(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 July 2017 (20.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/123791 A1

(51) International Patent Classification:
A61K 35/12 (2015.01) *A61P 19/00* (2006.01)
A61L 27/00 (2006.01) *A61P 25/00* (2006.01)
A61L 27/40 (2006.01) *A61P 43/00* (2006.01)

(21) International Application Number:
PCT/US2017/013231

(22) International Filing Date:
12 January 2017 (12.01.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/278,857 14 January 2016 (14.01.2016) US
62/298,872 23 February 2016 (23.02.2016) US

(71) Applicant: **OHIO STATE INNOVATION FOUNDATION** [US/US]; 1524 North High Street, Columbus, Ohio 43201 (US).

(72) Inventors: **ANAND, Rene**; c/o Ohio State Innovation Foundation, 1524 North High Street, Columbus, Ohio 43201 (US). **MCKAY, Susan**; c/o Ohio State Innovation Foundation, 1524 North High Street, Columbus, Ohio 43201 (US).

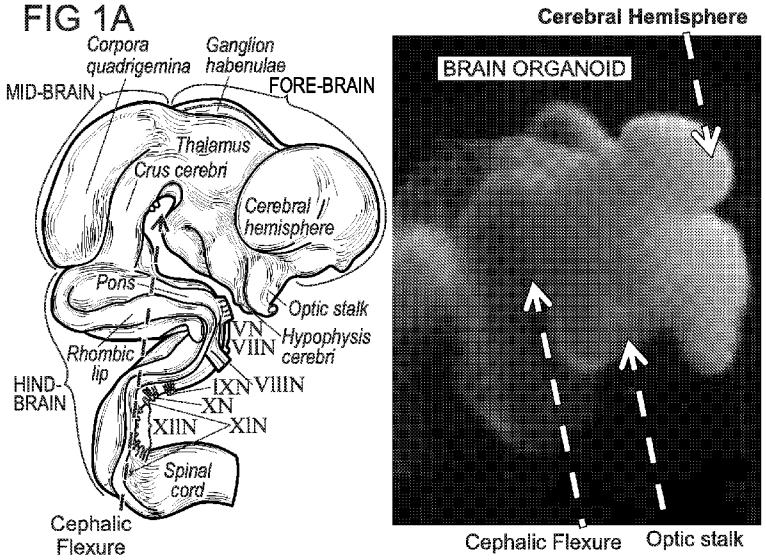
(74) Agent: **HUNTER-ENSOR, PH.D., Melissa**; Greenberg Traurig, LLP, One International Place, Boston, Massachusetts 02110 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: A NEURAL ORGANOID COMPOSITION AND METHODS OF USE



(57) Abstract: The present invention features a neural organoid that recapitulates *in vitro* most characteristics of the brain (e.g., human), and methods of using this neural organoid to study disease and to identify therapeutic agents for the treatment of neurological diseases and disorders.

WO 2017/123791 A1

Published:

— *with international search report (Art. 21(3))*

A NEURAL ORGANOID COMPOSITION AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of the following U.S. Provisional Application Nos.: 62/278,857, filed January 14, 2016 and 62/298,872, filed February 23, 2016, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Nearly one-third of adults will be affected by neurodevelopmental, neuropsychiatric or neurological disease (e.g., autism, anxiety, mood disorders, neurodegenerative disease) at least once in their life. The cost of brain disease to the US and European economies is estimated to be hundreds of billions of dollars per year. Neuroscience has typically relied on the experimental manipulation of living brains or tissue samples, but scientific progress has been limited by a number of factors. For ethical and technical reasons, most invasive techniques are impossible to use on humans. Experiments in animals are expensive and results obtained in animals must be verified in long and expensive human clinical trials. Improved experimental models of the human brain are urgently required to understand disease mechanisms and test potential therapeutics.

SUMMARY OF THE INVENTION

As described below, the present invention features a neural organoid that recapitulates *in vitro* most characteristics of the brain (e.g., human), and methods of using this neural organoid to study disease and to identify therapeutic agents for the treatment of neurological diseases and disorders.

In one aspect, the invention features an *in vitro* generated three-dimensional neural organoid derived from a human induced pluripotent stem cell (hIPSC), the organoid containing a first region expressing retinal or cortical markers and one or more additional neural regions, each expressing markers of the brain stem, cerebellum, and/or spinal cord. In one embodiment, the organoid comprises a cell expressing one or more neural markers and a cell expressing an astrocytic marker, oligodendrocyte marker, microglia marker, and/or vascular marker. In another embodiment, the hIPSC comprises a genetic mutation associated with a neurological defect. In another embodiment, the genetic mutation is in TSC1, TSC2, PSEN1, or APP.

In one aspect, the invention features an *in vitro* generated three-dimensional neural organoid derived from human induced pluripotent stem cells, the organoid containing a first cell type expressing neural markers, and a second cell type expressing an astrocytic marker, oligodendrocyte marker, microglia marker, or vascular marker. In one embodiment, the retinal marker is retina specific Guanylate Cyclases (GUY2D, GUY2F), Retina And Anterior Neural Fold Homeobox (RAX), and retina specific Amine Oxidase, Copper Containing 2 (RAX). In another embodiment, the neural marker is a cortical marker that is doublecortin, NeuN, FOXP2, CNTN4, and TBR1. In another embodiment, the neural marker is a marker of dopaminergic neurons selected from the group consisting of tyrosine hydroxylase, vesicular monoamine transporter 2 (VMAT2), dopamine active transporter (DAT) and Dopamine receptor D_2 (D2R). In another embodiment, the neural marker is ATOH1, PAX6, SOX2, LHX2, GRID2, or another cerebellar marker. In another embodiment, the neural marker is SOX2, NeuroD1, DCX, EMX2, FOXG1, PROX1, or another granule neuron marker. In another embodiment, the neural marker is FGF8, INSM1, GATA2, ASCL1, GATA3, or another brain stem marker. In another embodiment, the neural marker is a homeobox gene that is HOXA1, A2, A3, B4, A5, C8, or D13. In another embodiment, the neural marker is NKCC1, KCC2, or another GABAergic marker. In another embodiment, the astrocytic marker is GFAP, the oliogodendrocytic marker is OLIG2 or MBP, the microglia marker is AIF1 or CD4, and the vascular marker is NOS3.

In another aspect, the invention features a method for obtaining a neural organoid, the method includes selecting minimally adherent human induced pluripotent stem cells (hIPScs) from a mixed culture of hIPScs and gamma irradiated mouse embryonic fibroblast feeder cells (MEFs), and culturing the IPSCs under conditions that facilitate sphere formation to obtain an embryoid body (EB); transferring the EB to a plate and culture under conditions that induce neuroectodermal differentiation; culturing the EB in a three-dimensional matrix comprising growth factors for about 3-5 days under static conditions; culturing the EB in a three-dimensional matrix under conditions that facilitate the laminar flow of growth media, thereby obtaining a neural organoid.

In another aspect, the invention features a method for obtaining a neural organoid, the method involving culturing iPSCs alone or in the presence of irradiated MEFs; culturing the iPSCs from the previous step under conditions that promote germ layer differentiation in a low-attachment U-bottom plate in the presence of ROCK inhibitor and bFGF for about four days and then culturing the iPSCs in media lacking ROCK inhibitor or bFGF to form ; plating

the iPSCs from the previous step in a low-attachment plate under conditions that promote neural induction and selecting embryoid bodies displaying neuroectodermal outgrowth from the embryoid body; embedding the selected embryoid body in a 3-dimensional culture matrix and culturing under conditions that promote neural organoid development while gently oscillating the culture 2-3 times daily; and statically culturing the neural organoid.

In various embodiments of the above-aspects, beta mercaptoethanol is stored under conditions that minimize oxidation is added to the culture media at each step in the method. In other embodiments, the culture is gently oscillated for about 1-5 (e.g., 1, 2, 3, 4, 5) minutes twice daily to induce slow laminar flow of media within the culture. In other embodiments, the amount of 3-dimensional culture matrix is optimized to sequester morphogens and growth factor while permitting exchange of nutrients and gases. . In another embodiment, the embryoid body is embedded in about 10, 20, or 30 µl of 3-dimensional culture matrix. In other embodiment, the hIPSCs are selected by allowing the MEFs to adhere to a substrate, then removing the non-adherent hIPSCs. In other embodiment, the three-dimensional matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) sarcoma cells.

In another aspect, the invention features an *in vitro* derived neural organoid generated according to any previous aspect, wherein the organoid comprises a first region expressing retinal or cortical markers and one or more additional regions expressing markers of the midbrain, brain stem, cerebellum, and/or spinal cord.

Compositions and articles defined by the invention were isolated or otherwise manufactured. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By "amyloid precursor protein" is meant a protein having at least about 85% identity to NCBI Ref Seq. NP_001129488 or a fragment thereof, which is associated with Alzheimer's disease. In one embodiment, an APP sequence is duplicated in Alzheimer's disease. An exemplary APP sequence is provided below:

```
  1 mdqledllvl finyvptdgn agllaepqia mfcgrlnmhm nvqngkwdsd psgtktcidt
 61 kegilqycqe vypelqitnv veanqpvtiq nwckrgrkqc kthphfvipy rclvgefvsd
121 allvpdkckf lhqermdvce thlhwhtvak etcsekstnl hdygmllpcg idkfrgvefv
181 ccplaeesdn vdsadaeedd sdvwwggadt dyadgsedkv vevaeeeeva eveeeeaddd
241 eddedgdeve eeaeepyeea terttsiatt tttttesvee vvrevcseqa etgpcramis
301 rwyfdvtegk capffyggcg gnrnnfdtee ycmavcgsai pttaastpda vdkyletpgd
361 enehahfqka kerleakhre rmsqvmrewe eaerqaknlp kadkkaviqh fqekvesleq
421 eaanerqqlv ethmarveam lndrrrlale nyitalqavp prprhvfnml kkyvraeqkd
481 rqhtlkhfeh vrmvdpkkaa qirsqvmthl rviyermnqs lsllynvpav aeeiqdevde
541 llqkeqnysd dvlanmisep risygndalm psltetkttv ellpvngefs lddlqpwhsf
601 gadsvpante nevepvdarp aadrglttrp gsgltnikte eisevkmdae frhdsgyevh
661 hqklvffaed vgsnkgaiig lmvggvviat vivitlvmlk kkqytsihhg vvevdaavtp
721 eerhlskmqq ngyenptykf feqmqn
```

By "APP polynucleotide" is meant a nucleic acid molecule encoding an APP protein.

By "organoid" is meant an organized mass of cell types generated *in vitro* that mirrors at least to some degree the structure, marker expression, or function of a naturally occurring organ.

By "neural marker" is meant any protein or polynucleotide the expression of which is associated with a neural cell fate. Exemplary neural markers include markers associated with the cortex, retina, cerebellum, brain stem, granular neurons, dopaminergic, and GABAergic neurons. Exemplary cerebellar markers include but are not limited to ATOH1, PAX6, SOX2, LHX2, and GRID2. Exemplary markers of dopaminergic neurons include but are not limited to tyrosine hydroxylase, vesicular monoamine transporter 2 (VMAT2), dopamine active transporter (DAT) and Dopamine receptor D_2 (D2R). Exemplary cortical markers include, but are not limited to, doublecortin, NeuN, FOXP2, CNTN4, and TBR1. Exemplary retinal markers s include but are not limited to retina specific Guanylate Cyclases (GUY2D, GUY2F), Retina And Anterior Neural Fold Homeobox (RAX), and retina specific Amine Oxidase, Copper Containing 2 (RAX). Exemplary granular neuron markers include, but are not limited to SOX2, NeuroD1, DCX, EMX2, FOXG1, and PROX1. Exemplary brain stem markers include, but are not limited to FGF8, INSM1, GATA2, ASCL1, GATA3. Exemplary spinal cord markers include, but are not limited to homeobox genes including but not limited to HOXA1, A2, A3, B4, A5, C8, or D13. Exemplary GABAergic markers include, but are not limited to NKCC1 or KCC2. Exemplary astrocytic markers include, but are not limited to GFAP. Exemplary oliogodendrocytic markers include, but are not limited

to OLIG2 or MBP. Exemplary microglia markers include, but are not limited to AIF1 or CD4. Exemplary vascular markers include, but are not limited to NOS3.

By "TSC1 polypeptide" is meant a protein or fragment thereof having at least 85% amino acid identity to the sequence provided at NCBI Ref: NP_000359.1 that functions in brain development. An exemplary human amino acid sequence is provided below:

```
   1 MAQQANVGEL LAMLDSPMLG VRDDVTAVFK ENLNSDRGPM LVNTLVDYYL ETSSQPALHI
  61 LTTLQEPHDK HLLDRINEYV GKAATRLSIL SLLGHVIRLQ PSWKHKLSQA PLLPSLLKCL
 121 KMDTDVVVLT TGVLVLITML PMIPQSGKQH LLDFFDIFGR LSSWCLKKPG HVAEVYLVHL
 181 HASVYALFHR LYGMYPCNFV SFLRSHYSMK ENLETFEEVV KPMMEHVRIH PELVTGSKDH
 241 ELDPRRWKRL ETHDVVIECA KISLDPTEAS YEDGYSVSHQ ISARFPHRSA DVTTSPYADT
 301 QNSYGCATST PYSTSRLMLL NMPGQLPQTL SSPSTRLITE PPQATLWSPS MVCGMTTPPT
 361 SPGNVPPDLS HPYSKVFGTT AGGKGTPLGT PATSPPPAPL CHSDDYVHIS LPQATVTPPR
 421 KEERMDSARP CLHRQHHLLN DRGSEEPPGS KGSVTLSDLP GFLGDLASEE DSIEKDKEEA
 481 AISRELSEIT TAEAEPVVPR GGFDSPFYRD SLPGSQRKTH SAASSSQGAS VNPEPLHSSL
 541 DKLGPDTPKQ AFTPIDLPCG SADESPAGDR ECQTSLETSI FTPSPCKIPP PTRVGFGSGQ
 601 PPPYDHLFEV ALPKTAHHFV IRKTEELLKK AKGNTEEDGV PSTSPMEVLD RLIQQGADAH
 661 SKELNKLPLP SKSVDWTHFG GSPPSDEIRT LRDQLLLLHN QLLYERFKRQ QHALRNRRLL
 721 RKVIKAAALE EHNAAMKDQL KLQEKDIQMW KVSLQKEQAR YNQLQEQRDT MVTKLHSQIR
 781 QLQHDREEFY NQSQELQTKL EDCRNMIAEL RIELKKANNK VCHTELLLSQ VSQKLSNSES
 841 VQQQMEFLNR QLLVLGEVNE LYLEQLQNKH SDTTKEVEMM KAAYRKELEK NRSHVLQQTQ
 901 RLDTSQKRIL ELESHLAKKD HLLLEQKKYL EDVKLQARGQ LQAAESRYEA QKRITQVFEL
 961 EILDLYGRLE KDGLLKKLEE EKAEAAEAAE ERLDCCNDGC SDSMVGHNEE ASGHNGETKT
1021 PRPSSARGSS GSRGGGGSSS SSSELSTPEK PPHQRAGPFS SRWETTMGEA SASIPTTVGS
1081 LPSSKSFLGM KARELFRNKS ESQCDEDGMT SSLSESLKTE LGKDLGVEAK IPLNLDGPHP
1141 SPPTPDSVGQ LHIMDYNETH HEHS"
```

By "TSC1 polynucleotide" is meant any nucleic acid sequence encoding an TSC1 polypeptide or fragment thereof. An exemplary human TSC1 nucleic acid sequence is provided at NCBI Ref NM_000368

```
   1 acgacggggg aggtgctgta cgtccaagat ggcggcgccc tgtaggctgg agggactgtg
  61 aggtaaacag ctgaggggga ggagacggtg gtgaccatga aagacaccag gttgacagca
 121 ctggaaactg aagtaccagt tgtcgctaga acagtttggt agtggcccca atgaagaacc
 181 ttcagaacct gtagcacacg tcctggagcc agcacagcgc cttcgagcga gagaatggcc
 241 caacaagcaa atgtcgggga gcttcttgcc atgctggact cccccatgct gggtgtgcgg
 301 gacgacgtga cagctgtctt taaagagaac ctcaattctg accgtggccc tatgcttgta
 361 aacaccttgg tggattatta cctggaaacc agctctcagc cggcattgca catcctgacc
 421 accttgcaag agccacatga caagcacctc ttggacagga ttaacgaata tgtgggcaaa
 481 gccgccactc gtttatccat cctctcgtta ctgggtcatg tcataagact gcagccatct
 541 tggaagcata agctctctca agcacctctt ttgccttctt tactaaaatg tctcaagatg
 601 gacactgacg tcgttgtcct cacaacaggc gtcttggtgt tgataaccat gctaccaatg
 661 attccacagt ctgggaaaca gcatcttctt gatttctttg acatttttgg ccgtctgtca
 721 tcatggtgcc tgaagaaacc aggccacgtg gcggaagtct atctcgtcca tctccatgcc
 781 agtgtgtacg cactctttca tcgcctttat ggaatgtacc cttgcaactt cgtctccttt
 841 ttgcgttctc attacagtat gaaagaaaac ctggagactt ttgaagaagt ggtcaagcca
 901 atgatggagc atgtgcgaat tcatccggaa ttagtgactg gatccaagga ccatgaactg
 961 gaccctcgaa ggtggaagag attagaaact catgatgttg tgatcgagtg tgccaaaatc
1021 tctctggatc ccacagaagc ctcatatgaa gatggctatt ctgtgtctca ccaaatctca
1081 gcccgctttc ctcatcgttc agccgatgtc accaccagcc cttatgctga cacacagaat
1141 agctatgggt gtgctacttc tacccccttac tccacgtctc ggctgatgtt gttaaatatg
1201 ccagggcagc tacctcagac tctgagttcc ccatcgacac ggctgataac tgaaccacca
1261 caagctactc tttggagccc atctatggtt tgtggtatga ccactcctcc aacttctcct
1321 ggaaatgtcc cacctgatct gtcacaccct tacagtaaag tctttggtac aactgcaggt
1381 ggaaaaggaa ctcctctggg aaccccagca acctctcctc ctccagcccc actctgtcat
```

```
1441 tcggatgact acgtgcacat ttcactcccc caggccacag tcacaccccc caggaaggaa
1501 gagagaatgg attctgcaag accatgtcta cacagacaac accatcttct gaatgacaga
1561 ggatcagaag agccacctgg cagcaaaggt tctgtcactc taagtgatct tccagggttt
1621 ttaggtgatc tggcctctga agaagatagt attgaaaaag ataaagaaga agctgcaata
1681 tctagagaac tttctgagat caccacagca gaggcagagc ctgtggttcc tcgaggaggc
1741 tttgactctc ccttttaccg agacagtctc ccaggttctc agcggaagac ccactcggca
1801 gcctccagtt ctcagggcgc cagcgtgaac cctgagcctt tacactcctc cctggacaag
1861 cttgggcctg acacaccaaa gcaagccttt actcccatag acctgccctg cggcagtgct
1921 gatgaaagcc ctgcgggaga cagggaatgc cagacttctt tggagaccag tatcttcact
1981 cccagtcctt gtaaaattcc acctccgacg agagtgggct ttggaagcgg gcagcctccc
2041 ccgtatgatc atctttttga ggtggcattg ccaaagacag cccatcattt tgtcatcagg
2101 aagactgagg agctgttaaa gaaagcaaaa ggaaacacag aggaagatgg tgtgccctct
2161 acctccccaa tggaagtgct ggacagactg atacagcagg gagcagacgc gcacagcaag
2221 gagctgaaca agttgccttt acccagcaag tctgtcgact ggacccactt tggaggctct
2281 cctccttcag atgagatccg caccctccga gaccagttgc ttttactgca caaccagtta
2341 ctctatgagc gttttaagag gcagcagcat gccctccgga acaggcggct cctccgcaag
2401 gtgatcaaag cagcagctct ggaggaacat aatgctgcca tgaaagatca gttgaagtta
2461 caagagaagg acatccagat gtggaaggtt agtctgcaga aagaacaagc tagatacaat
2521 cagctccagg agcagcgtga cactatggta accaagctcc acagccagat cagacagctg
2581 cagcatgacc gagaggaatt ctacaaccag agccaggaat tacagacgaa gctggaggac
2641 tgcaggaaca tgattgcgga gctgcggata gaactgaaga aggccaacaa caaggtgtgt
2701 cacactgagc tgctgctcag tcaggtttcc caaaagctct caaacagtga gtcggtccag
2761 cagcagatgg agttcttgaa caggcagctg ttggttcttg gggaggtcaa cgagctctat
2821 ttggaacaac tgcagaacaa gcactcagat accacaaagg aagtagaaat gatgaaagcc
2881 gcctatcgga aagagctaga aaaaaacaga agccatgttc tccagcagac tcagaggctt
2941 gatacctccc aaaaacggat tttggaactg gaatctcacc tggccaagaa agaccacctt
3001 cttttggaac agaagaaata tctagaggat gtcaaactcc aggcaagagg acagctgcag
3061 gccgcagaga gcaggtatga ggctcagaaa aggataaccc aggtgtttga attggagatc
3121 ttagatttat atggcaggtt ggagaaagat ggcctcctga aaaaacttga agaagaaaaa
3181 gcagaagcag ctgaagcagc agaagaaagg cttgactgtt gtaatgacgg gtgctcagat
3241 tccatggtag ggcacaatga agaggcatct ggccacaacg gtgagaccaa gacccccagg
3301 cccagcagcg cccggggcag tagtggaagc agaggtggtg gaggcagcag cagcagcagc
3361 agcgagcttt ctaccccaga gaaaccccca caccagaggg caggcccatt cagcagtcgg
3421 tgggagacga ctatgggaga agcgtctgcc agcatcccca ccactgtggg ctcacttccc
3481 agttcaaaaa gcttcctggg tatgaaggct cgagagttat ttcgtaataa gagcgagagc
3541 cagtgtgatg aggacggcat gaccagtagc ctttctgaga gcctaaagac agaactgggc
3601 aaagacttgg gtgtggaagc caagattccc ctgaacctag atggccctca cccgtctccc
3661 ccgaccccgg acagtgttgg acagctacat atcatggact acaatgagac tcatcatgaa
3721 cacagctaag gaatgatggt caatcagtgt taacttgcat attgttggca cagaacagga
3781 ggtgtgaatg cacgtttcaa agctttcctg tttccagggt ctgagtgcaa gttcatgtgt
3841 ggaaatggga cggaggtcct ttggacagct gactgaatgc agaacggttt ttggatctgg
3901 cattgaaatg cctcttgacc ttcccctcca cccgccctaa ccccctctca tttacctcgc
3961 agtgtgttct aatccaaggg ccagttggtg ttcctcagta gctttacttt cttcctttcc
4021 cccccaaatg gttgcgtcct ttgaacctgt gcaatatgag gccaaattta atctttgagt
4081 ctaacacacc actttctgct ttcccgaagt tcagataact gggttggctc tcaattagac
4141 caggtagttt gttgcattgc aggtaagtct ggttttgtcc cttccaggag gacatagcct
4201 gcaaagctgg ttgtctttac atgaaagcgt ttacatgaga ctttccgact gctttttga
4261 ttctgaagtt cagcatctaa agcagcaggt ctagaagaac aacggtttat tcatacttgc
4321 attcttttgg cagttctgat aagcttccta gaaagttctg tgtaaacaga agcctgtttc
4381 agaaatctgg agctggcact gtggagacca cacacccttt gggaaagctc ttgtctcttc
4441 ttcccccact acctcttatt tatttggtgt ttgcttgaat gctggtacta ttgtgaccac
4501 aggctggtgt gtaggtggta aaacctgttc tccataggag ggaaggagca gtcactggga
4561 gaggttaccc gagaagcact tgagcatgag gaactgcacc tttaggccat ctcagcttgc
4621 tgggcctttt gttaaaccct tctgtctact ggcctccctt tgtgtgcata cgcctcttgt
4681 tcatgtcagc ttatatgtga cactgcagca gaaaggctct gaaggtccaa agagtttctg
4741 caaagtgtat gtgaccatca tttcccaggc cattagggtt gcctcactgt agcaggttct
4801 aggctaccag aagaggggca gctttttcat accaattcca actttcaggg gctgactctc
4861 cagggagctg atgtcatcac actctccatg ttagtaatgg cagagcagtc taaacagagt
4921 ccgggagaat gctggcaaag gctggctgtg tatacccact aggctgcccc acgtgctccc
4981 gagagatgac actagtcaga aaattggcag tggcagagaa tccaaactca acaagtgctc
5041 ctgaaagaaa cgctagaagc ctaagaactg tggtctggtg ttccagctga ggcaggggga
```

```
5101 tttggtagga aggagccagt gaacttggct ttcctgtttc tatctttcat taaaaagaat
5161 agaaggattc agtcataaag aggtaaaaaa ctgtcacggt acgaaatctt agtgcccacg
5221 gaggcctcga gcagagagaa tgaaagtctt ttttttttt ttttttttt agcatggcaa
5281 taaatattct agcatcccta actaaagggg actagacagt tagagactct gtcaccctag
5341 ctataccagc agaaaacctg ttcaggcagg ctttctgggt gtgactgatt cccagcctgt
5401 ggcagggcgt ggtcccaact actcagccta gcacaggctg gcagttggta ctgaattgtc
5461 agatgtggag tattagtgac accacacatt taattcagct ttgtccaaag gaaagcttaa
5521 aacccaatac agtctagttt cctggttccg ttttagaaaa ggaaaacgtg aacaaactta
5581 gaaagggaag gaaatcccat cagtgaatcc tgaaactggt tttaagtgct ttccttctcc
5641 tcatgcccaa gagatctgtg ccatagaaca agataccagg cacttaaagc cttttcctga
5701 attggaaagg aaaagaggcc caagtgcaaa agaaaaaaca ttttagaaac ggacagctta
5761 taaaaataaa gggaagaaag gaggcagcat ggagagaggc ctgtgctaga agctccatgg
5821 acgtgtctgc acagggtcct cagctcatcc atgcggcctg ggtgtccttt tactcagctt
5881 tataacaaat gtggctccaa gctcaggtgc ctttgagttc taggaggctg tgggttttat
5941 tcaactacgg ttgggagaat gagacctgga gtcatgttga aggtgcccaa cctaaaaatg
6001 taggctttca tgttgcaaag aactccagag tcagtagtta ggtttggttt ggttttggac
6061 atgataaacc tgccaagagt caacaggtca cttgatcatg ctgcagtggg tagttctaag
6121 gatggaaagg tgacagtatt actctcgaga ggcaattcag tcctgggcaa aggtattagt
6181 acaataagcg ttaagggcag agtctacctt gaaaccaatt aagcagcttg gtattcataa
6241 atattgggat tggatggcct ccatccagaa atcactatgg gtgagcatac ctgtctcagc
6301 tgtttggcca atgtgcataa cctactcgga tccccacctg acactaacca gagtcagcac
6361 aggccccgag gagcccgaag tctgctgctg tgcagcatgg aattccttta aaaaggtgca
6421 ctacagtttt agcggggagg gggataggaa gacgcagagc aaatgagctc cggagtccct
6481 gcaggtgaat aaacacacag atctgcatct gatagaactt tgatggattt tcaaaaagcc
6541 gttgacaagg ctctgctata cagtctataa aaattgttat tatgggattg gaagaaacac
6601 gtggtcatga atagaaaaaa aacaaaccca aaggtaggaa ggtcaaggtc atttcttaga
6661 tggagaagtt gtgaaagatg tccttggaga tgagttttag gaccagcatt actaaggcag
6721 gtgggcagac agtgacctct ctaggtgtgt ccacagagtt tttcaggaga gaaaactgcc
6781 tgacctttgg gactaagctg cggaatcttc ttactaagct tgaagagtgg agaggcgaga
6841 ggtgagctac tttgtgagcc aaagcttatg tgacatggtt ggggaaacag tccaaactgt
6901 tctgagaagg tgaactgtta cgacccagga caattagaaa aattcaccca ccatgccgca
6961 cattactggg taaaagcagg gcagcaggga acaaaactcc agactcttgg gccgtcccca
7021 tttgcaacag cacacatagt ttctggtata tttgttggga aagataaaac tctagcagtt
7081 gttgagggga ggatgtataa aatggtcatg gggatgaaag gatctctgag accacagagg
7141 ctcagactca ctgttaagaa tagaaaactg ggtatgcgtt tcatgtagcc agcagaactg
7201 aagtgtgctg tgacaagcca atgtgaattt ctaccaaata gtagagcata ccacttgaag
7261 aaggaaagaa ccgaagagca aacaaaagtt ctgcgtaatg agactcacct tttctcgctg
7321 aaagcactaa gaggtgggag gaggcctgca caggctggag gagggtttgg gcagagcgaa
7381 gacccggcca ggaccttggt gagatggggt gccgcccacc tcctgcggat actcttggag
7441 agttgttccc ccaggggggct ctgccccacc tggagaagga agctgcctgg tgtggagtga
7501 ctcaaatcag tatacctatc tgctgcacct tcactctcca gggtacatgc tttaaaaccg
7561 acccgcaaca agtattggaa aaatgtatcc agtctgaaga tgtttgtgta tctgtttaca
7621 tccagagttc tgtgacacat gccccccaga ttgctgcaaa gatcccaagg cattgattgc
7681 acttgattaa gcttttgtct gtaggtgaaa gaacaagttt aggtcgagga ctggccccta
7741 ggctgctgct gtgacccttg tcccatgtgg cttgtttgcc tgtccgggac tcttcgatgt
7801 gcccagggga gcgtgttcct gtctcttcca tgccgtcctg cagtccttat ctgctcgcct
7861 gagggaagag tagctgtagc tacaagggaa gcctgcctgg aagagccgag cacctgtgcc
7921 catggcttct ggtcatgaaa cgagttaatg atggcagagg agcttcctcc ccacttcgca
7981 gcgccacatt atccatcctc tgagataagt aggctggttt aaccattgga atggaccttt
8041 cagtggaaac cctgagagtc tgagaacccc cagaccaacc cttccctccc tttccccacc
8101 tcttacagtg tttggacagg agggtatggt gctgctctgt gtagcaagta ctttggctta
8161 tgaaagaggc agccacgcat tttgcactag gaagaatcag taatcacttt tcagaagact
8221 tctatggacc acaaatatat tacggaggaa cagattttgc taagacataa tctagtttta
8281 taactcaatc atgaatgaac catgtgtggc aaacttgcag tttaaagggg tcccatcagt
8341 gaaagaaact gatttttttt aacggactgc ttttagttaa attgaagaaa gtcagctctt
8401 gtcaaaaggt ctaaactttc ccgcctcaat cctaaaagca tgtcaacaat ccacatcaga
8461 tgccataaat atgaactgca ggataaaatg gtacaatctt agtgaatggg aattggaatc
8521 aaaagagttt gctgtccttc ttagaatgtt ctaaaatgtc aaggcagttg cttgtgttta
8581 actgtgaaca aataaaaatt tattgttttg cactacaaaa aaaaaa
```

