroid (hRNS) obtained by the method of any of claims 1, or 3-12.

~~45~~14. A cortico-raphe nuclei assembloid (hCS-hRNS) comprising a raphe nuclei spheroid (hRNS) fused to a cortical spheroid (hCS), wherein the hCS-hRNS comprises human serotonergic neurons projecting between the hRNS and hCS and forming a human neuromodulatory circuit, and wherein the hCS-hRNS is capable of being maintained in suspension culture without adhering to a surface for at least 4 weeks, obtained by the method of any of claims 2-12.

~~46. A cortico-raphe nuclei assembloid produced by the method of any of claims 23-44.~~

~~47~~15. A method of determining the effect of a candidate agent on serotonergic modulation on cortical neural circuits, the method comprising: contacting the candidate agent with the cortico-raphe nuclei assembloid (hCS-hRNS) of claim ~~45-46~~14; and determining the effect of the candidate agent on the ability of serotonergic neurons to modulate function of cortical neural circuits,

optionally wherein cells of the hCS-hRNS comprise at least one allele or genetic event associated with a neurologic or psychiatric disorder, further optionally wherein the neurologic or psychiatric disorder is selected from the group consisting of: schizophrenia, affective disorder and autism spectrum disorder (ASD).

~~48. The method of claim 47, wherein the candidate agent is a selective serotonin reuptake inhibitor (SSRI).~~