By "TSC2 polypeptide" is meant a protein or fragment thereof having at least 85% amino acid sequence identity to the sequence provided at NCBI Ref: NP_000539.2 that functions in brain development. An exemplary human amino acid sequence is provided below:

```
   1 MAKPTSKDSG LKEKFKILLG LGTPRPNPRS AEGKQTEFII TAEILRELSM ECGLNNRIRM
  61 IGQICEVAKT KKFEEHAVEA LWKAVADLLQ PERPLEARHA VLALLKAIVQ GQGERLGVLR
 121 ALFFKVIKDY PSNEDLHERL EVFKALTDNG RHITYLEEEL ADFVLQWMDV GLSSEFLLVL
 181 VNLVKFNSCY LDEYIARMVQ MICLLCVRTA SSVDIEVSLQ VLDAVVCYNC LPAESLPLFI
 241 VTLCRTINVK ELCEPCWKLM RNLLGTHLGH SAIYNMCHLM EDRAYMEDAP LLRGAVFFVG
 301 MALWGAHRLY SLRNSPTSVL PSFYQAMACP NEVVSYEIVL SITRLIKKYR KELQVVAWDI
 361 LLNIIERLLQ QLQTLDSPEL RTIVHDLLTT VEELCDQNEF HGSQERYFEL VERCADQRPE
 421 SSLLNLISYR AQSIHPAKDG WIQNLQALME RFFRSESRGA VRIKVLDVLS FVLLINRQFY
 481 EEELINSVVI SQLSHIPEDK DHQVRKLATQ LLVDLAEGCH THHFNSLLDI IEKVMARSLS
 541 PPPELEERDV AAYSASLEDV KTAVLGLLVI LQTKLYTLPA SHATRVYEML VSHIQLHYKH
 601 SYTLPIASSI RLQAFDFLLL LRADSLHRLG LPNKDGVVRF SPYCVCDYME PERGSEKKTS
 661 GPLSPPTGPP GPAPAGPAVR LGSVPYSLLF RVLLQCLKQE SDWKVLKLVL GRLPESLRYK
 721 VLIFTSPCSV DQLCSALCSM LSGPKTLERL RGAPEGFSRT DLHLAVVPVL TALISYHNYL
 781 DKTKQREMVY CLEQGLIHRC ASQCVVALSI CSVEMPDIII KALPVLVVKL THISATASMA
 841 VPLLEFLSTL ARLPHLYRNF AAEQYASVFA ISLPYTNPSK FNQYIVCLAH HVIAMWFIRC
 901 RLPFRKDFVP FITKGLRSNV LLSFDDTPEK DSFRARSTSL NERPKSLRIA RPPKQGLNNS
 961 PPVKEFKESS AAEAFRCRSI SVSEHVVRSR IQTSLTSASL GSADENSVAQ ADDSLKNLHL
1021 ELTETCLDMM ARYVFSNFTA VPKRSPVGEF LLAGGRTKTW LVGNKLVTVT TSVGTGTRSL
1081 LGLDSGELQS GPESSSSPGV HVRQTKEAPA KLESQAGQQV SRGARDRVRS MSGGHGLRVG
1141 ALDVPASQFL GSATSPGPRT APAAKPEKAS AGTRVPVQEK TNLAAYVPLL TQGWAEILVR
1201 RPTGNTSWLM SLENPLSPFS SDINNMPLQE LSNALMAAER FKEHRDTALY KSLSVPAAST
1261 AKPPPLPRSN TVASFSSLYQ SSCQGQLHRS VSWADSAVVM EEGSPGEVPV LVEPPGLEDV
1321 EAALGMDRRT DAYSRSSSVS SQEEKSLHAE ELVGRGIPIE RVVSSEGGRP SVDLSFQPSQ
1381 PLSKSSSSPE LQTLQDILGD PGDKADVGRL SPEVKARSQS GTLDGESAAW SASGEDSRGQ
1441 PEGPLPSSSP RSPSGLRPRG YTISDSAPSR RGKRVERDAL KSRATASNAE KVPGINPSFV
1501 FLQLYHSPFF GDESNKPILL PNESQSFERS VQLLDQIPSY DTHKIAVLYV GEGQSNSELA
1561 ILSNEHGSYR YTEFLTGLGR LIELKDCQPD KVYLGGLDVC GEDGQFTYCW HDDIMQAVFH
1621 IATLMPTKDV DKHRCDKKRH LGNDFVSIVY NDSGEDFKLG TIKGQFNFVH VIVTPLDYEC
1681 NLVSLQCRKD MEGLVDTSVA KIVSDRNLPF VARQMALHAN MASQVHHSRS NPTDIYPSKW
1741 IARLRHIKRL RQRICEEAAY SNPSLPLVHP PSHSKAPAQT PAEPTPGYEV GQRKRLISSV
1801 EDFTEFV
```

In one embodiment, a TSC2 polypeptide comprises a mutation affecting brain development. In another embodiment, a TSC2 polypeptide comprises ARG1743GLN where the Arginine in the 1743rd position from the N-terminal is replaced by a Glutamine. ARG1743GLN may also be termed as R1743Q.

By "TSC2 polynucleotide" is meant any nucleic acid sequence encoding a TSC2 polypeptide or fragment thereof. An exemplary human TSC2 nucleic acid sequence is provided at NCBI Ref NM_000548:

```
   1 tttccgccag agggcggcac agaactacaa ctcccagcaa gctcccaagg cggccctccg
  61 cgcaatgccg ctaccggaag tgcgggtcgc gcttccggcg gcgtcccggg gccaggggcg
 121 tgcgcctttc tccgcgtcgg ggcggcccgg agcgcggtgg cgcggcgcgg gaggggtttt
 181 ctggtgcgtc ctggtccacc atggccaaac caacaagcaa agattcaggc ttgaaggaga
 241 agtttaagat tctgttggga ctgggaacac cgaggccaaa tcccaggtct gcagagggta
 301 aacagacgga gtttatcatc accgcggaaa tactgagaga actgagcatg gaatgtggcc
 361 tcaacaatcg catccggatg atagggcaga tttgtgaagt cgcaaaaacc aagaaatttg
 421 aagagcacgc agtggaagca ctctggaagg cggtcgcgga tctgttgcag ccggagcggc
```

```
 481 cgctggaggc ccggcacgcg gtgctggctc tgctgaaggc catcgtgcag gggcagggcg
 541 agcgtttggg ggtcctcaga gccctcttct ttaaggtcat caaggattac ccttccaacg
 601 aagaccttca cgaaaggctg gaggttttca aggccctcac agacaatggg agacacatca
 661 cctacttgga ggaagagctg gctgactttg tcctgcagtg gatggatgtt ggcttgtcct
 721 cggaattcct tctggtgctg gtgaacttgg tcaaattcaa tagctgttac ctcgacgagt
 781 acatcgcaag gatggttcag atgatctgtc tgctgtgcgt ccggaccgcg tcctctgtgg
 841 acatagaggt ctccctgcag gtgctggacg ccgtggtctg ctacaactgc ctgccggctg
 901 agagcctccc gctgttcatc gttaccctct gtcgcaccat caacgtcaag gagctctgcg
 961 agccttgctg gaagctgatg cggaacctcc ttggcaccca cctgggccac agcgccatct
1021 acaacatgtg ccacctcatg gaggacagag cctacatgga ggacgcgccc ctgctgagag
1081 gagccgtgtt ttttgtgggc atggctctct ggggagccca ccggctctat tctctcagga
1141 actcgccgac atctgtgttg ccatcatttt accaggccat ggcatgtccg aacgaggtgg
1201 tgtcctatga gatcgtcctg tccatcacca ggctcatcaa gaagtatagg aaggagctcc
1261 aggtggtggc gtgggacatt ctgctgaaca tcatcgaacg gctccttcag cagctccaga
1321 ccttggacag cccggagctc aggaccatcg tccatgacct gttgaccacg gtggaggagc
1381 tgtgtgacca gaacgagttc cacgggtctc aggagagata ctttgaactg gtggagagat
1441 gtgcggacca gaggcctgag tcctccctcc tgaacctgat ctcctataga gcgcagtcca
1501 tccacccggc caaggacggc tggattcaga acctgcaggc gctgatggag agattcttca
1561 ggagcgagtc ccgaggcgcc gtgcgcatca aggtgctgga cgtgctgtcc tttgtgctgc
1621 tcatcaacag gcagttctat gaggaggagc tgattaactc agtggtcatc tcgcagctct
1681 cccacatccc cgaggataaa gaccaccagg tccgaaagct ggccacccag ttgctggtgg
1741 acctggcaga gggctgccac acacaccact tcaacagcct gctggacatc atcgagaagg
1801 tgatggcccg ctccctctcc ccacccccgg agctggaaga aagggatgtg gccgcatact
1861 cggcctcctt ggaggatgtg aagacagccg tcctggggct tctggtcatc cttcagacca
1921 agctgtacac cctgcctgca agccacgcca cgcgtgtgta tgagatgctg gtcagccaca
1981 ttcagctcca ctacaagcac agctacaccc tgccaatcgc gagcagcatc cggctgcagg
2041 cctttgactt cctgttgctg ctgcgggccg actcactgca ccgcctgggc ctgcccaaca
2101 aggatggagt cgtgcggttc agcccctact gcgtctgcga ctacatggag ccagagagag
2161 gctctgagaa gaagaccagc ggcccccttt ctcctcccac agggcctcct ggcccggcgc
2221 ctgcaggccc cgccgtgcgg ctggggtccg tgccctactc cctgctcttc cgcgtcctgc
2281 tgcagtgctt gaagcaggag tctgactgga aggtgctgaa gctggttctg ggcaggctgc
2341 ctgagtccct gcgctataaa gtgctcatct ttacttcccc ttgcagtgtg gaccagctgt
2401 gctctgctct ctgctccatg ctttcaggcc caaagacact ggagcggctc cgaggcgccc
2461 cagaaggctt ctccagaact gacttgcacc tggccgtggt tccagtgctg acagcattaa
2521 tctcttacca taactacctg gacaaaacca aacagcgcga gatggtctac tgcctggagc
2581 agggcctcat ccaccgctgt gccagccagt gcgtcgtggc cttgtccatc tgcagcgtgg
2641 agatgcctga catcatcatc aaggcgctgc ctgttctggt ggtgaagctc acgcacatct
2701 cagccacagc cagcatggcc gtcccactgc tggagttcct gtccactctg gccaggctgc
2761 cgcacctcta caggaacttt gccgcggagc agtatgccag tgtgttcgcc atctccctgc
2821 cgtacaccaa cccctccaag tttaatcagt acatcgtgtg tctggcccat cacgtcatag
2881 ccatgtggtt catcaggtgc cgcctgccct tccggaagga ttttgtccct ttcatcacta
2941 agggcctgcg gtccaatgtc ctcttgtctt ttgatgacac ccccgagaag gacagcttca
3001 gggcccggag tactagtctc aacgagagac ccaagagtct gaggatagcc agacccccca
3061 aacaaggctt gaataactct ccacccgtga aagaattcaa ggagagctct gcagccgagg
3121 ccttccggtg ccgcagcatc agtgtgtctg aacatgtggt ccgcagcagg atacagacgt
3181 ccctcaccag tgccagcttg gggtctgcag atgagaactc cgtggcccag gctgacgata
3241 gcctgaaaaa cctccacctg gagctcacgg aaacctgtct ggacatgatg gctcgatacg
3301 tcttctccaa cttcacggct gtcccgaaga ggtctcctgt gggcgagttc ctcctagcgg
3361 gtggcaggac caaaacctgg ctggttggga acaagcttgt cactgtgacg acaagcgtgg
3421 gaaccgggac ccggtcgtta ctaggcctgg actcgggggа gctgcagtcc ggcccggagt
3481 cgagctccag ccccggggtg catgtgagac agaccaagga ggcgccggcc aagctggagt
3541 cccaggctgg gcagcaggtg tcccgtgggg cccgggatcg ggtccgttcc atgtcggggg
3601 gccatggtct tcgagttggc gccctggacg tgccggcctc ccagttcctg ggcagtgcca
3661 cttctccagg accacggact gcaccagccg cgaaacctga gaaggcctca gctggcaccc
3721 gggttcctgt gcaggagaag acgaacctgg cggcctatgt gcccctgctg acccagggct
3781 gggcggagat cctggtccgg aggcccacag ggaacaccag ctggctgatg agcctggaga
3841 acccgctcag ccctttctcc tcggacatca acaacatgcc cctgcaggag ctgtctaacg
3901 ccctcatggc ggctgagcgc ttcaaggagc accgggacac agccctgtac aagtcactgt
3961 cggtgccggc agccagcacg gccaaacccc ctcctctgcc tcgctccaac acagtggcct
4021 ctttctcctc cctgtaccag tccagctgcc aaggacagct gcacaggagc gtttcctggg
4081 cagactccgc cgtggtcatg gaggagggaa gtccgggcga ggttcctgtg ctggtggagc
```

```
4141 ccccagggtt ggaggacgtt gaggcagcgc taggcatgga caggcgcacg gatgcctaca
4201 gcaggtcgtc ctcagtctcc agccaggagg agaagtcgct ccacgcggag gagctggttg
4261 gcaggggcat ccccatcgag cgagtcgtct cctcggaggg tggccggccc tctgtggacc
4321 tctccttcca gccctcgcag cccctgagca agtccagctc ctctcccgag ctgcagactc
4381 tgcaggacat cctcggggac cctggggaca aggccgacgt gggccggctg agccctgagg
4441 ttaaggcccg gtcacagtca gggaccctgg acggggaaag tgctgcctgg tcggcctcgg
4501 gcgaagacag tcggggccag cccgagggtc ccttgccttc cagctccccc cgctcgccca
4561 gtggcctccg gccccgaggt tacaccatct ccgactcggc cccatcacgc aggggcaaga
4621 gagtagagag ggacgcctta aagagcagag ccacagcctc caatgcagag aaagtgccag
4681 gcatcaaccc cagtttcgtg ttcctgcagc tctaccattc ccccttcttt ggcgacgagt
4741 caaacaagcc aatcctgctg cccaatgagt cacagtcctt tgagcggtcg gtgcagctcc
4801 tcgaccagat cccatcatac gacacccaca agatcgccgt cctgtatgtt ggagaaggcc
4861 agagcaacag cgagctcgcc atcctgtcca atgagcatgg ctcctacagg tacacggagt
4921 tcctgacggg cctgggccgg ctcatcgagc tgaaggactg ccagccggac aaggtgtacc
4981 tgggaggcct ggacgtgtgt ggtgaggacg gccagttcac ctactgctgg cacgatgaca
5041 tcatgcaagc cgtcttccac atcgccaccc tgatgcccac caaggacgtg gacaagcacc
5101 gctgcgacaa gaagcgccac ctgggcaacg actttgtgtc cattgtctac aatgactccg
5161 gtgaggactt caagcttggc accatcaagg gccagttcaa ctttgtccac gtgatcgtca
5221 ccccgctgga ctacgagtgc aacctggtgt ccctgcagtg caggaaagac atggagggcc
5281 ttgtggacac cagcgtggcc aagatcgtgt ctgaccgcaa cctgcccttc gtggcccgcc
5341 agatggccct gcacgcaaat atggcctcac aggtgcatca tagccgctcc aaccccaccg
5401 atatctaccc ctccaagtgg attgcccggc tccgccacat caagcggctc cgccagcgga
5461 tctgcgagga agccgcctac tccaacccca gcctacctct ggtgcaccct ccgtcccata
5521 gcaaagcccc tgcacagact ccagccgagc ccacacctgg ctatgaggtg ggccagcgga
5581 agcgcctcat ctcctcggtg gaggacttca ccgagtttgt gtgaggccgg ggccctccct
5641 cctgcactgg ccttggacgg tattgcctgt cagtgaaata aataaagtcc tgaccccagt
5701 gcacagacat agaggcacag attgcagtca gacaaaaaaa aaaaaaaaaa a
```

By "PSEN1 polypeptide" is meant a protein or fragment thereof having at least 85% amino acid sequence identity to the sequence provided at NCBI Ref: NP_000012.1 having enzymatic activity or functioning in regulating beta amyloid levels. An exemplary human amino acid sequence is provided below:

```
  1 MTELPAPLSY FQNAQMSEDN HLSNTVRSQN DNRERQEHND RRSLGHPEPL SNGRPQGNSR
 61 QVVEQDEEED EELTLKYGAK HVIMLFVPVT LCMVVVVATI KSVSFYTRKD GQLIYTPFTE
121 DTETVGQRAL HSILNAAIMI SVIVVMTILL VVLYKYRCYK VIHAWLIISS LLLLFFFSFI
181 YLGEVFKTYN VAVDYITVAL LIWNFGVVGM ISIHWKGPLR LQQAYLIMIS ALMALVFIKY
241 LPEWTAWLIL AVISVYDLVA VLCPKGPLRM LVETAQERNE TLFPALIYSS TMVWLVNMAE
301 GDPEAQRRVS KNSKYNAEST ERESQDTVAE NDDGGFSEEW EAQRDSHLGP HRSTPESRAA
361 VQELSSSILA GEDPEERGVK LGLGDFIFYS VLVGKASATA SGDWNTTIAC FVAILIGLCL
421 TLLLLAIFKK ALPALPISIT FGLVFYFATD YLVQPFMDQL AFHQFYI
```

In one embodiment, a PSEN1 polypeptide encompasses a mutation (e.g., ALA246GLU). In one embodiment, the PSEN1 polypeptide comprises an Alanine corresponding to the Alanine in the 246th position from the N-terminal in the exemplary PSEN1 polypeptide replaced by a Glutamic acid. ALA246GLU may also be termed as A246E.

By "PSEN1 polynucleotide" is meant any nucleic acid sequence encoding a PSEN1 polypeptide or fragment thereof. An exemplary human PSEN1 nucleic acid sequence is provided at NCBI Ref NM_000021:

```
  1 aaatgacgac aacggtgagg gttctcgggc ggggcctggg acaggcagct ccggggtccg
 61 cggtttcaca tcggaaacaa aacagcggct ggtctggaag gaacctgagc tacgagccgc
121 ggcggcagcg gggcggcggg gaagcgtata cctaatctgg gagcctgcaa gtgacaacag
```

```
 181 cctttgcggt ccttagacag cttggcctgg aggagaacac atgaaagaaa gaacctcaag
 241 aggctttgtt ttctgtgaaa cagtatttct atacagttgc tccaatgaca gagttacctg
 301 caccgttgtc ctacttccag aatgcacaga tgtctgagga caaccacctg agcaatactg
 361 tacgtagcca gaatgacaat agagaacggc aggagcacaa cgacagacgg agccttggcc
 421 accctgagcc attatctaat ggacgacccc agggtaactc ccggcaggtg gtggagcaag
 481 atgaggaaga agatgaggag ctgacattga aatatggcgc caagcatgtg atcatgctct
 541 ttgtccctgt gactctctgc atggtggtgg tcgtggctac cattaagtca gtcagctttt
 601 atacccggaa ggatgggcag ctaatctata ccccattcac agaagatacc gagactgtgg
 661 gccagagagc cctgcactca attctgaatg ctgccatcat gatcagtgtc attgttgtca
 721 tgactatcct cctggtggtt ctgtataaat acaggtgcta taaggtcatc catgcctggc
 781 ttattatatc atctctattg ttgctgttct ttttttcatt catttacttg ggggaagtgt
 841 ttaaaaccta taacgttgct gtggactaca ttactgttgc actcctgatc tggaattttg
 901 gtgtggtggg aatgatttcc attcactgga aaggtccact tcgactccag caggcatatc
 961 tcattatgat tagtgccctc atggccctgg tgtttatcaa gtacctccct gaatggactg
1021 cgtggctcat cttggctgtg atttcagtat atgatttagt ggctgttttg tgtccgaaag
1081 gtccacttcg tatgctggtt gaaacagctc aggagagaaa tgaaacgctt tttccagctc
1141 tcatttactc ctcaacaatg gtgtggttgg tgaatatggc agaaggagac ccggaagctc
1201 aaaggagagt atccaaaaat tccaagtata atgcagaaag cacagaaagg gagtcacaag
1261 acactgttgc agagaatgat gatggcgggt tcagtgagga atgggaagcc cagagggaca
1321 gtcatctagg gcctcatcgc tctacacctg agtcacgagc tgctgtccag gaactttcca
1381 gcagtatcct cgctggtgaa gacccagagg aaaggggagt aaaacttgga ttgggagatt
1441 tcattttcta cagtgttctg gttggtaaag cctcagcaac agccagtgga gactggaaca
1501 caaccatagc ctgtttcgta gccatattaa ttggtttgtg ccttacatta ttactccttg
1561 ccattttcaa gaaagcattg ccagctcttc caatctccat cacctttggg cttgttttct
1621 actttgccac agattatctt gtacagcctt ttatggacca attagcattc catcaatttt
1681 atatctagca tatttgcggt tagaatccca tggatgtttc ttctttgact ataacaaaat
1741 ctggggagga caaaggtgat tttcctgtgt ccacatctaa caaagtcaag attcccggct
1801 ggacttttgc agcttccttc caagtcttcc tgaccacctt gcactattgg actttggaag
1861 gaggtgccta tagaaaacga ttttgaacat acttcatcgc agtggactgt gtccctcggt
1921 gcagaaacta ccagatttga gggacgaggt caaggagata tgataggccc ggaagttgct
1981 gtgccccatc agcagcttga cgcgtggtca caggacgatt tcactgacac tgcgaactct
2041 caggactacc gttaccaaga ggttaggtga agtggtttaa accaaacgga actcttcatc
2101 ttaaactaca cgttgaaaat caacccaata attctgtatt aactgaattc tgaacttttc
2161 aggaggtact gtgaggaaga gcaggcacca gcagcagaat ggggaatgga gaggtgggca
2221 ggggttccag cttccctttg attttttgct gcagactcat ccttttaaa tgagacttgt
2281 tttcccctct ctttgagtca agtcaaatat gtagattgcc tttggcaatt cttcttctca
2341 agcactgaca ctcattaccg tctgtgattg ccatttcttc ccaaggccag tctgaacctg
2401 aggttgcttt atcctaaaag ttttaacctc aggttccaaa ttcagtaaat tttggaaaca
2461 gtacagctat ttctcatcaa ttctctatca tgttgaagtc aaatttggat tttccaccaa
2521 attctgaatt tgtagacata cttgtacgct cacttgcccc agatgcctcc tctgtcctca
2581 ttcttctctc ccacacaagc agtctttttc tacagccagt aaggcagctc tgtcgtggta
2641 gcagatggtc ccattattct agggtcttac tctttgtatg atgaaaagaa tgtgttatga
2701 atcggtgctg tcagccctgc tgtcagacct tcttccacag caaatgagat gtatgcccaa
2761 agacggtaga attaaagaag agtaaaatgg ctgttgaagc actttctgtc ctggtatttt
2821 gtttttgctt ttgccacaca gtagctcaga atttgaacaa atagccaaaa gctggtggtt
2881 gatgaattat gaactagttg tatcaacaca aagcaagagt tggggaaagc catatttaac
2941 ttggtgagct gtgggagaac ctggtggcag aaggagaacc aactgccaag gggaaagaga
3001 aggggcctcc agcagcgaag gggatacagt gagctaatga tgtcaaggag gagtttcagg
3061 ttattctcgt cagctccaca aatgggtgct ttgtggtctc tgcccgcgtt acctttcctc
3121 tcaatgtacc tttgtgtgaa ctgggcagtg gaggtgcctg ctgcagttac catggagttc
3181 aggctctggg cagctcagtc aggcaaaaca cacaaacagc catcagcctg tgtgggctca
3241 gggcacctct ggacaaaggc ttgtggggca taaccttctt taccacagag agcccttagc
3301 tatgctgatc agaccgtaag cgtttatgag aaacttagtt tcctcctgtg gctgaggagg
3361 ggccagcttt ttcttctttt gcctgctgtt ttctctccca atctatgata tgatatgacc
3421 tggtttgggg ctgtctttgg tgtttagaat atttgttttc tgtcccagga tatttcttat
3481 aagaacctaa cttcaagagt agtgtgcgag tactgatctg aatttaaatt aaaattggct
3541 tatattaggc agtcacagac aggaaaaata agagctatgc aaagaaaggg ggatttaaag
3601 tagtaggttc tatcatctca attcattttt ttccatgaaa tcccttcttc caagattcat
3661 tccctctctc agacatgtgc tagcatgggt attatcattg agaaagcaca gctacagcaa
3721 agccacctga atagcaattt gtgattggaa gcattcttga gggatcccta atctagagta
3781 atttatttgt gtaaggatcc caaatgtgtt gcacctttca tgatacattt cttctctgaa
```

```
3841 gagggtacgt ggggtgtgtg tatttaaatc catcctatgt attactgatt gtcctgtgta
3901 gaaagatggc aattattctg tctctttctc caagtttgag ccacatctca gccacattgt
3961 tagacagtgt acagagaacc tatctttcct ttttttttt ttaaaggaca ggattttgct
4021 gtgttgccca ggctagactt gaactcctgg gctcaagtaa tccacctcag cctgagtagc
4081 tgagactaca gcccatctta tttctttaaa tcattcatct caggcagaga acttttccct
4141 caaacattct ttttagaatt agttcagtca ttcctaaaac atccaaatgc tagtcttcca
4201 ccatgaaaaa tagattgtca ctggaaagaa cagtagcaat ttccataagg atgtgccttc
4261 actcacacgg gacaggcggt ggttatagag tcgggcaaaa ccagcagtag agtatgacca
4321 gccaagccaa tctgcttaat aaaaagatgg aagacagtaa ggaaggaaag tagccactaa
4381 gagtctgagt ctgactgggc tacagaataa agggtattta tggacagaat gtcattacat
4441 gcctatggga ataccaatca tatttggaag atttgcagat tttttttcag agaggaaaga
4501 ctcaccttcc tgtttttggt tctcagtagg ttcgtgtgtg ttcctagaat cacagctctg
4561 actccaaatg actcaatttc tcaattagaa aaagtagaag ctttctaagc aacttggaag
4621 aaaacagtca taagtaagca atttgttgat tttactacag aagcaacaac tgaagaggca
4681 gtgtttttac tttcagactc cgggattccc attctgtagt ctctctgctt ttaaaaaccc
4741 tccttttgca atagatgccc aaacagatga tgtttattac ttgttattta cgtggcctca
4801 gacagtgtat gtattctcga tataacttgt agagtgtgaa atataagttt aactaccaaa
4861 taaggtctcc cagggttaga tgactgcggg aagcctttga tcccaacccc caaggctttg
4921 tatatttgat catttgtgat ctaaccctgg aagaaaaaga gctcagaaac cactatgaaa
4981 aaatttgttc agtgttttct gtgttcccgt aggttctgga gtctgaggat gcaaagatga
5041 ataagataaa ttctcagaat gtagttataa tctcttgttt tctggtatat gccatctttc
5101 tttaacttct ctaaaatatt gggtatttgt caaataacca cttttaacag ttaccattac
5161 tgagggctta tacattggtg ttataaaagt gacttgattc agaaatcaat ccattcagta
5221 aagtactcct tctctaaatt tgctgttatg tctataagga acagtttgac ctgcccttct
5281 cctcacctcc tcacctgcct tccaacattg aatttggaag gagacgtgaa aattggacat
5341 ttggttttgc ccttgggctg gaaactatca tataatcata agtttgagcc tagaagtgat
5401 ccttgtgatc ttctcacctc tttaaattcc cacaacacaa gagattaaaa acagaggttt
5461 cagctcttca tagtgcgttg tgaaatggct ggccagagtg taccaacaaa gctgtcatcg
5521 ggctcacagc tcagagacat ctgcatgtga tcatctgcat agtcctctcc tctaacggga
5581 aacacctcag atttgcatat aaaaaagcac cctggtgctg aaatgaaccc ctttcttgaa
5641 catcaaagct gtctcccaca gccttgggca gcagggtgcc tcttagtgga tgtgctgggt
5701 ccaccctgag ccctgacatg tggtggcagc attgccagtt ggtctgtgtg tctgtgtagc
5761 agggacgatt tcccagaaag caattttcct tttgaaatac gtaattgttg agactaggca
5821 gtttcaaagt cagctgcata tagtagcaag tacaggactg tcttgttttt ggtgtccttg
5881 gaggtgctgg ggtgagggtt tcagtgggat catttactct cacatgttgt ctgccttctg
5941 cttctgtgga cactgctttg tacttaattc agacagactg tgaatacacc ttttttataa
6001 atacctttca aattcttggt aagatataat tttgatagct gattgcagat tttctgtatt
6061 tgtcagatta ataaagactg catgaatcca aaaaaaaaaa aaaaaaa
```

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in expression levels, a 25% change, a 40% change, or even a 50% or greater change in expression levels. "

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean " includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is

recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

"Detect" refers to identifying the presence, absence or amount of the analyte to be detected.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include neurological conditions, including tuberous sclerosis or Alzheimer's Disease.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide of this invention is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. In one embodiment, the preparation is at least 75%. In other embodiments, at least about 90-99%, by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By “marker” is meant any protein or polynucleotide analyte having an expression level or activity associated with a particular cell type. In one embodiment, transcriptomics are used to measure the levels of markers associated with cell fate, cell differentiation, and cell specific structure or function.

As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, purchasing, or otherwise acquiring the agent.

By “reference” is meant a standard or control condition.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

As used herein, the terms “treat,” treating,” “treatment,” and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard

deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig 1A is a micrograph showing a 4X dark field image of Brain Organoid Structures typical of approximately 5 week *in utero* development achieved in 12 weeks *in vitro*. Average size: 2-3 mm long.

FIG 1B shows immuno-fluorescence images of sections of iPSC-derived human brain organoid after approximately 12 weeks in culture. Z-stack of thirty three optical sections, 0.3 microns thick, obtained using laser confocal imaging with a 40X lens. Stained with **Top panel:** beta III tubulin (green: axons); MAP2 (red:dendrites);Hoechst (blue: nuclei); **Bottom panel:** Doublecortin (red)

FIG. 2 is a micrograph showing immunohistochemical staining of brain organoid section with the midbrain marker tyrosine hydroxylase. Paraformaldehyde fixed sections of a 8- week old brain organoid was stained with an Ab to tyrosine hydroxylase and detected with Alexa 488 conjugated secondary Abs (green) and counter stained with Hoechst to mark cell nuclei (blue). spinning disc confocal image (40X lens) of section stained with an antibody that binds tyrosine hydroxylase and Hoechst (scale bar: 10μm).

FIG. 3: Spinning disc confocal image (40X lens) of section. Astrocytes stained with GFAP (red) and mature neurons with NeuN (green).

FIG. 4 is a schematic showing in the upper panel a Developmental Expression Profile for transcripts as Heat Maps of NKCC1 and KCC2 expression at week 1, 4 and 12 of organoid culture as compared to approximate known profiles (lower panel). NKCC1: Na(+)-K(+)-Cl(-) cotransporter isoform 1. KCC2: K(+)-Cl(-) cotransporter isoform 2.

Fig 5A is a schematic showing GABAergic chloride gradient regulation by NKCC1 and KCC2.

FIG. 5B provides a table showing a representative part of the entire transcriptomic profile of brain organoids in culture for ~12 weeks measured using a transcriptome sequencing approach that is commercially available as AmpliSeq. This technique highlighted the expression of neuronal markers for diverse populations of neurons and other cell types that are comparable to those expressed in an adult human brain reference (HBR) purchased from Clontech and also the publicly available embryonic human brain (BRAINSCAN) atlas of the Allen Institute database.

FIG. 5C provides a table showing Ampliseq gene expression data comparing gene expression in an organoid (column 2) after ~12 weeks in culture *in vitro* versus Human Brain Reference (column 3). A concordance of greater than 98% was observed.

FIG. 5D provides a table showing Ampliseq gene expression data comparing organoids generated during two independent experiments after ~12 weeks in culture (column 2 and 3). Gene expression reproducibility between the two organoids was greater than 99%. Note that values are RPKM (Reads Per Kilo Base per Million reads) in the tables and <1 is background.

FIG. 6A is a schematic showing results of developmental transcriptomics. Brain organoid development *in vitro* follows KNOWN Boolean logic for the expression pattern of transcription factors during initiation of developmental programs of the brain. Time Points: 1, 4 and 12 Weeks. PITX3 and NURR1 (NR4A) are transcription factors that initiate midbrain development (early; at week 1), DLK1, KLHL1, PTPRU, and ADH2 respond to these two transcription factors to further promote midbrain development (mid; at week 4 &12), and TH, VMAT2, DAT and D2R define dopamine neuron functions mimicking in vivo development expression patterns. The organoid expresses genes previously known to be involved in the development of dopaminergic neurons (Blaess S, Ang SL. Genetic control of midbrain dopaminergic neuron development.Wiley Interdiscip Rev Dev Biol. 2015 Jan 6. doi: 10.1002/wdev.169).

FIG. 6B is a table showing Ampliseq gene expression data for genes not expressed in organoid (column 2) and Human Brain Reference (column 3). This data indicates that the organoids generated do not express genes that are characteristic of non-neural tissues. This gene expression concordance is less than 5% for approximately 800 genes that are considered highly enriched or specifically expressed in a non-neural tissue. The olfactory receptor genes expressed in the olfactory epithelium shown are a representative example. Gene expression for most genes in table is zero.

FIG. 7 includes schematics showing developmental heat maps of transcription factors (TF) expressed in cerebellum development and of specific Markers GRID 2.

FIG. 8 provides a schematic and a developmental heat map of transcription factors expressed in Hippocampus Dentate Gyrus.

FIG. 9 provides a schematic and a developmental heat map of transcription factors expressed in GABAergic Interneuron Development. GABAergic Interneurons develop late in vitro.

FIG. 10 provides a schematic and a developmental heat map of transcription factors expressed in Serotonergic Raphe Nucleus Markers of the Pons.

FIG. 11 provides a schematic and a developmental heat map of transcription factor transcriptomics. Hox genes involved in spinal cord cervical, thoracic and lumbar region segmentation are expressed at discrete times in utero. The expression pattern of these Hox gene in organoids as a function of in vitro developmental time (1 week; 4 weeks; 12 weeks)

FIG. 12 is a graph showing the replicability of brain organoid development from two independent experiments. Transcriptomic results were obtained by Ampliseq analysis of normal 12 week old brain organoids.

FIG. 13 provides a schematic and gene expression quantification of markers for astrocytes, oligodendrocytes, microglia and vasculature cells.

FIG. 14 includes scatter plots of Ampliseq whole genome transcriptomics data from technical replicates for Normal (WT), Tuberous Sclerosis (TSC2) and TSC2 versus WT at ~1 week in culture. Approximately 13, 000 gene transcripts are represented in each replicate.

FIG. 15 shows developmental heat maps of transcription factors (TF) expressed in retina development and other specific Markers. Retinal markers are described, for example, in Farkas et al. BMC Genomics 2013, 14:486.

FIG. 16 shows developmental heat maps of transcription factors (TF) and Markers expressed in radial glial cells and neurons of the cortex during development

FIG. 17 is a schematic showing the brain organoid development in vitro. iPSC stands for induced pluripotent stem cells. NPC stands for neural progenitor cell.

FIG. 18 is a graph showing the replicability of brain organoid development from two independent experiments.

FIG. 19 is a table showing the change in the expression level of certain genes in TSC2 (ARG1743GLN) organoid. About 13,000 gene were analyzed, among which 995 genes are autism related and 121 genes are cancer related.

FIG. 20 is a schematic showing the analysis of gene expression in TSC2 (ARG1743GLN) organoid.

FIGS. 21A and 21B are two tables showing the change in the expression level of certain genes in APP gene duplication (ALA246GLU) organoid.

DETAILED DESCRIPTION OF THE INVENTION

The invention features an induced pluripotent stem cell (iPSC) derived organoid useful as an *in vitro* model to study genetic, molecular, and cellular abnormalities associated with human disorders. This organoid recapitulates *in vitro* the development, physiology, and other characteristics of the brain (e.g., human, rodent). The invention further provides methods of using this neural organoid to study disease and to identify therapeutic agents for the treatment of neurological diseases and disorders.

The invention is based, at least in part on methods useful for engineering a human brain organoid that after ~12 weeks of culture *in vitro* exhibits a level of development comparable to that of a human embryonic brain after about 5 weeks *in utero*. These organoids express markers characteristic of a large variety of neurons. The organoids also include markers for astrocytic, oligodendritic, microglial, and vascular cells. These organoids form all the major regions of the brain including the retina, cortex, midbrain, brain stem, and the spinal cord in a single brain structure which expresses >98% of the genes known to be expressed in the human brain. This organoid is useful as a platform to enable screening of therapeutic agents for efficacy, safety, and toxicity prior to in vivo use in humans.

In particular embodiments, organoids are derived from iPSCs of fibroblast origin. The full development of major parts of brain: retina, cortex, midbrain, hindbrain, and spinal cord within 12 weeks can be observed in these organoids. These organoids may be formed on 96-well plates. Interactive milieu of brain circuits are present in these organoids. Neural niche effects, such as exchange of miRNAs and proteins by exosomes among neurons as well as glial cells, are maintained in these organoids. Results from two independent experiments show greater than 99% reproducibility in gene expression patterns. These have been matched to a human brain reference. Technical replicates from three independent iPSC lines show greater than 99% gene expression patterns. Results from three independent brain organoids, one of which is derived from a female, show greater than 99% gene pattern similarity except for specific diseases pathology. The organoid model is under development to reach an FDA metric for clinical diagnostic use and drug development.

Screening Assays

Neural organoids can be used for toxicity and efficacy screening of agents that treat or prevent the development of a neurological condition. In one embodiment, an organoid generated according to the methods described herein is contacted with a candidate agent. The viability of the organoid (or various cells within the organoid) is compared to the viability of an untreated control organoid to characterize the toxicity of the candidate compound. Assays for measuring cell viability are known in the art, and are described, for example, by Crouch et al. (J. Immunol. Meth. 160, 81–8); Kangas et al. (Med. Biol.62, 338–43, 1984); Lundin et al., (Meth. Enzymol.133, 27–42, 1986); Petty et al. (Comparison of J. Biolum. Chemilum.10, 29–34, .1995); and Cree et al. (AntiCancer Drugs 6: 398–404, 1995). Cell viability can be assayed using a variety of methods, including MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) (Barltrop, Bioorg. & Med. Chem. Lett.1: 611, 1991; Cory et al., Cancer Comm. 3, 207–12, 1991; Paull J. Heterocyclic Chem. 25, 911, 1988). Assays for cell viability are also available commercially. These assays include but are not limited to CELLTITER-GLO® Luminescent Cell Viability Assay (Promega), which uses luciferase technology to detect ATP and quantify the health or number of cells in culture, and the CellTiter-Glo® Luminescent Cell Viability Assay, which is a lactate dehyrodgenase (LDH) cytotoxicity assay (Promega).

In another embodiment, the organoid comprises a genetic mutation that effects neurodevelopment, activity, or function. Polypeptide or polynucleotide expression of cells within the organoid can be compared by procedures well known in the art, such as Western blotting, flow cytometry, immunocytochemistry, *in situ* hybridization, fluorescence *in situ* hybridization (FISH), ELISA, microarray analysis, RT-PCR, Northern blotting, or colorimetric assays, such as the Bradford Assay and Lowry Assay.

In one working example, one or more candidate agents are added at varying concentrations to the culture medium containing an organoid. An agent that promotes the expression of a polypeptide of interest expressed in the cell is considered useful in the invention; such an agent may be used, for example, as a therapeutic to prevent, delay, ameliorate, stabilize, or treat an injury, disease or disorder characterized by a defect in neurodevelopment or neurological function. Once identified, agents of the invention may be used to treat or prevent a neurological condition.

In another embodiment, the activity or function of a cell of the organoid is compared in the presence and the absence of a candidate compound. Compounds that desirably alter the activity or function of the cell are selected as useful in the methods of the invention.

Test Compounds and Extracts

In general, agents useful in the invention are identified from large libraries of natural product or synthetic (or semi-synthetic) extracts or chemical libraries or from polypeptide or nucleic acid libraries, according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Agents used in screens may include known those known as therapeutics for the treatment of neurological conditions. Alternatively, virtually any number of unknown chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as the modification of existing polypeptides.

Libraries of natural polypeptides in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, Fla.), and PharmaMar, U.S.A. (Cambridge, Mass.). Such polypeptides can be modified to include a protein transduction domain using methods known in the art and described herein. In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:6909, 1993; Erb *et al.*, *Proc. Natl. Acad. Sci. USA* 91:11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37:2678, 1994; Cho *et al.*, *Science* 261:1303, 1993; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop *et al.*, *J. Med. Chem.* 37:1233, 1994. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of polypeptides, chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds.

Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, N.H.) and Aldrich Chemical (Milwaukee, Wis.). Alternatively, chemical compounds to be used as candidate compounds can be synthesized from readily available starting materials using standard synthetic techniques and methodologies known to those of ordinary skill in the art. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds identified by the methods described herein are known in the art and include, for example, those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T. W. Greene and P. G. M. Wuts, Protective Groups in Organic Synthesis, 2nd ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.*, *Proc Natl Acad Sci USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990; Devlin, *Science* 249:404-406, 1990; Cwirla *et al. Proc. Natl. Acad. Sci.* 87:6378-6382, 1990; Felici, *J. Mol. Biol.* 222:301-310, 1991; Ladner *supra.*).

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their activity should be employed whenever possible.

When a crude extract is found to have the desired activity further fractionation of the positive lead extract is necessary to isolate molecular constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that treats or prevents a neurological defect. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful as therapeutics are chemically modified according to methods known in the art.

Kits

In one embodiment, the invention provides for kits comprising an organoid of the invention. In another embodiment, the invention provides reagents for obtaining an organoid described herein, alone or in combination with directions for the use of such reagents. Associated with such kits may be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: Generation of human induced pluripotent stem cell-derived neural organoids.

Human induced pluripotent stem cell-derived neural organoids were generated as follows.

Preparation of MEFs

- Plate irradiated murine embryonic fibroblasts (MEFs) on gelatin coated substrate in MEF media at a density of 2 x 10^5 cells per well. Place the plate in the 37°C incubator overnight.

Passaging induced pluripotent stem cells (iPSCs):

- Wash MEFs with prewarmed PBS. Replace media with 1 ml iPSC media/ROCK inhibitor per well.
- Remove the iPSC plate from the incubator. Feed iPSC cells with iPSC media. Using a sterile StemPro EZPassage tool, cut and resuspend the iPSC colonies. Gently resuspend cells, and divide and transfer to the MEF containing wells (1:1)

1. Making Embryoid Bodies (EBs):

- Coat a 100mm culture dish with 0.1% gelatin. Put in 37oC incubator for 20 minutes. Remove gelatin, and let the dish air dry in BSC till ready to use.
- Two wells of a 6 well plate should provide enough cells for a 96 well plate. Wash wells containing iPSCs and MEFs with prewarmed PBS that lacks Ca2+/Mg2+. Remove the PBS solution and replace with 1ml/well of ACCUTASE™, a prewarmed cell detachment solution of proteolytic and collagenolytic enzymes. Incubate plates at 37°C incubator for 20 minutes until all of the cells are detached.
- Add prewarmed iPSC media to each well and gently triturate to break up visible colonies.
- Add additional pre-warmed media to 15 mls, and move the cells onto a gelatin-coated culture plate at 37°C incubator for 60 minutes to allow MEFs to adhere to the coated surface. The iPSCs present in the cell suspension are counted.
- Centrifuge the suspension at 300xg for 5 minutes at room temperature. Discard the supernatant and resuspend the cells in EB media with ROCK inhibitor (50uM final concentration) to a volume of 9,000 cells/150 µl.
- Plate 150 µl in a LIPIDURE® low-attachment U-bottom 96-well plate incubate at 37°C. The LIPIDURE coating contains MPC Polymer, a biocompatible polymer composed by Phosphoryl Choline.

2. Initiation of germ layer differentiation:

- EBs are fed every other day by gently replacing three fourths of the EB media without disturbing the EB forming at the bottom of the well. It is important that the interactions among the iPSC cells within the EB are not perturbed by shear stress during pipetting. For the first four days, the EB media includes 50uM ROCK inhibitor and 4ng/ml bFGF. For the remaining two to three days, no ROCK inhibitor or bFGF is added to the EB.

3. Induction of primitive neuroepithelia:

- EBs in the LIPIDURE® 96 well plate are transferred on the sixth or seventh day to two 24 well plates containing 500µl/well Neural Induction media. Two EBs are gently plated in each well.
- After 2 days, the media is changed. The EBs should take on a "halo" around their perimeter, indicating neuroectodermal differentiation. Only EBs having a "halo" are selected for embedding in matrigel. Other EBs are discarded.

4. **Matrigel Embedding:**

- Sterilize plastic paraffin film (PARAFILM) rectangles [5cmx7cm] using 3% hydrogen peroxide and create a series of dimples in the rectangles. This may be accomplished, for example, by centering the rectangles onto an empty sterile 200ul tip box press, and pressing the rectangles gently to dimple it with the impression of the holes in the box. Spray the boxes with ethanol, and let them stay in the BSC to dry.
- Thaw frozen Matrigel matrix aliquots (500 µl) on ice in the refrigerator for 2-3 hours until equilibrated at 4°C.
- A single EB from Step 3 is transferred to each dimple of the film. A 7cm X 5cm rectangle should be hold 20 EBs. 20µl aliquots of Matrigel are transferred onto the EB after removing extra media with a pipette . Incubate at 37°C for 30 min to allow the Matrigel to polymerize. The 20µl droplet of viscous Matrigel was found to form an optimal 3D environment that supports the proper growth of the brain organoid from EBs by sequestering the gradients of morphogens and growth factors secreted by cells within the EB early, yet permitting exchange of essential nutrients and gases. Gentle oscillation by hand twice a day for a few minutes within a tissue culture

incubator (37°C/5%CO_2) further allows optimal exchange of gases and nutrients to the embedded EBs.

- Add Differentiation Media 1 a 100mm tissue culture dish. Invert the film containing the EB in Matrigel onto the media and incubate at 37°C for 16 hours.
- After 16 hours, the EB/Matrigel droplets are transferred from the film into culture dishes containing media. Static culture at 37°C is continued for 4 days to form stable neural organoids.

5. Organoid Development:

Organoids are gently transferred to culture dishes containing differentiation media 2. The flasks are set on an orbital shaker rotating at 40 rpm within the 37°C/5% incubator. Without wishing to be bound by theory, these conditions were selected to minimize disturbance of diffusion gradients among early progenitors of neurons of different lineages that are may affect patterning during development of the brain organoids into more complex and complete structures that include the retina, cortex, midbrain, hindbrain and spinal cord; to provide optimum exchange of gases within the matrix for survival of organoids and prevent apoptosis; provide nutrients to diffuse into the matrix optimally; and allow efflux of waste products effectively mimicking the function of the cerebrospinal fluid. The media is changed in the flasks every 3-4 days to provide sufficient time for morphogen and growth factor gradients to act on targets within the recipient cells forming relevant structures of the brains. The change of media is done with care to avoid unnecessary perturbations to the morphogen/secreted growth factor gradients setting up in the outer most periphery of the organoids as the structures grow into larger organoids.

FIG. 16 ilustrates the brain organoid development in vitro. Based on transcriptomic analysis, iPSC cells form a body of cells after 3D culture, which becomes neural progenitor cells (NPC) after neural differentiation media treatment. Neurons can be observed in the cell culture in about one week. In about four (4) weeks, neurons of multiple lineage appear. In about twelve (12) weeks, the organoid develops to a stage that has different types of cells, including microglia, oligodendrocyte, astrocyte, neural precursor, neurons, and interneurons.

Example 2: Human induced pluripotent stem cell-derived neural organoids express characteristics of human brain development.

After ~12 weeks in culture *in vitro*, transcriptomic and immunohistochemical analysis indicate that organoids generated according to the methods delineated in Example 1, contain

cells expressing markers characteristic of neurons, astrocytes, oligodendrocytes, microglia, and vasculature (FIGs. 1-14) and all major brain structures of neuroectodermal derivation. Morpologically by bright field imaging, the organoids include readily identifiable neural structures including cerebral cortex, cephalic flexture, and optic stalk (Grey's anatomy text book). Their gene expression pattern is >98 % concordant with those of the adult human brain reference (Clontech). They also express genes in a developmentally organized manner previously described (for the midbrain mescencephalic dopaminergic neurons, for example; Blaese et al., 2015). They also stain for multiple neural specific markers (dendrites, axons, nuclei), cortical neurons (Doublecortin) midbrain dopamine neurons (Tyrosine Hydroxylase) and astrocytes (GFAP by immunohistology).

All human organoids were derived from iPSCs of fibroblast origin (from System Biosciences, Inc). The development of a variety of brain structures was characterized in the organoids. Retinal markers are shown in FIG. 15. Doublecortin (DCX) a microtubule associated protein expressed during cortical development was observed (FIG. 1A and FIG. 1B, FIG. 16. Midbrain development was characterized using a marker for tyrosine hydroxylase (FIG. 2). Transcriptomics was used to detect the expression of the midbrain markers DLK1, KLHL1, and PTPRU (FIG. 6A). Staining with GFAP was used to identify the presence of astrocytes in the organoids (FIG. 3). The presence of mature neurons was characterized with staining for NeuN (FIG. 3). The presence of NKCC1 and KCC2, a neuron-specific membrane protein, was observed (FIG. 4). A schematic of the roles of NKCC1 and KCC2 is provided at FIG. 5A. FIG. 5B indicates that a variety of markers that are expressed during human brain development are also expressed in the organoids generated as described in Example 1.

Markers expressed within the organoids are consistent with the presence of the following cell types: excitatory, inhibitory, cholinergic, dopaminergic, serotonergic, astrocytic, oligodendritic, microglial, vasculature. These markers are consistent with those identified by the Human Brain Reference (HBR) from Clontech (FIG. 5C) and were reproducible in independent experiments (FIG. 5D). Markers characteristic of tissues outside the brain were not observed (FIG. 6B).

Tyrosine hydroxylase, which is an enzyme used in the synthesis of dopamine, was observed in the organoids using immunocytochemistry (FIG. 5B) and transcriptomics (FIG. 6A). The expression of other dopaminergic markers, including vesicular monoamine transporter 2 (VMAT2), dopamine active transporter (DAT) and Dopamine receptor D_2

(D2R) were observed using transcriptomic analysis. FIG. 7 delineates the expression of markers characteristic of cerebellar development. FIG. 8 provides a list of markers identified using transcriptomics that are characteristic of neurons present in the hippocampus dentate gyrus. spinal cord was observed after 12 weeks of in vitro culture. FIG. 9 provides a list of markers identified using transcriptomics that are characteristic of GABAergic interneuron development. FIG. 10 provides a list of markers identified using transcriptomics that are characteristic of the brain stem, in particular, markers associated with the serotonergic raphe nucleus of the pons. FIG. 11 lists the expression of various Hox genes that are expressed during the development of the cervical, thoracic and lumbar regions of the spinal cord.

FIG. 12 shows that results are reproducible between experiments. The expression of markers detected using transcriptomics is summarized in FIG. 13.

In sum, the results reported herein support that the invention provides an *in vitro* cultured organoid that resembles a ~5 week old human fetal brain based on size and specific morphological features with great likeness to the optical stock, the cerebral hemisphere, and cephalic flexure in a ~2-3mm organoid that can be grown in culture dishes. High resolution morphology analysis was carried out using immunohistological methods on sections and confocal imaging of the organoid to establish the presence of neurons, axons, dendrites, laminar development of cortex, and the presence of midbrain marker.

This organoid includes an interactive milieu of brain circuits as represented by the laminar organization of the cortical structures in Fig. X and thus supports formation of native neural niches in which exchange of miRNA and proteins by exosomes can occur among different cell types.

The brain organoids were evaluated at weeks 1, 4 and 12 by transcriptomics. The organoid is reproducible and replicable (FIGs. 5C, 5D, FIG. 12, and FIG. 18). Brain organoids generated in two independent experiments and subjected to transcriptomic analysis showed >99% replicability of the expression pattern and comparable expression levels of most genes with <2-fold variance among some of them.

Gene expression patterns were analyzed using whole genome transcriptomics as a function of time in culture. Results reported herein indicates that known developmental order of gene expression *in vivo* occurs, but on a somewhat slower timeline. Using the transcription factors NURR1 and PITX3 that are uniquely expressed in the development of mesencephalic neurons in the midbrain as examplars, we show that their temporal expression patterns in vitro replicate known in vivo gene expression patterns (Fig 6A). Similarly, the

transition from GABA mediating excitation to inhibition, occurs following the switch over of the expression of the Na(+)-K(+)-2Cl(--)) cotransporter NKCC1 (SLC12A2), which increases intracellular chloride ions, to the K(+)-Cl(-) cotransporter KCC2 (SLC12A5) (Owens and Kriegstein, 2002), which decreases intracellular chloride ions (Blaesse et al., 2009). We have data on the development of the brain organoids in culture in which the expression profile of NKCC1 and KCC2 changes in a manner consistent with an embryonic brain transitioning from GABA being excitatory to being inhibitory (Fig. 4 & 5) and can be monitored by developmental transcriptomics.

The organoids described above were obtained using the following methods and materials.

Cells:

- Human iPSCs, feeder-dependent (System Bioscience. WT SC600A-W)
- CF-1 mouse embryonic fibroblast feeder cells, gamma-irradiated (Applied StemCell, Inc #ASF- 1217)

Growth Media and supplements

- DMEM non-essential amino acids (MEM-NEAA, Invitrogen #11140-050)
- Phosphate Buffered Saline, sterile (Invitrogen #14040-091)
- Phosphate Buffered Saline, Ca++ and Mg++ free (Invitrogen #14190-094)
- Gentamicin Reagent Solution (Invitrogen #15750-060)
- Antibiotic-Antimycotic (Invitrogen #15240-062)
- 2-mercaptoethanol (EmbryoMAX, EMBMillipore#ES-007-E)
- Basic fibroblast growth factor (FGF, PeproTech #051408-1)
- Heparin (Sigma, #H3149-25KU) • Insulin solution (Sigma #I9278-5ml)
- Dimethyl sulfoxide (#D9170-5VL) • ROCK Inhibitor Y27632 (Millipore#SCM075)
- Gelatin solution, Type B (Sigma#G1393-100ml)
- Matrigel Matrix (BD Bioscience #354234), NOT Growth Factor Reduced Matrigel
- Accutase (Sigma #A6964)
- Hydrogen Peroxide (Fisher #H325-500)
- Ethanol
- Sterile H2O

Media composition:

MEF Media: DMEM media supplemented with:

- 10% Feta Bovine Serum
- 100units/ml penicillin
- 100 microgram/ml streptomycin
- 0.25 microgram/ml Fungizone
- IPSC Media: DMEM/F12 media supplemented with:
- 20% KnockOut Replacement Serum
- 3% Fetal Bovine Serum o 2mM Glutamax
- 1X Minimal Essential Medium Nonessential Amino Acids
- 20nanogram/ml basic Fibroblast Growth Factor

EB Media: Dulbecco's Modified Eagle's Medium (DMEM) (DMEM)/Ham's F-12 media (commercially available from Invitrogen) supplemented with:

- 20% KnockOut Replacement Serum
- 3% Fetal Bovine Serum o 2mM Glutamax
- 1X Minimal Essential Medium Nonessential Amino Acids
- 55microM beta-mercaptoethanol
- 4ng/ml basic Fibroblast Growth Factor
- Neural Induction Media: DMEM/F12 media supplemented with:
- 1:50 dilution N2 Supplement
- 1:50 dilution GlutaMax
- 1:50 dilution MEM-NEAA
- 10microgram/ml Heparin

Differentiation Media 1: DMEM/F12 media: Neurobasal media (1:1) (each of which is commercially available from Invitrogen) supplemented with:

- 1:200 dilution N2 supplement
- 1:100 dilution B27 – vitamin A
- 2.5microgram/ml insulin
- 55microM beta-mercaptoethanol kept under nitrogen mask and frozen at -20°C.
- 100units/ml penicillin
- 100microgram/ml streptomycin
- 0.25microgram/ml Fungizone

DIFFERENTIATION MEDIA 2: DMEM/F12 media: Neurobasal media (1:1) supplemented with:

- 1:200 dilution N2 supplement
- 1:100 dilution B27 + vitamin A
- 2.5microgram/ml Insulin
- 55uM beta-mercaptoethanol kept under nitrogen mask and frozen at -20°C . Without wishing to be bound by theory, beta-mercaptoethanol provides a redox condition for proper iPSC health and growth into EBs in the 20% oxygen environment, which likely promotes production of toxic reactive oxygen species, in the incubator and any loss of its redox capacity due to improper storage conditions may impair proper development of organoids from EBs derived from iPSC.
- 100units/ml penicillin
- 100microgram/ml streptomycin
- 0.25microgram/ml Fungizone

DIFFERENTIATION MEDIA 3: DMEM/F12 media: Neurobasal media (1:1) supplemented with:

- 1:200 dilution N2 supplement o 1:100 dilution B27 + vitamin A
- 2.5microgram/ml insulin
- 55microM beta-mercaptoethanol kept under nitrogen mask and frozen at -20oC. Without intending to be bound by theory, beta-mercaptoethanol may contribute to the development of midbrain structures in brain organoids from EBs
- 100units/ml penicillin
- 100microgram/ml streptomycin
- 0.25microgram/ml Fungizone
- melatonin
- TSH

Equipment:

- StemPro EZPassage (Invitrogen#23181-010) Without wishing to be bound by theory, the EZPassage tool cuts uniform squares of iPSCs which lead to more uniform iPSc colonies for subcloning. The uniformity enhances downstream homogeneity when making EBs.
- Tissue Culture Flasks, 115cm2 reclosable (TPP #TP90652)
- Tissue Culture Flask, 150cm2 reclosable (TPP#TP90552)
- Lipidure coat plate, 96 wells, U-bottom (LCU96)
- Lipidure coat MULTI dish, 24 well (510101619)
- Parafilm (Sigma #P7793) Sterile Filtration Units for 150ml/250ml solutions (TPP99150, TPP99250)

- Benchtop Tissue Culture Centrifuge CO2 incubator, maintained at 37°C and 5% CO2

Example 3: Tuberous sclerosis complex model

Tuberous sclerosis complex (TSC) is a genetic disorder that causes non-malignant tumors to form in many different organs, including the brain. TSC strongly impacts quality of life because patients have seizures, developmental delay, intellectual disability and autism. Two genes have been identified that can cause tuberous sclerosis complex. The TSC1 gene is located on chromosome 9 and is called the hamartin gene. The other gene, TSC2, is located on chromosome 16 and is called the tuberin gene.

We have derived a human brain organoid from iPSC cells derived from a patient with a gene variant of the TSC2 gene (ARG1743GLN) from iPSCs (Cat# GM25318 Coriell Institute Repository, NJ). This organoid serves as a genetic model of a tuberous sclerosis TSC2 mutant. Both normal and TSC2 mutant models were subject to genome wide transcriptomic analysis using the Ampliseq analysis to assess changes in gene expression and how well they correlated with known clinical pathology associated with TSC patients (Fig. 14).

The whole genome transcriptomic data shows that of all the genes expressed (~13, 000), less than 1 dozen show >2-fold variance in the replicates for both WT and TSC2. This is additional supporting evidence for the robustness and replicability of our brain organoids derivation process at 1 week in culture. TS patients clinically have tumors typically in multiple organs including their brains, lungs, heart, kidneys and skin (Harmatomas). In the comparison of WT versus TSC2, the genes that show >2-fold to 300-fold difference, include those correlated with 1) tumor formation and 2) autism mapped using whole genome and exome sequencing strategies (SFARI site data base) (FIG. 19 and 20).

FIG. 19 shows Ampliseq gene expression data for genes in the Simon Foundation (SFARI) data base compared between replicates of organoids from the TSC2 (Arg1743Gln) model (column 2 and 3) and the WT (normal) model (column 3 and 4). Highlighted are autism genes and genes associated with other clinical symptoms with fold change (column 5) and SFARI data base status or known tumor forming status.

Thus, the transcriptomic data correlates well with known clinical phenotypes of tumors, autism and other clinical symptoms in Tuberous Sclerosis patients and demonstrates the utility of the human brain organoid development model.

Example 4: Alzheimer's Disease APP1 Gene Duplication Human Brain Organoid

Model

Alzheimer's is a common form of dementia, associated with memory loss and other intellectual abilities that interfere with daily life. Alzheimer's disease accounts for 60 to 80 percent of dementia cases. Two abnormal structures called plaques and tangles are thought to damage and kill nerve cells. Plaques are deposits of a protein fragment called beta-amyloid that build up in the spaces between nerve cells. Tangles are twisted fibers of another protein called tau that build up inside cells.

A human brain organoid was generated from iPSC cells derived from a patient with a variant of the amyloid precursor protein (APP) gene in which the gene is duplicated from a 60 years old woman with early onset of AD. The iPSC was obtained from Coriell Institute in NJ.

The PSEN1 gene provides encodes a protein called presenilin 1. This protein is one part (subunit) of a complex called gamma- (γ-) secretase. Presenilin 1 carries out the major function of the complex, which is to cleave other proteins into smaller peptides by proteolysis, and presenilin 1 is described as the proteolytic subunit of γ-secretase.

The γ-secretase complex is located in the membrane that surrounds cells, where it cleaves many different proteins that span the cell membrane (transmembrane proteins). This cleavage is an important step in several chemical signaling pathways that transmit signals from outside the cell into the nucleus. One of these pathways, known as Notch signaling, is essential for the normal maturation and division of hair follicle cells and other types of skin cells. Notch signaling is also involved in normal immune system function.

The γ-secretase complex may be best known for its role in processing amyloid precursor protein (APP), which is made in the brain and other tissues. γ-secretase cuts APP into smaller peptides, including soluble amyloid precursor protein (sAPP) and several versions of amyloid-beta (β) peptide. Evidence suggests that sAPP has growth-promoting properties and may play a role in the formation of nerve cells (neurons) in the brain both before and after birth. Other functions of sAPP and amyloid-β peptide are under investigation.

The utility of the brain organoid model system was tested by engineering a genetic brain organoid model of an Alzheimer's patient with an APP mutation. Both normal and the APP mutant models were subject to whole genome transcriptomic analysis to assess changes in gene expression at 4 week in culture and how well they correlated with known clinical pathology associated with AD patients.

FIGs. 21A and 21B show the Ampliseq gene expression comparison for genes in SFARI database between replicates of organoids from the AD (APP) model (column 2 and 3) and the WT (normal) model (column 4 and 5) with fold change (column 6). These are representative examples of genes whose expression are dysregulated in the Alzeimer's Disease model.

The whole genome transcriptomic data shows that of all the genes expressed (~13, 000 at 4 week in culture), only ~1800 show >2-fold variance in the replicates for both WT and APP. This is additional supporting evidence for the robustness and replicability of the brain organoids derivation process.

In summary, because about eighteen hundreds of dysregulated genes map to databases dedicated to Alzheimer's disease, a new gene regulatory network perturbed by the APP mutation was identified as an "Alzheimer's network". The implications are that the hundreds of gene variants correlated with autism identified by genomics likely represent only a few Alzheimer's networks suggesting that identifying the nodes in these networks will vast simplify identifying therapeutic targets for AD.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. An *in vitro* generated three-dimensional neural organoid derived from a human induced pluripotent stem cell (hIPSC), the organoid comprising a first region expressing retinal or cortical markers and one or more additional neural regions, each expressing markers of the brain stem, cerebellum, and/or spinal cord.

2. The organoid of claim 1, wherein the organoid comprises a cell expressing one or more neural markers and a cell expressing a marker selected from the group consisting of astrocytic markers, oligodendrocyte markers, microglia markers, and/or vascular markers.

3. The organoid of claim 1, wherein the hIPSC comprises a genetic mutation associated with a neurological defect.

4. The organoid of claim 1, wherein the genetic mutation is in TSC1, TSC2, PSEN1, or APP.

5. An *in vitro* generated three-dimensional neural organoid derived from human induced pluripotent stem cells, the organoid comprising a first cell type expressing neural markers, and a second cell type expressing an astrocytic marker, oligodendrocyte marker, microglia marker, or vascular marker.

6. The neural organoid of any one of claims 2-5, wherein the neural marker is a retinal marker selected from the group consisting of retina specific Guanylate Cyclases (GUY2D, GUY2F), Retina And Anterior Neural Fold Homeobox (RAX), and retina specific Amine Oxidase, Copper Containing 2 (RAX).

7. The neural organoid of any one of claims 2-5, wherein the neural marker is a cortical marker selected from the group consisting of doublecortin, NeuN, FOXP2, CNTN4, and TBR1.

8. The neural organoid of any one of claims 2-5, wherein the neural marker is a marker of dopaminergic neurons selected from the group consisting of tyrosine hydroxylase, vesicular

monoamine transporter 2 (VMAT2), dopamine active transporter (DAT) and Dopamine receptor D_2 (D2R).

9. The neural organoid of any one of claims 2-5,, wherein the neural marker is ATOH1, PAX6, SOX2, LHX2, GRID2, or another cerebellar marker.

10. The neural organoid of any one of claims 2-5, wherein the neural marker is SOX2, NeuroD1, DCX, EMX2, FOXG1, PROX1, or another granule neuron marker.

11. The neural organoid of any one of claims 2-5,, wherein the neural marker is FGF8, INSM1, GATA2, ASCL1, GATA3, or another brain stem marker.

12. The neural organoid of any one of claims 2-5, wherein the neural marker is a homeobox gene selected from the group consisting of HOXA1, A2, A3, B4, A5, C8, or D13.

13. The neural organoid of any one of claims 2-5, wherein the neural marker is NKCC1, KCC2, or another GABAergic marker.

14. The neural organoid of any one of claims 2-5, wherein the astrocytic marker is GFAP, the oliogodendrocytic marker is OLIG2 or MBP, the microglia marker is AIF1 or CD4, and the vascular marker is NOS3.

15. A method for obtaining a neural organoid, the method comprising

(a) select minimally adherent human induced pluripotent stem cells (hIPScs) from a mixed culture of hIPScs and gamma irradiated mouse embryonic fibroblast feeder cells (MEFs), and culture the IPSCs under conditions that facilitate sphere formation to obtain an embryoid body (EB);

(b) transfer the EB to a plate and culture under conditions that induce neuroectodermal differentiation;

(c) culture the EB in a three-dimensional matrix comprising growth factors for about 3-5 days under static conditions;

(d) culture the EB in a three-dimensional matrix under conditions that facilitate the laminar flow of growth media, thereby obtaining a neural organoid.

16. A method for obtaining a neural organoid, the method comprising

(a) culturing iPSCs alone or in the presence of irradiated MEFs;

(b) culturing the iPSCs from step (a) under conditions that promote germ layer differentiation in a low-attachment U-bottom plate in the presence of ROCK inhibitor and bFGF for about four days and then culturing the iPSCs in media lacking ROCK inhibitor or bFGF to form ;

(c) plating the iPSCs from (b) in a low-attachment plate under conditions that promote neural induction and selecting embryoid bodies displaying neuroectodermal outgrowth from the embryoid body;

(d) embedding the selected embryoid body in a 3-dimensional culture matrix and culturing under conditions that promote neural organoid development while gently oscillating the culture 2-3 times daily; and

(e) statically culturing the neural organoid.

17. The method of claim 15 or 16 wherein beta mercaptoethanol stored under conditions that minimize oxidation is added to the culture media at each of steps a-e.

18. The method of claim 16 wherein the culture is gently oscillated for about 2 minutes twice daily to induce slow laminar flow of media within the culture.

19. The method of claim 15 or 16, wherein the amount of 3-dimensional culture matrix is optimized to sequester morphogens and growth factor while permitting exchange of nutrients and gases.

20. The method of claim 19, wherein the embryoid body is embedded in about 10, 20, or 30 µl of 3-dimensional culture matrix.

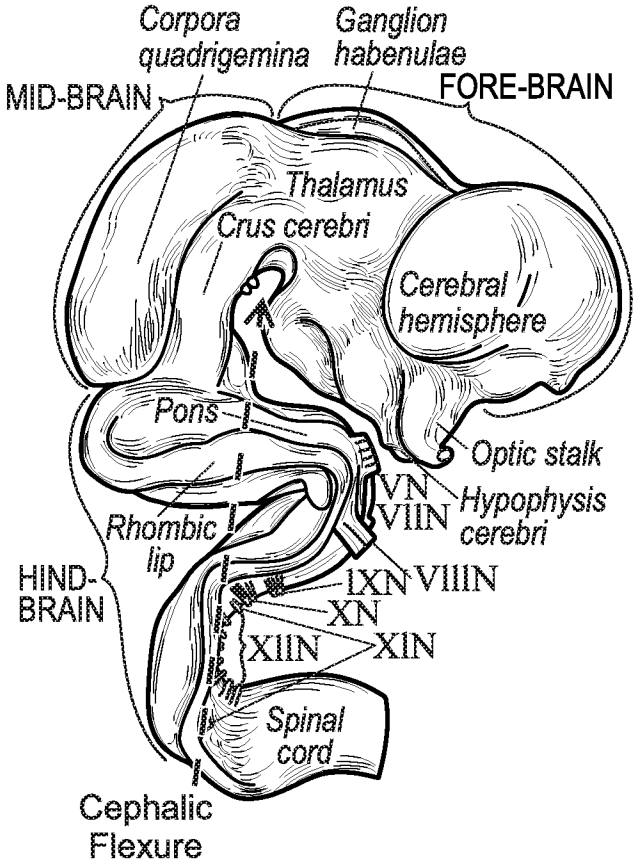
21. The method of claim 15, wherein the hIPSCs are selected by allowing the MEFs to adhere to a substrate, then removing the non-adherent hIPSCs.

22. The method of claim 15 or 16, wherein the three-dimensional matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) sarcoma cells.

23. An *in vitro* derived neural organoid generated according to the method of claim 15 or 16, wherein the organoid comprises a first region expressing retinal or cortical markers and one or more additional regions expressing markers of the midbrain, brain stem, cerebellum, and/or spinal cord.

24. The organoid of claim 23, wherein the organoid comprises a mutation associated with Alzheimer's disease or tuberous sclerosis.

FIG 1A


Corpora quadrigemina
Ganglion habenulae
MID-BRAIN
FORE-BRAIN
Thalamus
Crus cerebri
Cerebral hemisphere
Pons
Optic stalk
VN
VIIN
Hypophysis cerebri
Rhombic lip
HIND-BRAIN
IXN
VIIIN
XN
XIIN
XIN
Spinal cord
Cephalic Flexure

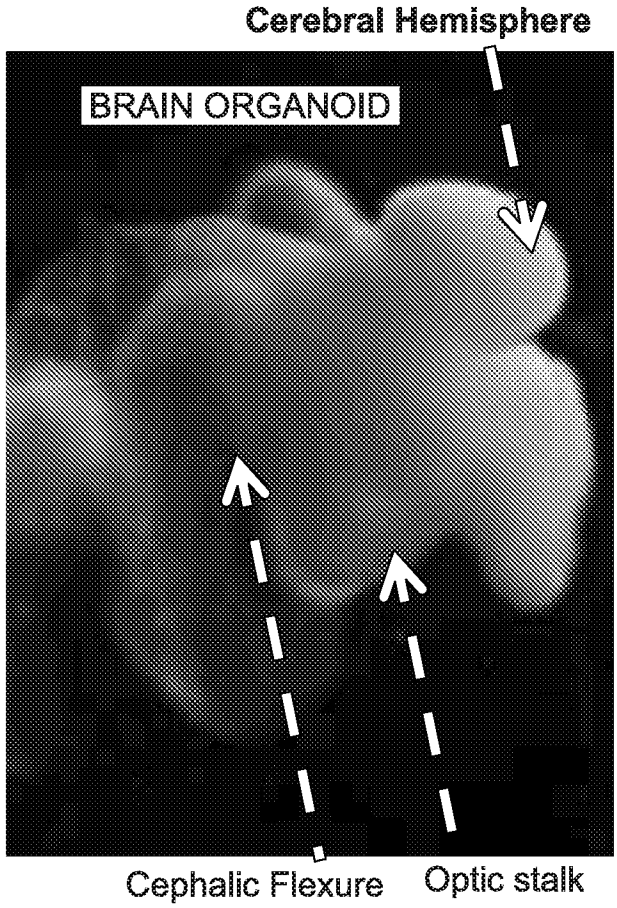

Cerebral Hemisphere
BRAIN ORGANOID
Cephalic Flexure
Optic stalk

FIG. 1B

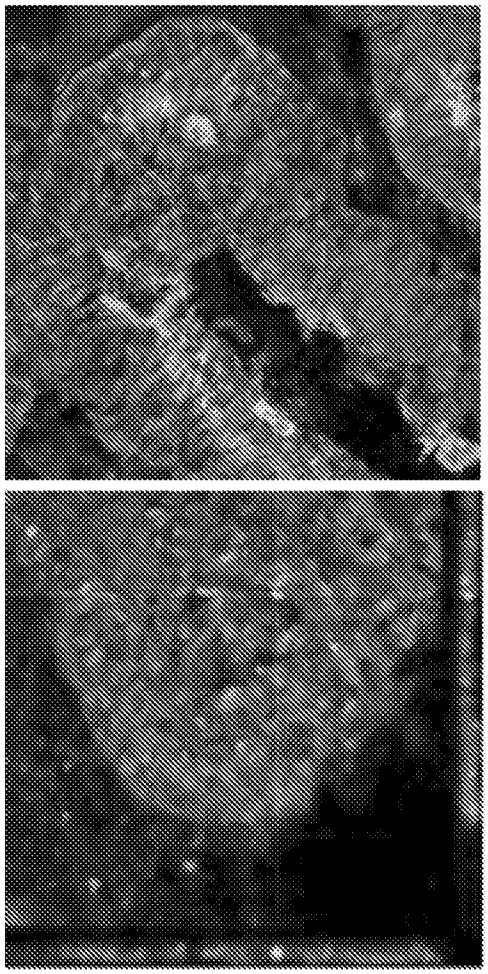


FIG. 2

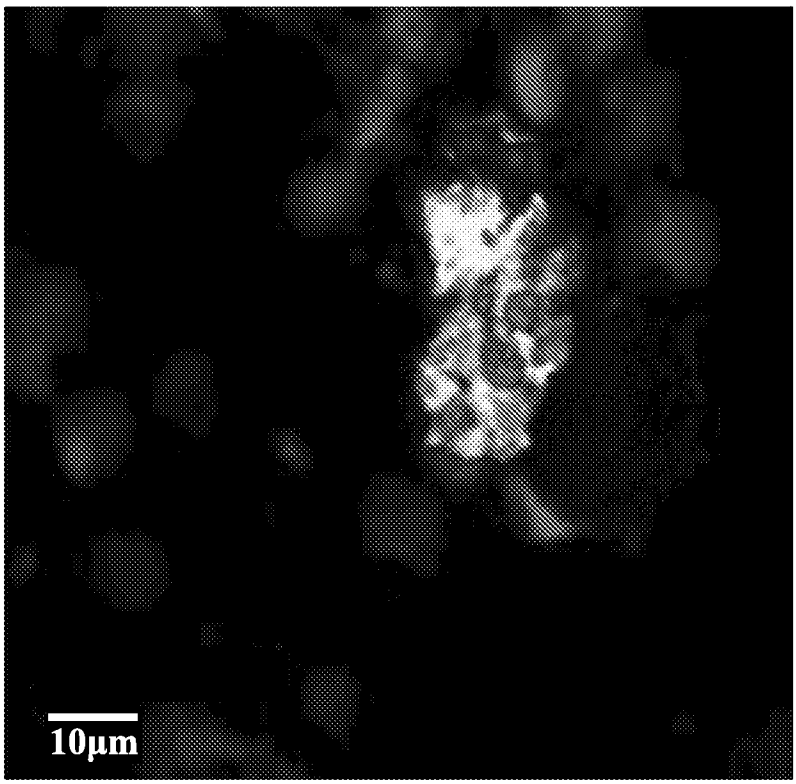


FIG. 3

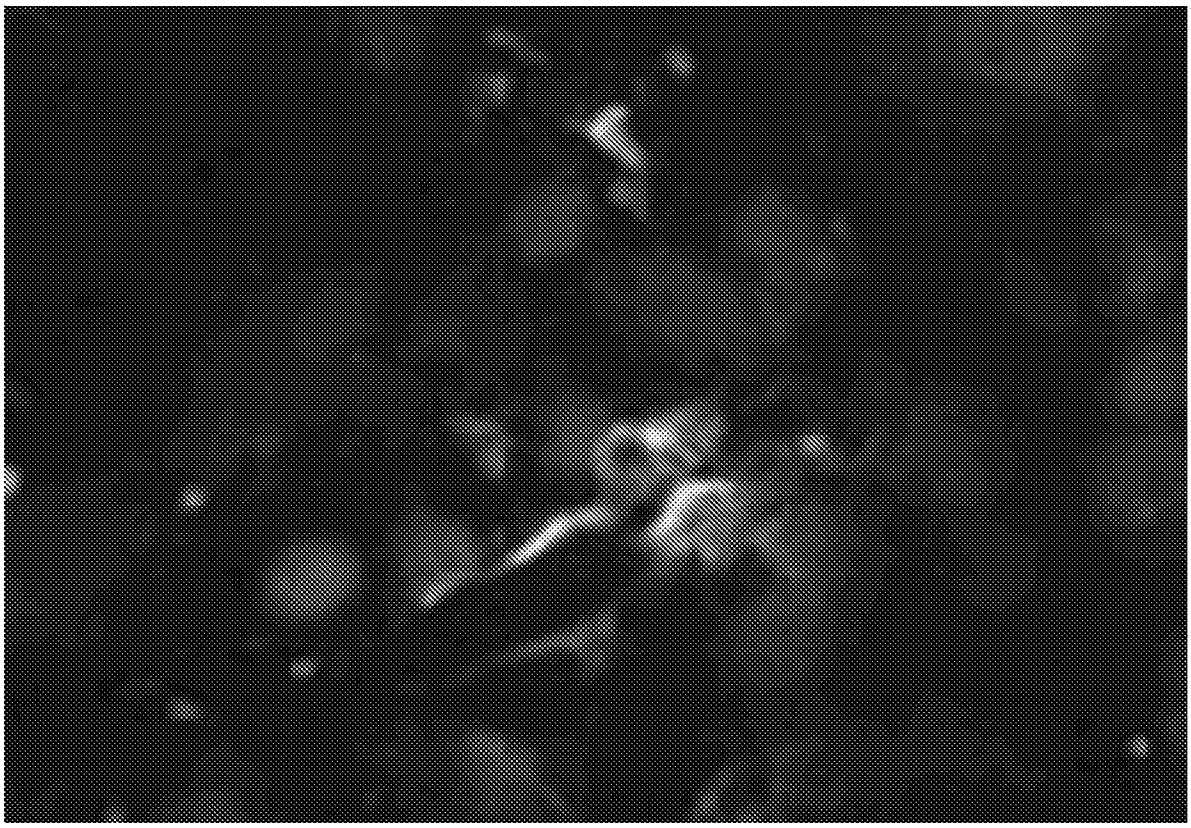
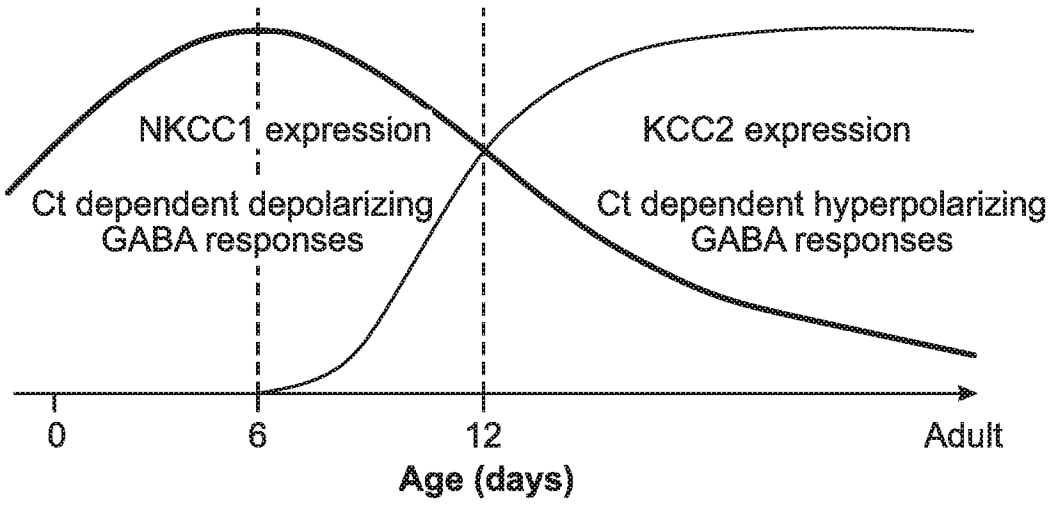


FIG. 4

	1 week	4 weeks	12 Weeks	
SLC12A2	31.792	54.439	35.969	NKCC1
SLC12A5	0	0.446	1.628	KCC2



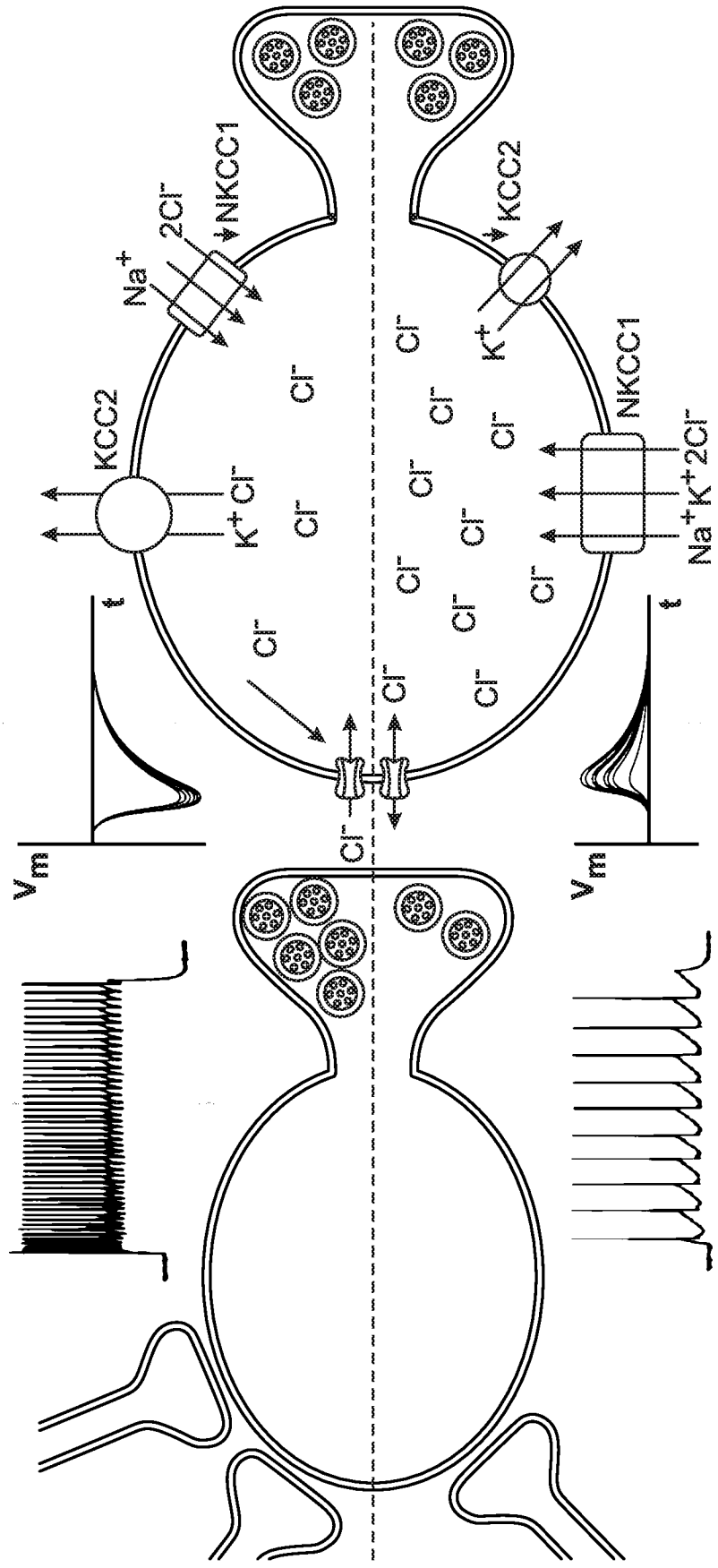

Mature
Immature
NKCC1
KCC2
KCC2
NKCC1
Na^+
$2Cl^-$
K^+
K^+Cl^-
$Na^+K^+2Cl^-$
Cl^-
V_m
t

FIG. 5A

FIG. 5B

Class	Genes										
Excitatory	GRIA1	GRIA2	GRIA3	GRIA4	GRIN2A	GRIN2B	GRIN2C	SLC1A1	SLC1A2		
Inhibitory	GABRA1	GABRA2	GABRA3	GABRR1	GABRR2	SLC6A13	GABRA6				
Cholinergic	CHRM1	CHRM2	CHRM3	CHRNA1	CHRNA3	CHRNA4	CHRNB2	CHRNB3	CHRNB4	VAT1	
Dopaminergic	TH	DAT	DRD1	DRD2	DRD3	DRD4	COMT	DDC	SLC18A1	SLCA18A2	
Serotonergic	HTR1A	HTR1B	HRT1C	HTR1D	HTR2A	HTR2C	MAOA	DDC	SLC6A2	SLC6A4	
Astrocytic	GFAP										
Oligodendritic	OLIG1	OLIG2	MOBP	MBB	PLP1	OMD	MOGS				
Microgial	AIF1	CD4	ICAM								
Vasculature	NOS3	ANGPT1	EDN3	VEGFA							

FIG. 5C

COMPARISON: Organoid versus Human Brain Reference; CONCORDANCE >98%

GENE	12 week	HBR
A1BG	0.977	9
A1CF	0.651	0
A2M	28.97	827
A2M-AS1	3.418	64
A2ML1	0.163	0
A2MP1	0	0
A4GALT	1.79	60
A4GNT	0	0
AA06	0	32
AAAS	26.854	174
AACS	20.019	331
AACSP1	0.651	2
AADAC	3.581	0
AADACL2	0	0
AADACL3	0	0
AADACL4	0	0
AADAT	20.344	114
AAED1	6.347	52
AAGAB	48.664	601
AAK1	10.091	2501
AAMDC	23.762	118
AAMP	50.779	939
AANAT	0	0
AAR2	41.828	359
AARD	0.814	15
AARS	256.664	4850
AARS2	13.02	145
AASDH	7.324	72
AASDHPPT	147.781	1081
AASS	9.44	63
AATF	65.265	657
AATK	11.393	1503
ABAT	105.953	5228
ABCA1	51.756	175
ABCA10	3.255	153
ABI1	77.308	1601
ABI2	47.362	941
ABI3	1.139	158
ABI3BP	62.498	129
ABL1	196.77	742
ABL2	43.13	1031
ABLIM1	7.161	1547
ABLIM2	0.651	559
ABLIM3	0.814	211
ABO	0.163	6
ABP1	0.814	0
ABR	4.72	432
ABRA	0	5
ABRACL	41.665	201
ABT1	28.482	234
ABTB1	17.74	2568
ABTB2	9.603	199
ACAA1	24.088	425
ACAA2	57.452	254
ACACA	42.316	491
ACACB	18.88	244
ACAD10	14.16	136
ACAD11	43.618	280
ACAD8	25.39	580
ACAD9	23.762	369
ACADL	1.953	22
ACADM	32.714	270
ACADS	13.183	173
ACADSB	7.649	257
ACTA1	28.482	17
ACTA2	119.95	702
ACTB	2844.786	47548
ACTBL2	0.326	0
ACTC1	84.795	10
ACTG1	5171.035	35006
ACTG1P4	1.302	104
ACTG2	3.418	46
ACTL10	5.371	34
ACTL6A	14.322	39
ACTL6B	6.185	352
ACTL7A	0	0
ACTL7B	0	0
ACTL8	0.163	0
ACTL9	0	0
ACTN1	97.164	618
ACTN2	14.811	358
ACTN3	0.814	31
ACTN4	224.113	2508
ACTR10	156.407	1718
ACTR1A	95.862	1353
ACTR1B	42.479	1514
ACTR2	334.949	4120
ACTR3	198.235	1318
ACTR3B	6.673	199
ACTR3BP2	0.326	0
ACTR3C	0	22
ACTR5	7.161	92
ACTR6	32.551	432

FIG. 5D

COMPARISON: Two Independent Organoid Samples; Reproducibility >99%

A1BG	0.977	2.934
A1CF	0.651	1.334
A2M	28.97	42.28
A2M-AS1	3.418	4.735
A2ML1	0.163	0
A2MP1	0	0
A4GALT	1.79	5.535
A4GNT	0	0
AA06	0	0.267
AAAS	26.854	35.144
AACS	20.019	18.406
AACSP1	0.651	0.133
AADAC	3.581	3.001
AADACL2	0	0
AADACL3	0	0
AADACL4	0	0
AADAT	20.344	10.803
AAED1	6.347	11.403
AAGAB	48.664	36.078
AAK1	10.091	8.002
AAMDC	23.762	24.207
AAMP	50.779	61.419
AANAT	0	0.2
AAR2	41.828	55.017
AARD	0.814	3.401
AARS	256.664	309.094
AARS2	13.02	19.206
AASDH	7.324	2.734
AASDHPPT	147.781	51.883
AASS	9.44	11.737
AATF	65.265	43.413
ABCD3	48.826	38.545
ABCD4	43.781	43.947
ABCE1	133.621	103.432
ABCF1	62.009	61.285
ABCF2	70.147	43.147
ABCF3	13.183	24.207
ABCG1	0.977	2.334
ABCG2	1.628	1.6
ABCG4	3.092	2.134
ABCG5	0	0.467
ABCG8	0	0.333
ABHD1	0.163	1.334
ABHD10	15.462	21.073
ABHD11	8.463	17.405
ABHD12	63.637	34.21
ABHD12B	0	0
ABHD13	4.557	17.072
ABHD14A	6.347	15.872
ABHD14B	23.437	57.551
ABHD15	7.975	12.004
ABHD16A	29.459	29.009
ABHD16B	4.069	3.268
ABHD2	131.017	266.815
ABHD3	22.623	21.14
ABHD4	28.482	65.487
ABHD5	18.88	19.806
ABHD6	15.136	28.209
ABHD8	13.834	18.872
ABI1	77.308	87.96
ABI2	47.362	59.685
ABI3	1.139	2.667
ACP5	2.767	3.401
ACP6	30.435	26.208
ACPL2	79.912	63.819
ACPP	7.649	9.536
ACPT	0	0.067
ACR	3.255	3.801
ACRBP	2.767	2.801
ACRC	1.79	1.534
ACRV1	0	0.067
ACSBG1	0.488	0.267
ACSBG2	0	0.067
ACSF2	38.736	28.342
ACSF3	12.044	14.138
ACSL1	40.038	27.942
ACSL3	153.315	44.614
ACSL4	74.216	53.35
ACSL5	1.302	1.4
ACSL6	0.651	2.067
ACSM1	0	0.067
ACSM2A	0	0.867
ACSM2B	0	0.267
ACSM3	4.557	4.935
ACSM4	0	0
ACSM5	0	0
ACSS1	50.291	32.943
ACSS2	0.488	0
ACSS3	11.067	11.537
ACTA1	28.482	3.468
ACTA2	119.95	176.187
ACTB	2844.786	3943.537
ACTBL2	0.326	0.6

FIG. 6A

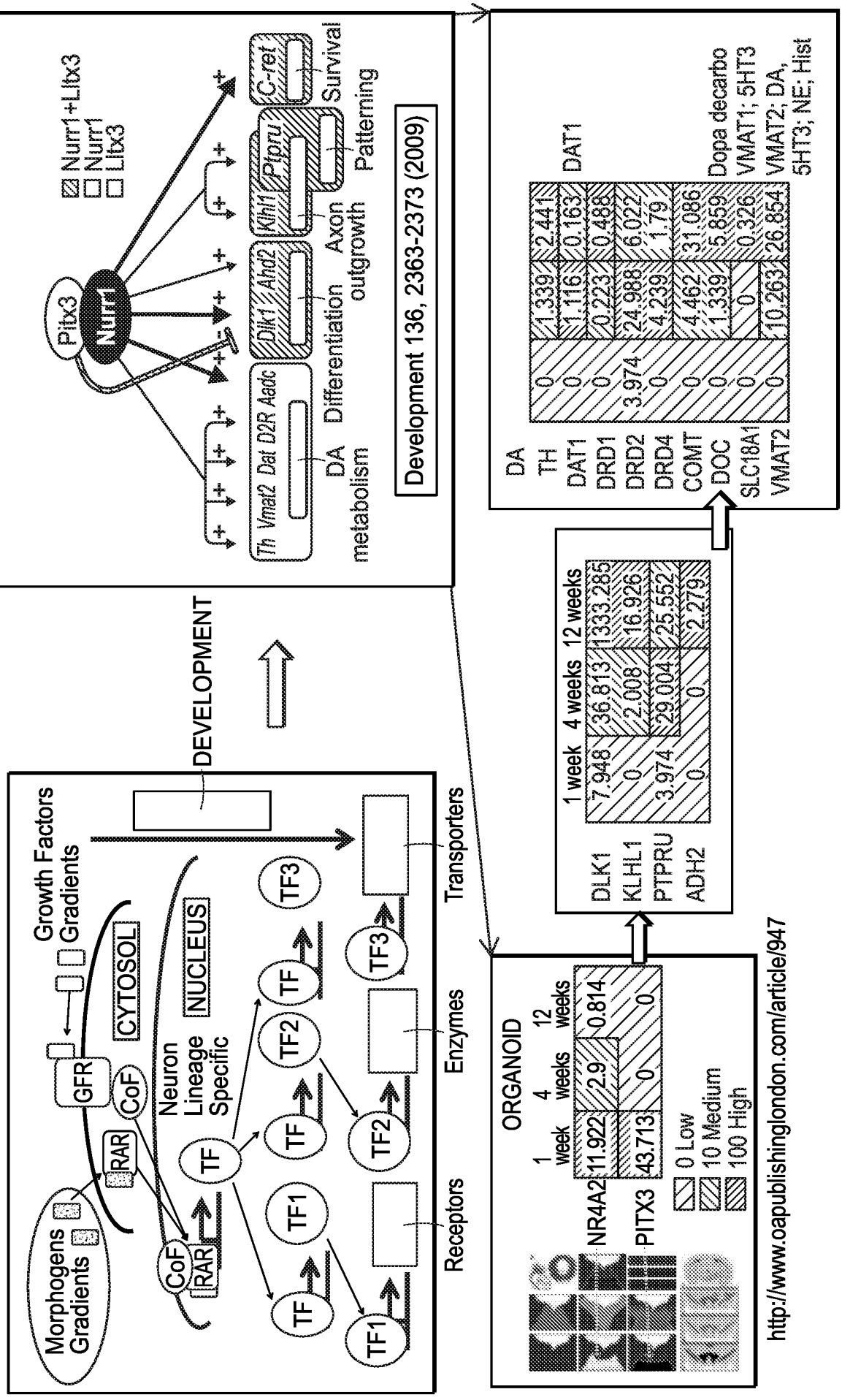

Morphogens Gradients
Growth Factors Gradients
GFR
CoF
RAR
CYTOSOL
NUCLEUS
Neuron Lineage Specific
TF
TF1
TF2
TF3
Receptors
Enzymes
Transporters
DEVELOPMENT
Pitx3
Nurr1
Nurr1+Litx3
Nurr1
Litx3
Th Vmat2 Dat D2R Aadc
Dlk1 Ahd2
Klhl1 Ptpru
C-ret
DA metabolism
Differentiation
Axon outgrowth
Survival
Patterning
Development 136, 2363-2373 (2009)
DA
TH
DAT1
DRD1
DRD2
DRD4
COMT
DOC
SLC18A1
VMAT2
DAT1
Dopa decarbo
VMAT1; 5HT3
VMAT2; DA, 5HT3; NE; Hist
1 week 4 weeks 12 weeks
DLK1 7.948 36.813 1333.285
KLHL1 0 2.008 16.926
PTPRU 3.974 29.004 25.552
ADH2 0 0 2.279
ORGANOID
1 week
4 weeks
12 weeks
NR4A2 11.922 2.9 0.814
PITX3 43.713 0 0
0 Low
10 Medium
100 High
http://www.oapublishinglondon.com/article/947

FIG. 6B-1 COMPARISON: Organoid & BRAIN versus OTHER ORGANS Disconcordance > 95%

BPIFA1	0	0	**UPPER AIRWAYS** Antimicrobial;
BPIFA1	0	0	
BPIFA3	0	0	
BPIFA4P	0	0	
BPIFB1	0.488		
BPIFB2	0	0	
BPIFB3	0	0	
BPIFB4	0	0	
BPIFB6	0	0	
BPIFC	0	0	
ADH1A	0		**LIVER** Alcohol dehydrogenase 1A;
ADIPOQ	0	0	**ADIPOSE** adiponectin adipose
AMELX	0.488	0	**TOOTH** Amelogenins are involved in biomineralization during tooth enamel development
AMELY	0	0	
BPIFA1	0	0	**UPPER AIRWAYS** Antimicroial protein expressed in the upper airways and nasopharyngeal regions
BPIFA2	0	0	
BPIFA3	0	0	
BPIFA4P	0	0	
BPIFB1	0.488	0	
BPIFB2	0	0	
BPIFB3	0	0	
BPIFB4	0	0	
BPIFB6	0	0	
BPIFC	0	0	
C17orf68	0.163	0	**TESTIS** chromosome 17 open reading frame 74; testis
C1orf68	0	0	**SKIN** chromosome 1 open reading frame 68; skin
C8A	0	0	**Macrophage** C8 is a component of the complement system and contains three polypeptides, alpha, beta and gamma
C8B	0	0	
C8G	0	0	
CST1	0	0	
CST11	0	0	
CST13P	0	0	
CST2	0	0	
CST4	0	0	**TESTIS** The protein is an S-type cyctatin, based on its high level of expression in saliva, tears and seminal plasma
CST5	0	0	**SALIVARY**; Salivary gland

FIG. 6B-1	FIG. 6B-2	FIG. 6B-3

FIG. 6B

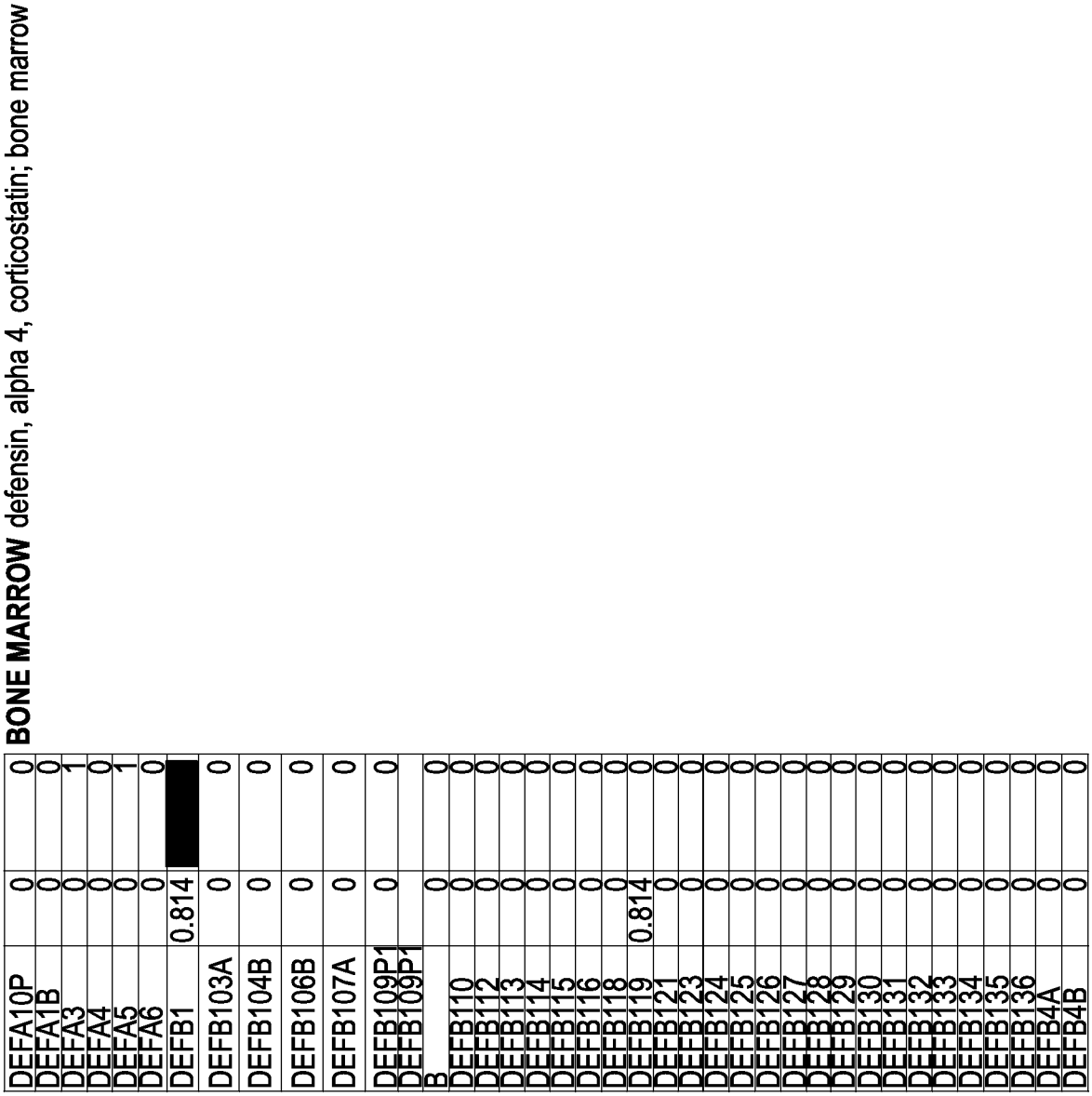

BONE MARROW defensin, alpha 4, corticostatin; bone marrow
DEFA10P 0
DEFA1B 0
DEFA3 0
DEFA4 0
DEFA5 0
DEFA6 0
DEFB1 0.814
DEFB103A 0
DEFB104B 0
DEFB106B 0
DEFB107A 0
DEFB109P1 0
DEFB109P1B 0
DEFB110 0
DEFB112 0
DEFB113 0
DEFB114 0
DEFB115 0
DEFB116 0
DEFB118 0
DEFB119 0.814
DEFB121 0
DEFB123 0
DEFB124 0
DEFB125 0
DEFB126 0
DEFB127 0
DEFB128 0
DEFB129 0
DEFB130 0
DEFB131 0
DEFB132 0
DEFB133 0
DEFB134 0
DEFB135 0
DEFB136 0
DEFB4A 0
DEFB4B 0

FIG. 6B-2

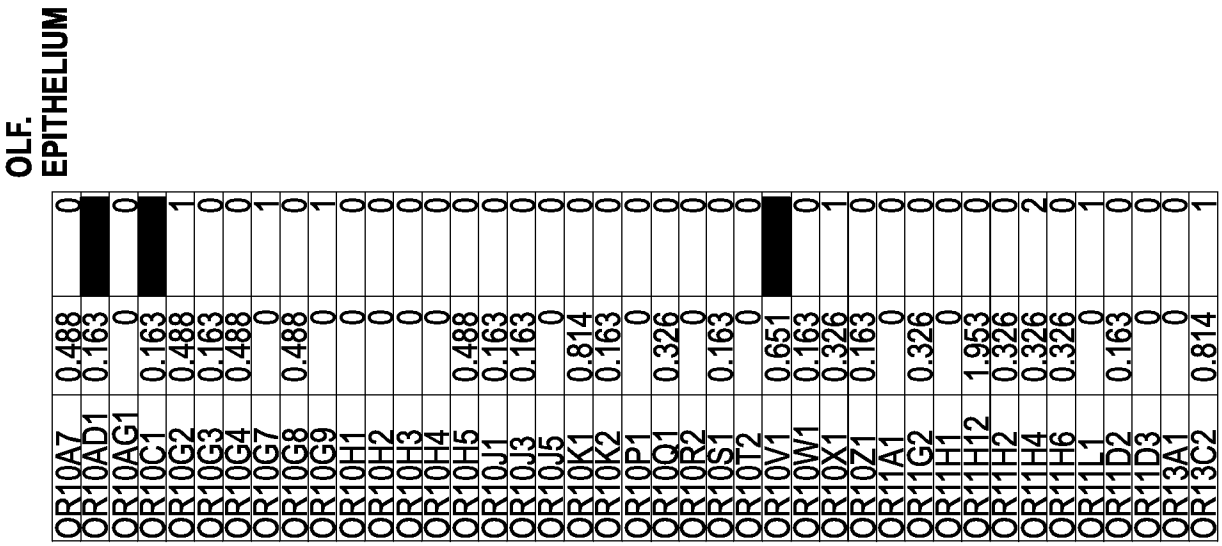
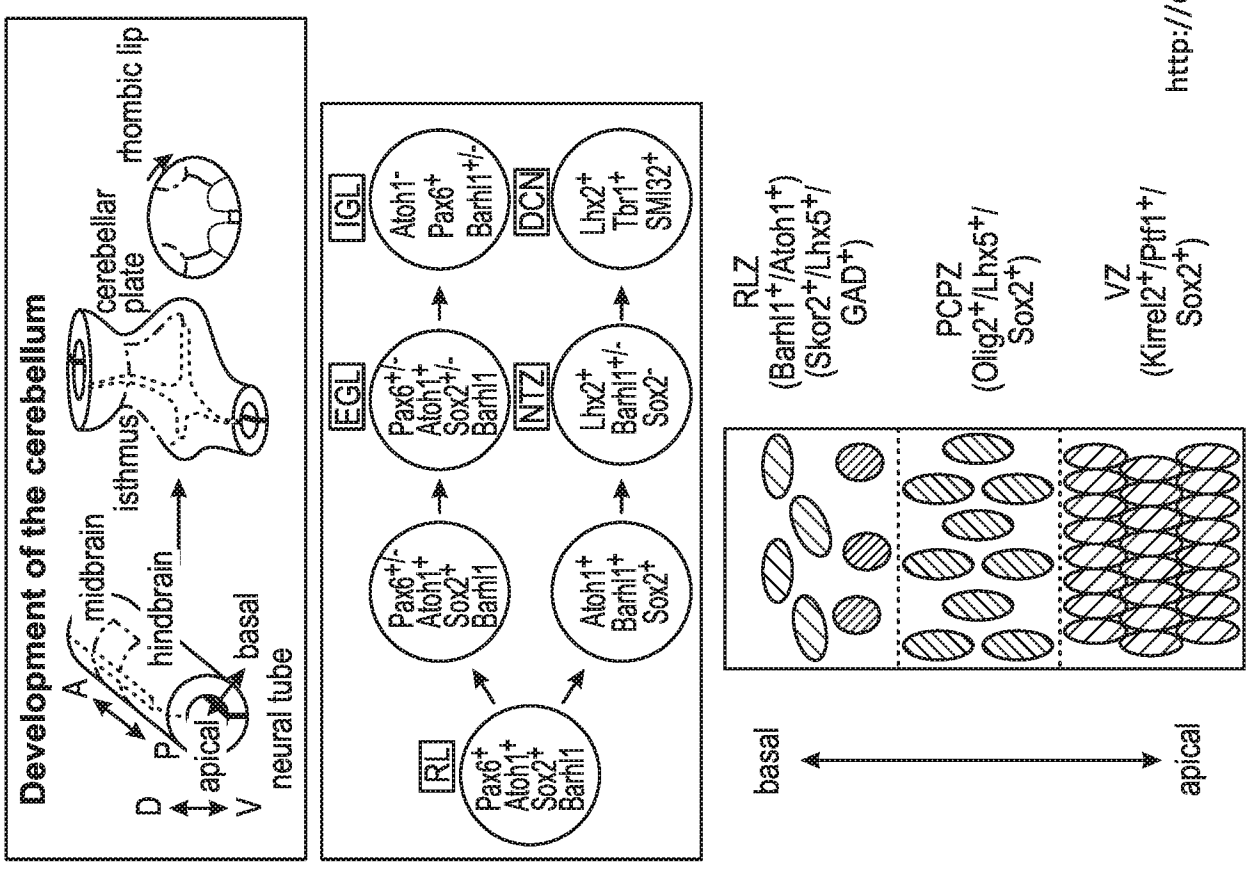

OLF. EPITHELIUM
OR10A7 0.488 0
OR10AD1 0.163 0
OR10AG1 0 0
OR10C1 0.163 0
OR10G2 0.488 1
OR10G3 0.163 0
OR10G4 0.488 0
OR10G7 0 1
OR10G8 0.488 0
OR10G9 0 1
OR10H1 0 0
OR10H2 0 0
OR10H3 0 0
OR10H4 0 0
OR10H5 0.488 0
OR10J1 0.163 0
OR10J3 0.163 0
OR10J5 0 0
OR10K1 0.814 0
OR10K2 0.163 0
OR10P1 0 0
OR10Q1 0.326 0
OR10R2 0 0
OR10S1 0.163 0
OR10T2 0 0
OR10V1 0.651 0
OR10W1 0.163 0
OR10X1 0.326 1
OR10Z1 0.163 0
OR11A1 0 0
OR11G2 0.326 0
OR11H1 0 0
OR11H12 1.953 0
OR11H2 0.326 0
OR11H4 0.326 2
OR11H6 0.326 0
OR11L1 0 1
OR11D2 0.163 0
OR11D3 0 0
OR13A1 0 0
OR13C2 0.814 1

FIG. 6B-3



	S ORGANOIDS 1 week	4 weeks	12 Weeks	HUMAN BRAIN HBR	
ATOH1	11.922	0.223	0	0	
PAX6	11.922	161.086	32.714	9.26	
BARHL1	0	1.339	0.814	5.56	
SOX2	39.739	185.181	58.917	55.3	
LHX2	7.948	252.784	17.903	16	
TBR1	11.922	0.223	0.326	29	
KIRREL2	3.974	20.749	13.671	1.3	
PTF1A	0	0	1.302	0.125	
GRID2	0	0	14.811	35.7	purkinje
PCP2	0	0.446	0.326	48	L7 purkinje

http://dx.doi.org/10.1016/j.celrep.2014.12.051

FIG. 7

FIG. 8

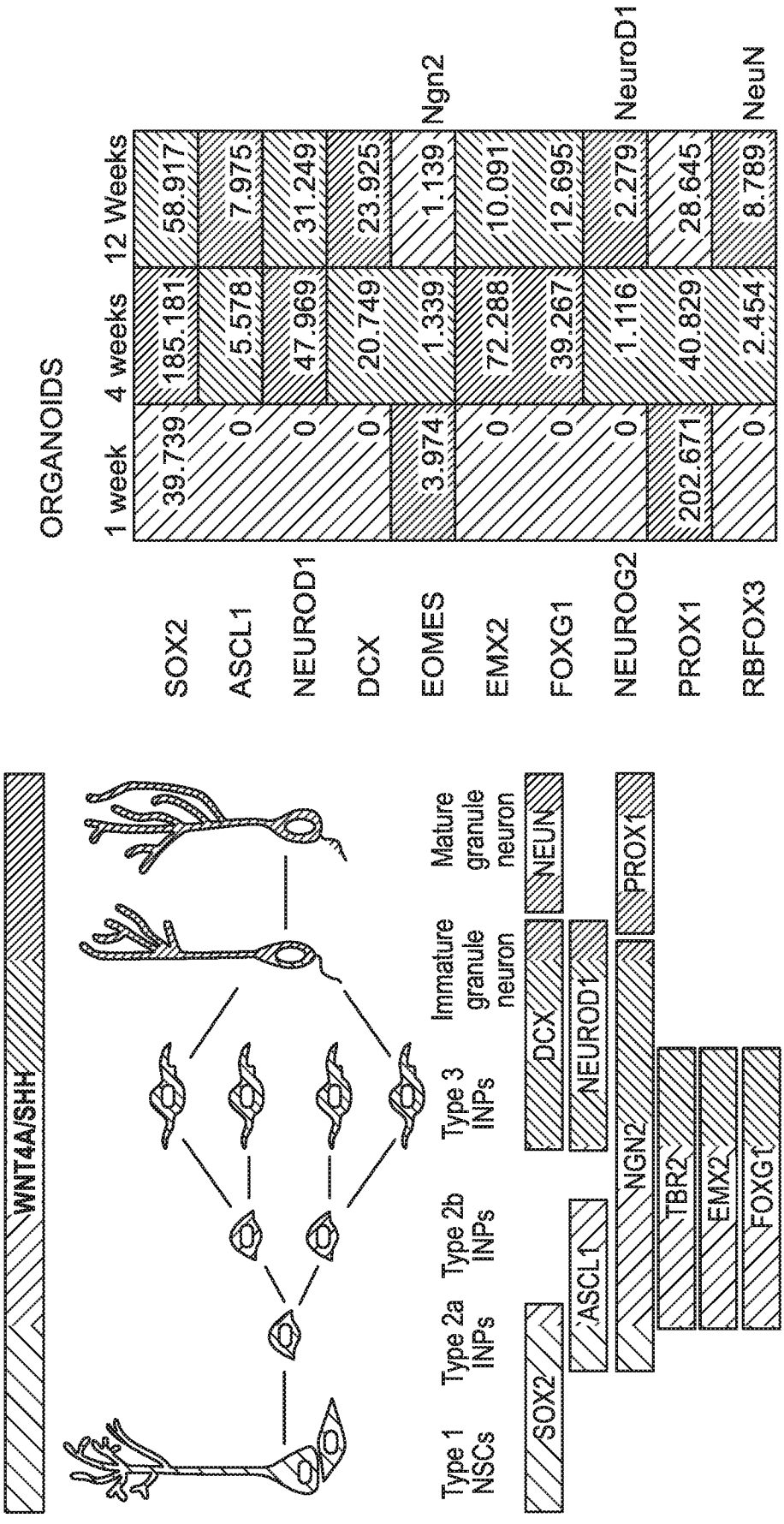
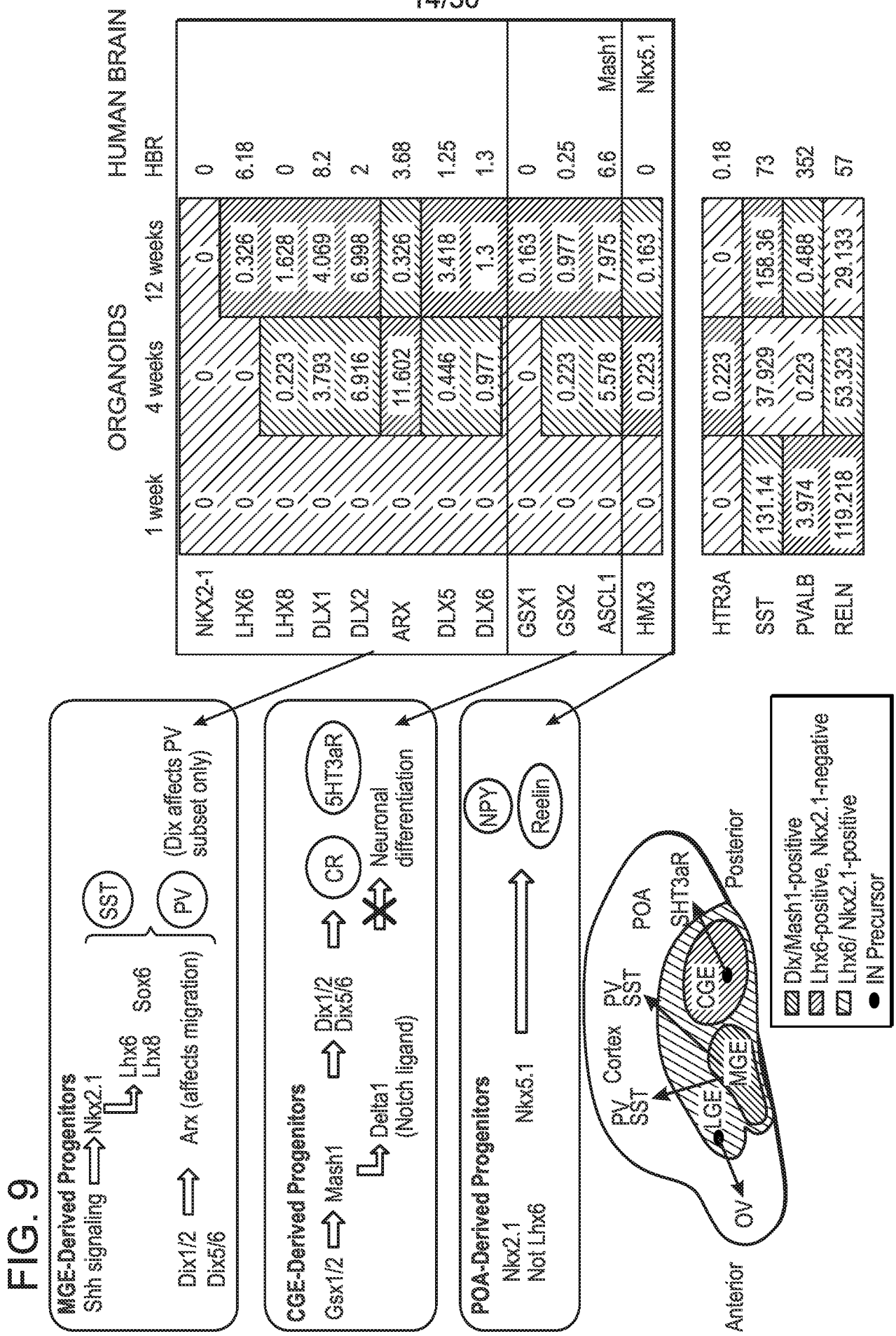
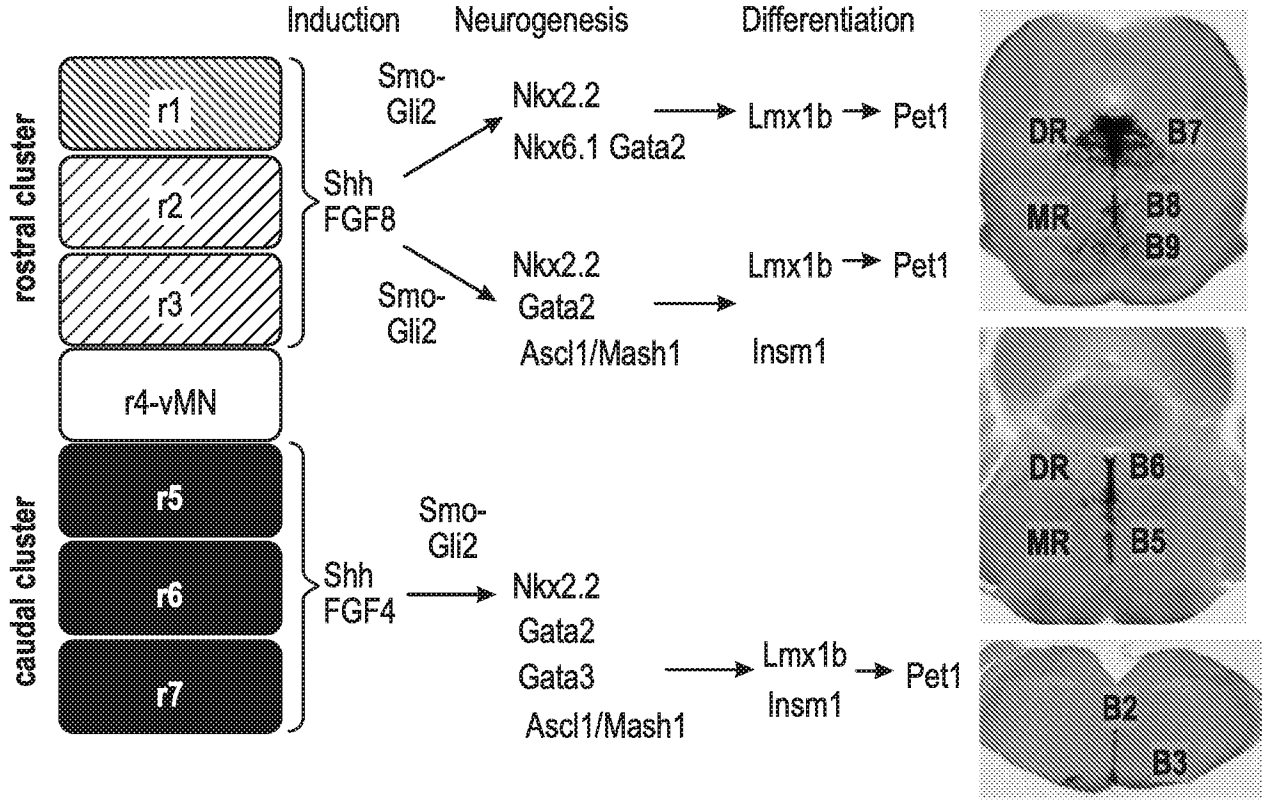

ORGANOIDS
1 week
4 weeks
12 Weeks
SOX2
39.739
185.181
58.917
ASCL1
0
5.578
7.975
NEUROD1
0
47.969
31.249
DCX
0
20.749
23.925
EOMES
3.974
1.339
1.139
EMX2
0
72.288
10.091
FOXG1
0
39.267
12.695
NEUROG2
0
1.116
2.279
PROX1
202.671
40.829
28.645
RBFOX3
0
2.454
8.789
Ngn2
NeuroD1
NeuN
WNT4A/SHH
Type 1 NSCs
Type 2a INPs
Type 2b INPs
Type 3 INPs
Immature granule neuron
Mature granule neuron
SOX2
ASCL1
NGN2
TBR2
EMX2
FOXG1
DCX
NEUROD1
PROX1
NEUN

FIG. 9


ORGANOIDS
HUMAN BRAIN
1 week
4 weeks
12 weeks
HBR
NKX2-1 0 0 0 0
LHX6 0 0 0.326 6.18
LHX8 0 0 1.628 0
DLX1 0 0.223 4.069 8.2
DLX2 0 3.793 6.998 2
ARX 0 6.916 0.326 3.68
DLX5 0 11.602 3.418 1.25
DLX6 0 0.446 1.3 1.3
GSX1 0 0.977 0.163 0
GSX2 0 0 0.977 0.25
ASCL1 0 0.223 7.975 6.6
HMX3 0 0.223 0.163 0
Mash1
Nkx5.1
HTR3A 0 0.223 0 0.18
SST 131.14 37.929 158.36 73
PVALB 3.974 0.223 0.488 352
RELN 119.218 53.323 29.133 57
MGE-Derived Progenitors
Shh signaling
Nkx2.1
Lhx6
Lhx8
Sox6
SST
PV
(Dix affects PV subset only)
Dix1/2
Dix5/6
Arx (affects migration)
CGE-Derived Progenitors
Gsx1/2
Mash1
Dix1/2
Dix5/6
CR
5HT3aR
Delta1
(Notch ligand)
Neuronal differentiation
POA-Derived Progenitors
Nkx2.1
Not Lhx6
Nkx5.1
NPY
Reelin
Anterior
Posterior
Cortex
PV
SST
POA
5HT3aR
CGE
LGE
MGE
OV
Dlx/Mash1-positive
Lhx6-positive, Nkx2.1-negative
Lhx6/ Nkx2.1-positive
IN Precursor



	ORGANOIDS			HUMAN BRAIN
	1 week	4 weeks	12 weeks	HBR
FGF4	0	0	0	0
FGF8	0	41.72	3.418	0.3
SHH	0	0.892	0.488	0.75
NKX2-2	0	0	0.488	16
NKX6-1	3.974	0	1.3	0.6
LMX1B	0	0	0.326	0.06
INSM1	11.922	66.48	55.17	12.37
GATA2	0	4.46	3.092	1.8
ASCL1	0	5.578	7.975	6.6
GATA3	87.427	0	10.254	0.125

DOI:10.1111/j.1460-9568.2011.07910.x

FIG. 10

FIG. 11-1

FIG. 11

FIG. 11-1	FIG. 11-2	FIG. 11-3

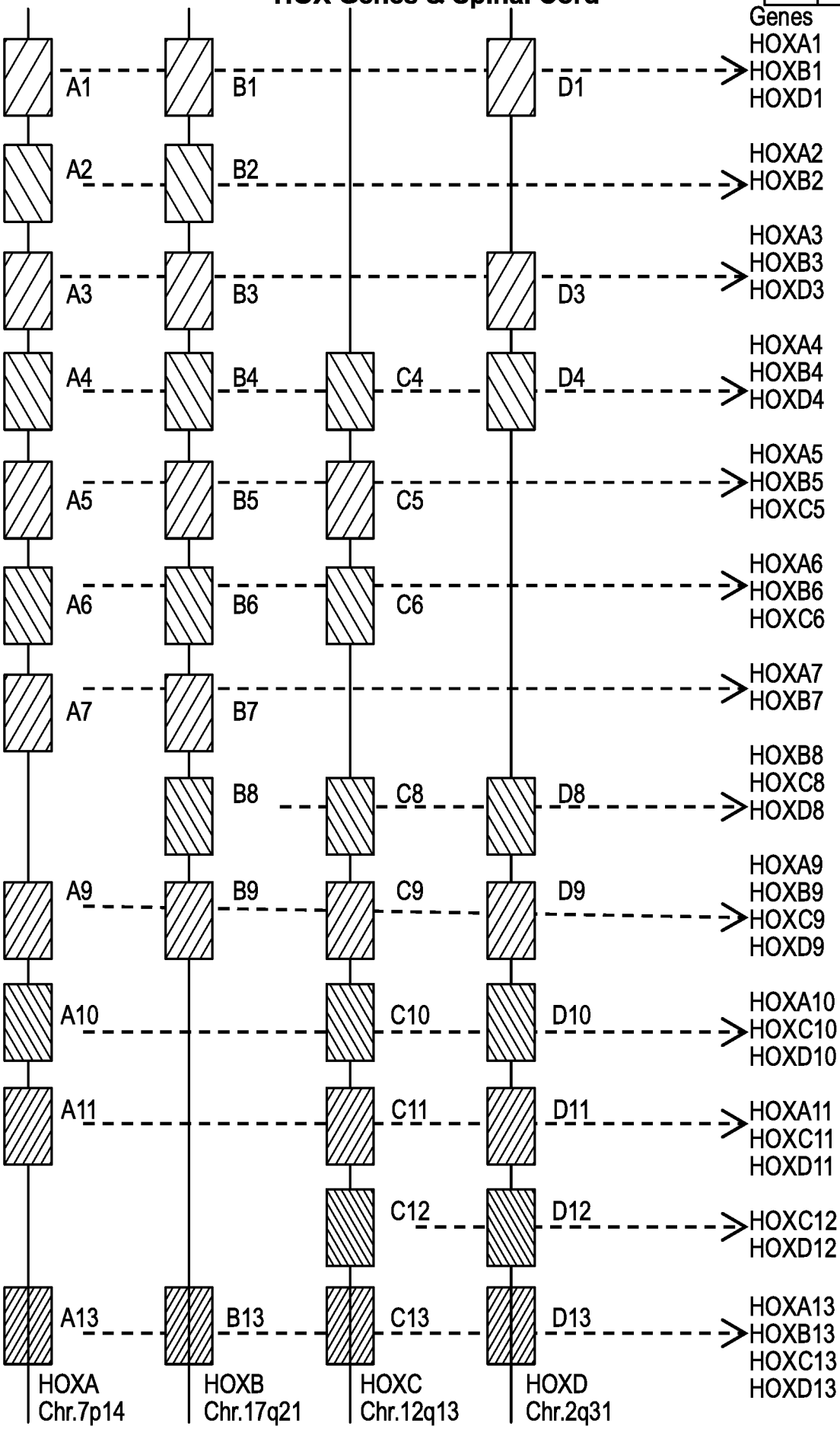


FIG. 11-2

HOX Genes & Spinal Cord

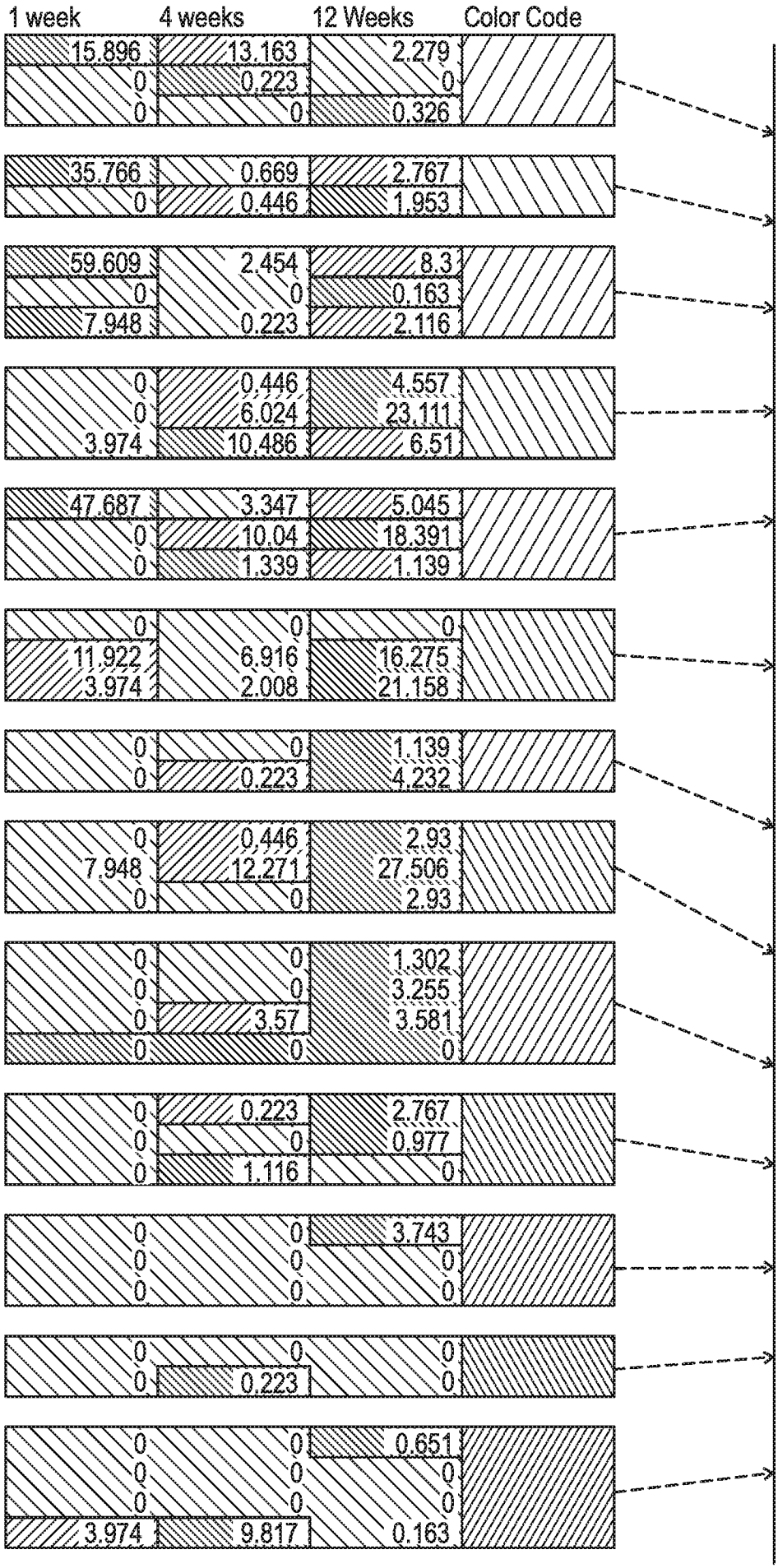
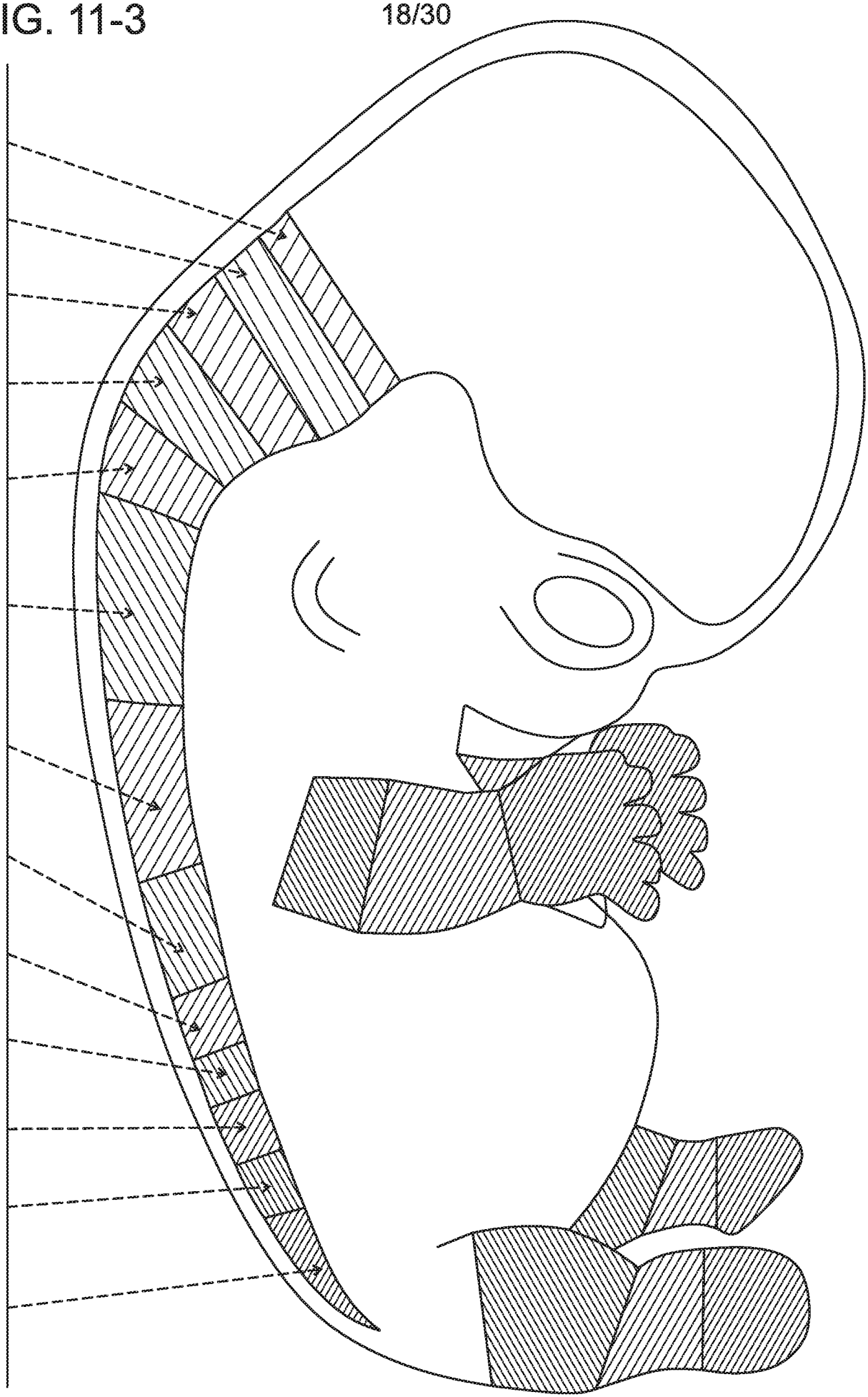


FIG. 11-3



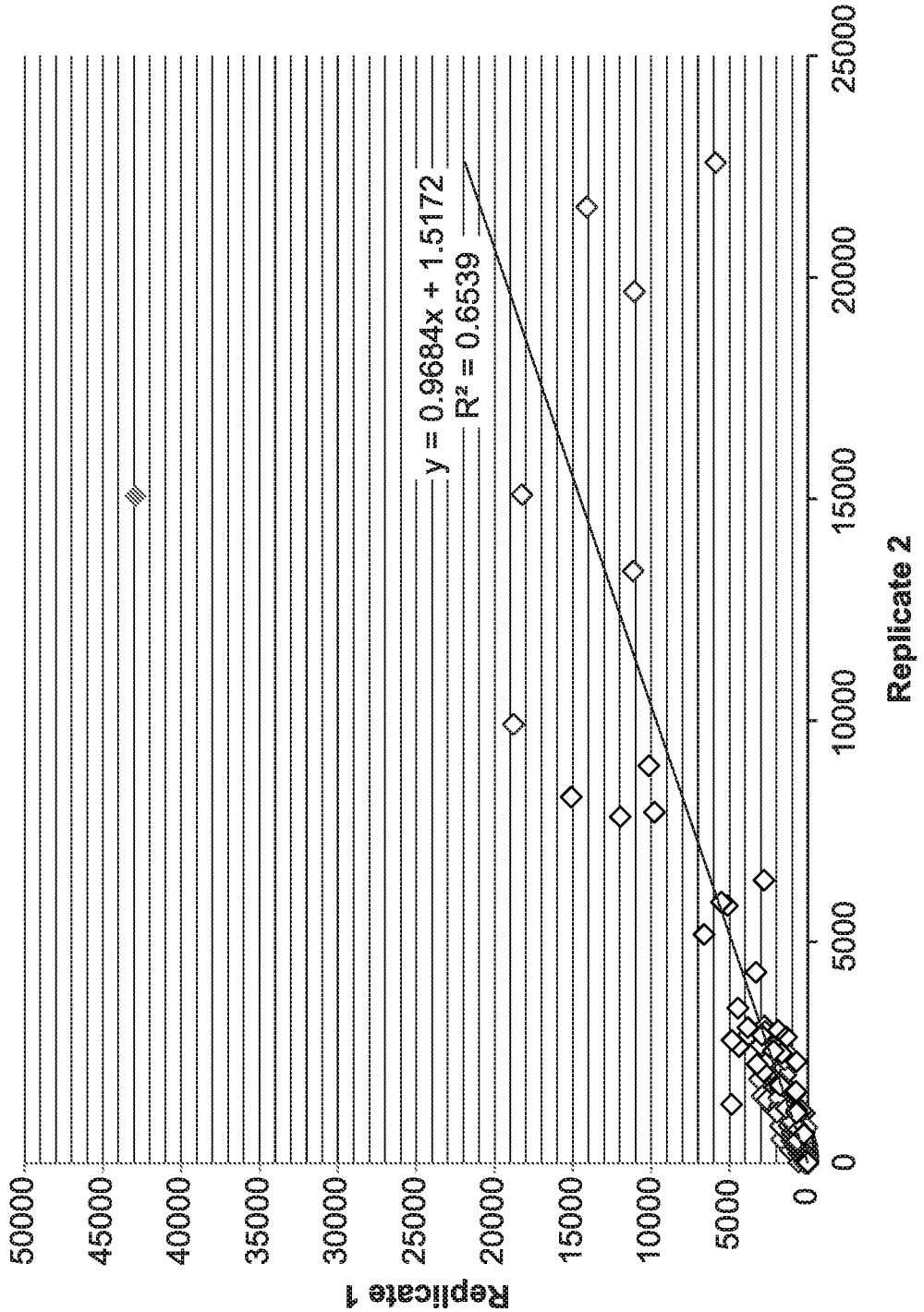

y = 0.9684x + 1.5172
R² = 0.6539
Replicate 1
Replicate 2
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000

FIG. 12

FIG. 13

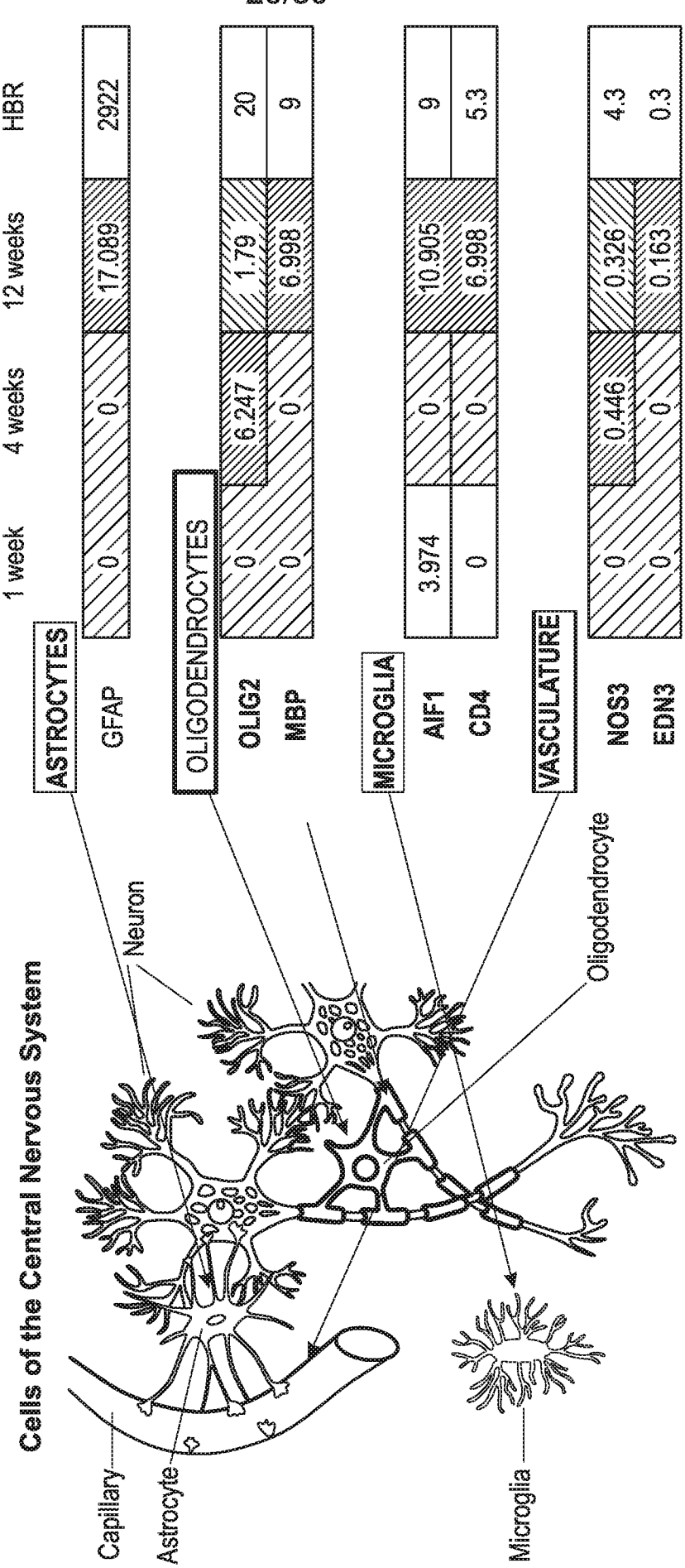

ORGANOID
HUMAN BRAIN
1 week
4 weeks
12 weeks
HBR
ASTROCYTES
GFAP
0
0
17.089
2922
OLIGODENDROCYTES
OLIG2
0
6.247
1.79
20
MBP
0
0
6.998
9
MICROGLIA
AIF1
3.974
0
10.905
9
CD4
0
0
6.998
5.3
VASCULATURE
NOS3
0
0.446
0.326
4.3
EDN3
0
0
0.163
0.3
Cells of the Central Nervous System
Neuron
Oligodendrocyte
Capillary
Astrocyte
Microglia

FIG. 14

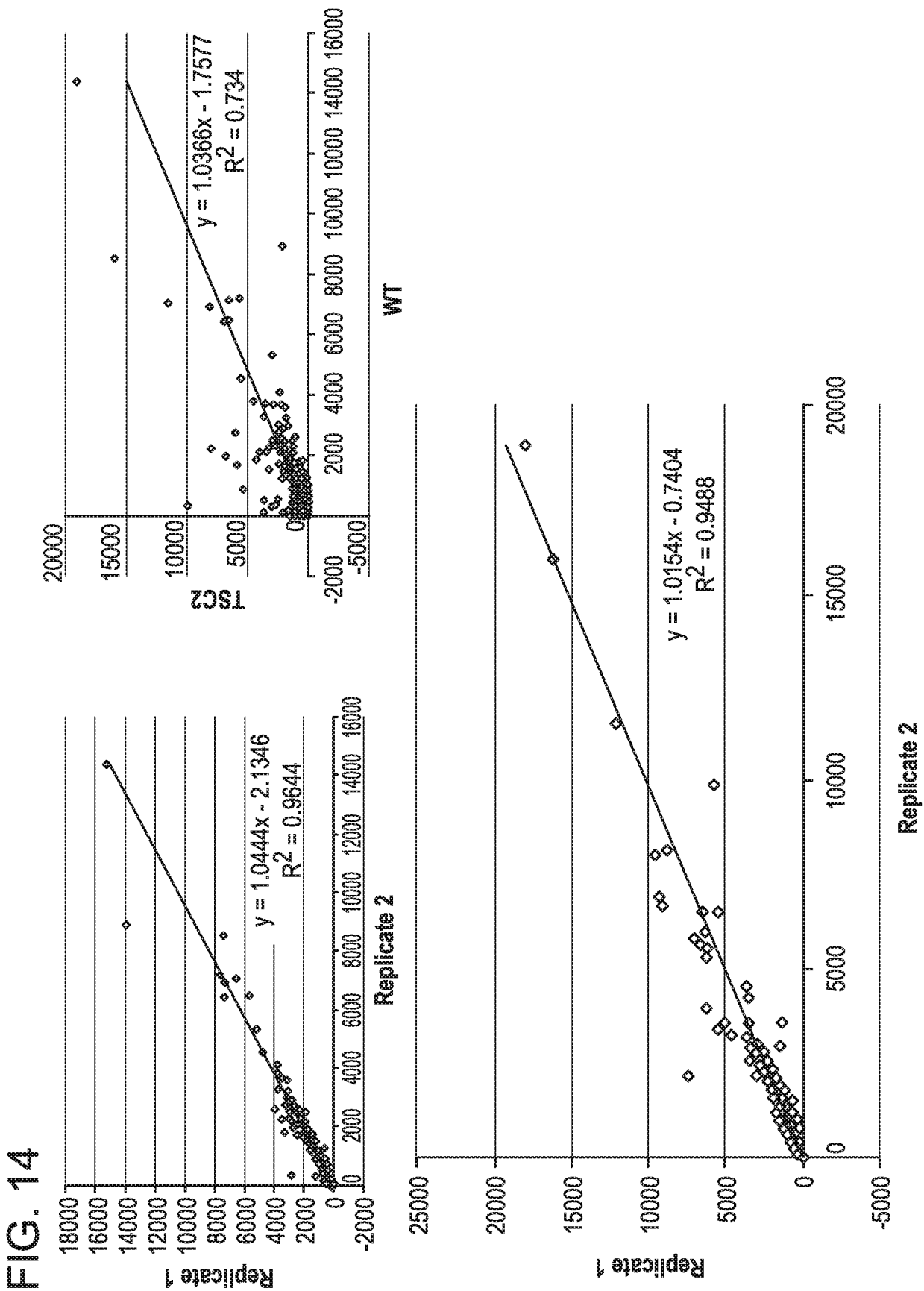

Replicate 1
Replicate 2
y = 1.0444x - 2.1346
R2 = 0.9644
TSC2
WT
y = 1.0366x - 1.7577
R2 = 0.734
Replicate 1
Replicate 2
y = 1.0154x - 0.7404
R2 = 0.9488

FIG. 15

Gene	1 Week	Weeks	12 Week
AOC2	0	0.892	6.998
GUCY2D	0	0	3.743
GUCY2F	0	2.454	0
RAX	0	180.496	25.39
RS1	0	0	3.092
CRX	3.974	0.446	6.836
RP1	0	1.116	2.441
RPGR	11.922	12.717	20.019
PDC	0	0	28.97
PDE6B	0	1.785	3.418
RD3	0	3.57	7.649
VSX1	0	21.195	3.743

FIG. 16

Gene	1 week	4 week	12 week
TNC	15.896	0.892	99.117
PTPRZ1	47.687	85.674	52.732
FAM107A	0	27.443	7.649
HOPX	0	0	0.651
ITGB5	51.661	14.056	63.8
FOXP2	71.531	63.81	27.18
THBS1	3.974	15.841	39.224
CNTN4	0	15.618	25.227
VSTM2L	3.974	0.446	7.812
CPNE8	59.609	1.785	14.648
TBR1	11.922	0.223	0.326

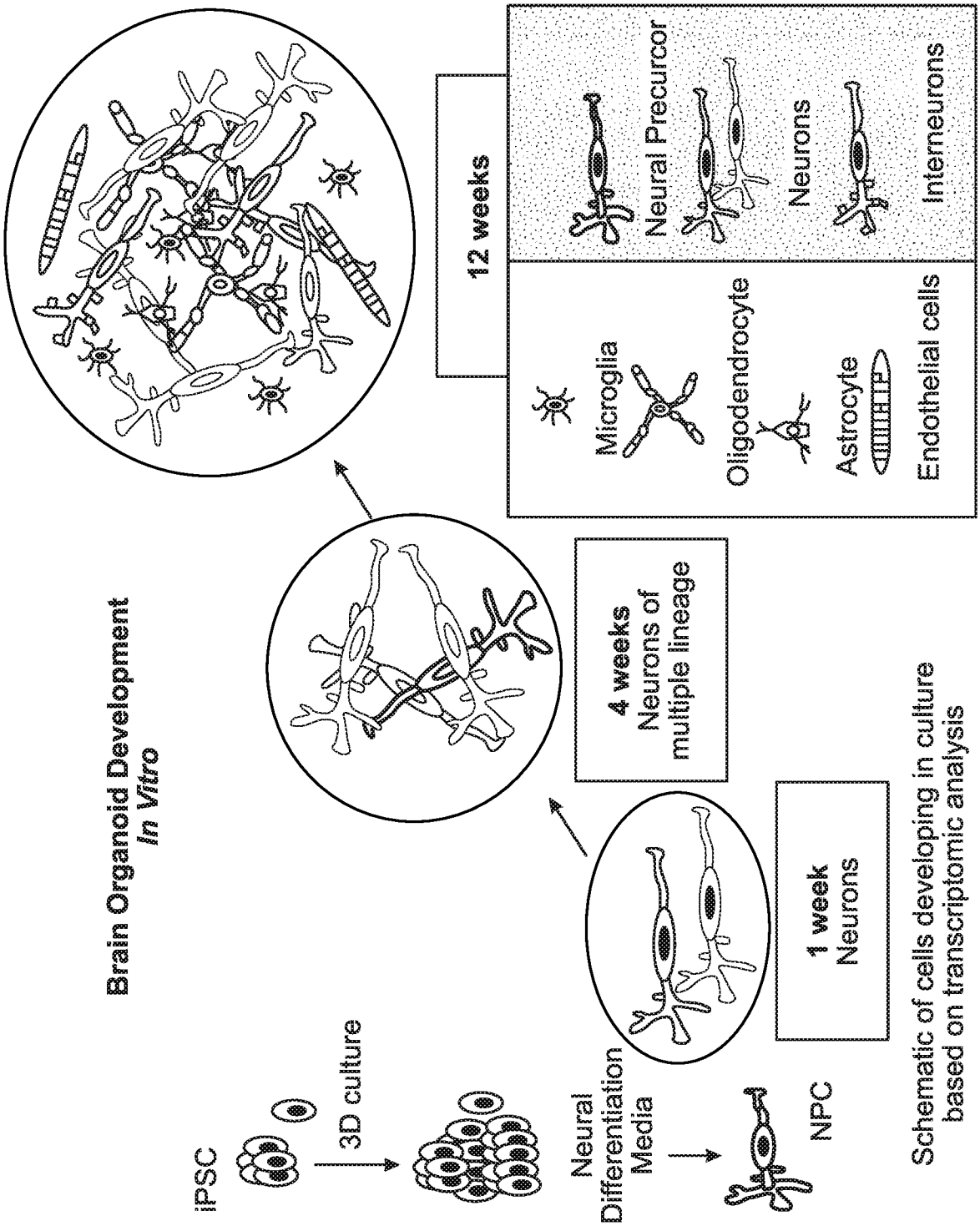

Brain Organoid Development
In Vitro
iPSC
3D culture
Neural
Differentiation
Media
NPC
1 week
Neurons
4 weeks
Neurons of
multiple lineage
12 weeks
Microglia
Oligodendrocyte
Astrocyte
Endothelial cells
Neural Precurcor
Neurons
Interneurons
Schematic of cells developing in culture
based on transcriptomic analysis

FIG. 17

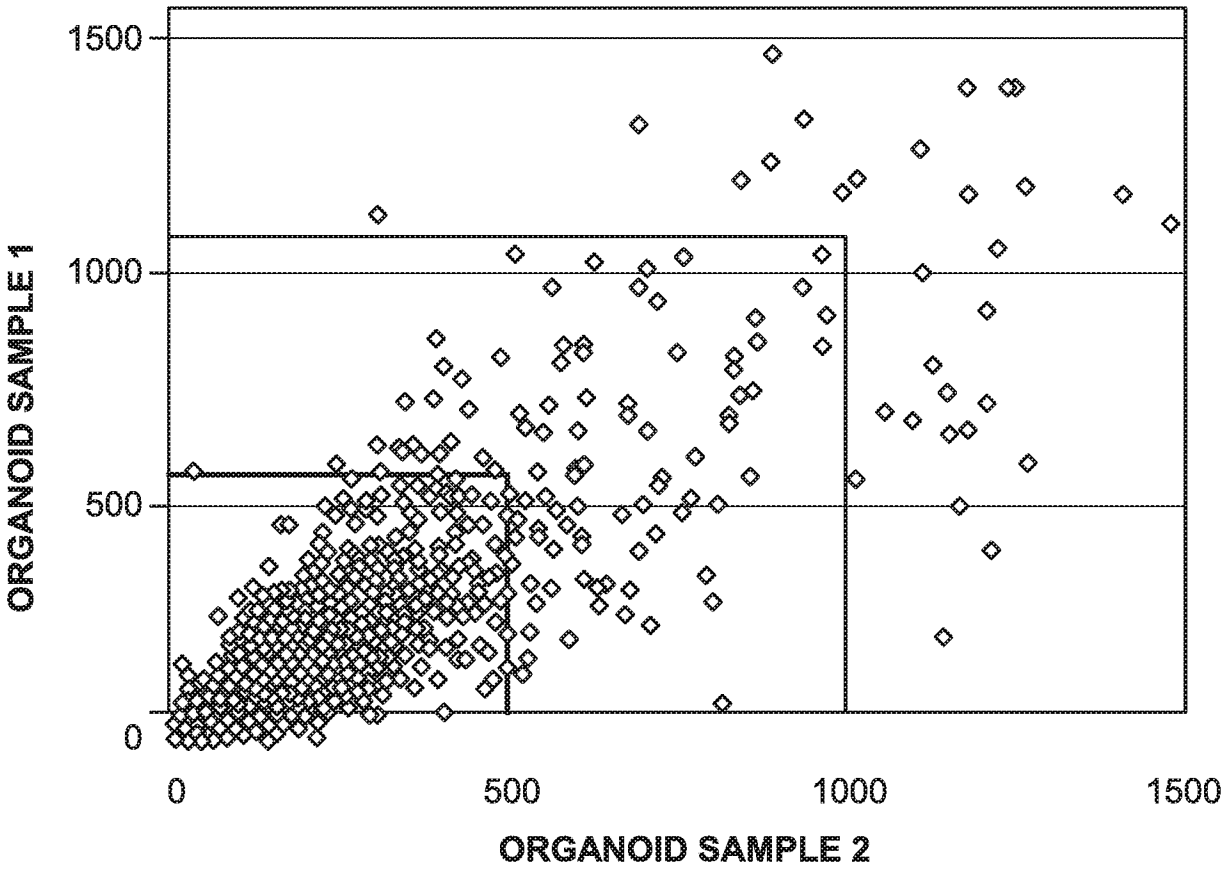

1500
1000
500
0
ORGANOID SAMPLE 1
0
500
1000
1500
ORGANOID SAMPLE 2

FIG. 18

AUTISM ASSOCIATED GENES					CHANGE	COMMENTS
Gene	TSC2	TSC2	WT	WT		
ADNP	76.944	56.94	37.033	30.873	2	high confidence
POGZ	144.045	156.206	211.255	251.871	1.3	high confidence
ANKRD11	44.576	60.249	93.05	103.95	2	strong candidate
BCL11A	15.048	11.167	31.235	29.418	2	strong candidate
NRXN1	1.136	0.827	4.302	3.015	3.5	strong candidate
RELN	76.944	54.183	117.551	138.877	2	strong candidate
HDAC4	17.13	16.682	31.515	39.085	2	syndromic
DMD	24.134	18.888	1.122	1.559	10	syndromic
PCDH19	7.666	6.618	42.083	44.179	6	syndromic

FIG. 19A

AUTISM ASSOCIATED GENES

Gene	TSC2	TSC2	WT	WT	CHANGE	COMMENTS	
ATP1B2	10.884	8.272	43.766	41.372	4	Microcephaly	21q11.2-q22.3-SFARI
ADAMTS1	52.148	78.034	279.897	226.299	4	Metalloproteinase	17pter-p13.1-SFARI
ADAMTS15	33.692	47.289	138.592	294.179	4	Metallopeptidase	11q24.2-q25-SFARI
ABAT	12.682	29.366	108.573	79.626	4	Catabolism of GABA	3q13.11-q13.31-SFARI
ALCAM	50.444	43.567	109.602	92.516	2	Activated leukocyte cell adhesion	16p13.3-p13.12-SFARI
AMBP	2.082	6.48	16.112	15.606	4	Alpha-1-Microglobulin/Bikunin Precursor	11q12.1-q12.2-SFARI
APLNR	555.263	424.223	28.429	28.17	15	Apelin Receptor	Zn++Deficiency?
APOC3	9.748	24.265	496.296	259.875	20	Dyslipidemia	11q22.1-q25-SFARI
ARSI	3.502	4.274	30.767	60.811	10	Arylsulfatase	5q33.1-q35.3-SFARI
ATR7B	71.455	67.142	331.799	4.5.447	5	Cu++ Transport	13q11-q34-SFARI
CDR1	851.208	931.168	5323.372	6246.464	6	Cerebellar Degeneration	Xq27.1-q28-SFARI
DHCR7	62.274	83.273	244.454	201.871	3	Smith-Lemli-Optiz syndrome	Xq27.1-q28-SFARI

FIG. 19B

OTHER KNOWN CLINICAL SYMPTOMS					CHANGE	COMMENTS
Gene	TSC2	TSC2	WT	WT		
AGT	2.461	16.131	106.423	77.131	8	Hypertension
AGTR1	0.473	0.276	18.142	5.509	12	Hypertension
ALB	1.893	3.171	11.746	11.518	4	Zn++Deficiency
AMBP	2.082	6.48	16.112	15.606	4	Kidney failure
HBE1	0.662	1.241	393.988	438.441	400	Ascariasis/Pb poisioning
HGD	0.379	0	26.559	18.504	20	PARKINSONS/Alkaptonuria

FIG. 19C

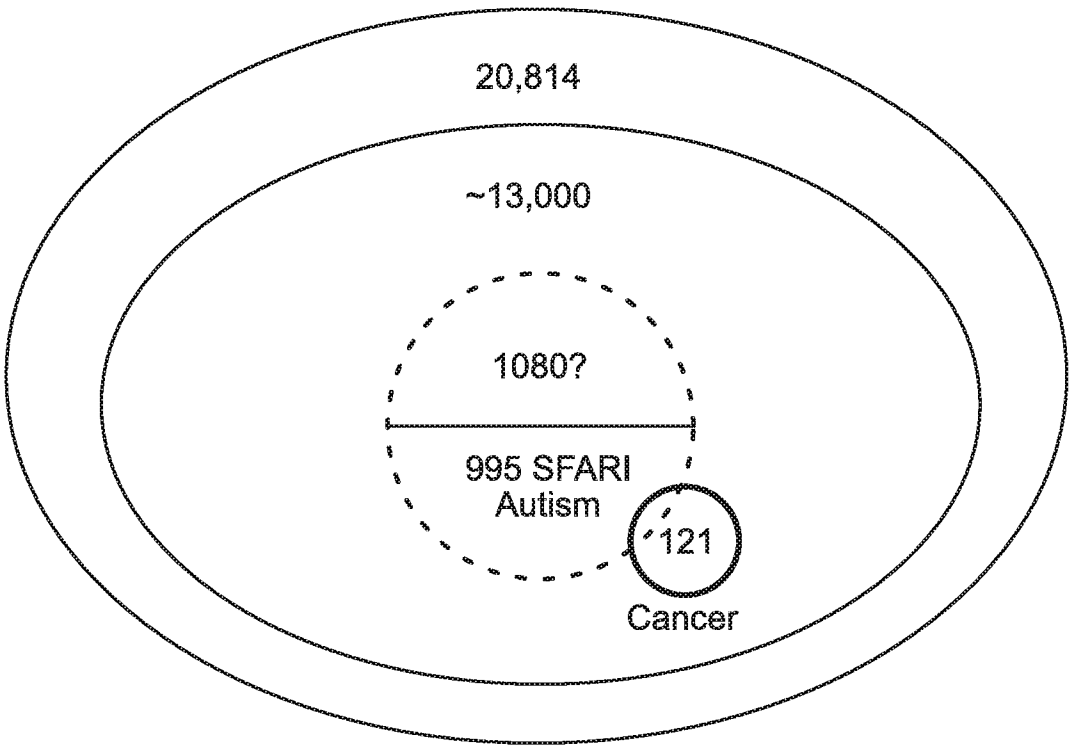


FIG. 20

	AD	AD	WT	WT	CHANGE		COMMENT
A2M	29.679	38.222	6.512	13.156	3.5	Lipid	alpha2-macroglobulin in late-onset Alzheimer's disease
ABCA2	41.686	62.111	36.771	28.38	2	Lipid	ABCA2 is a strong genetic risk factor for early-onset Alzheimer's disease
ABCA5	38.741	71.874	21.641	20.11	2.5	Lipid	ABCA2 is a strong genetic risk factor for early-onset Alzheimer's disease
ABTB2	20.39	26.589	5.171	5.075	4	NV	Neurovascular dysfunction
ASIC2	1.359	2.7	0	0.564	2	PH	Acid sensing channel in AD
ACSL6	2.266	3.531	0	0.564	3	Tau	A novel Alzheimer disease locus located near the gene encoding tau protein
ADA	18.577	10.386	34.473	41.536	2	Adenosine	Adenosine deficiency in AD
ADAMTSL4	9.515	9.348	30.643	28.756	3	LAOD	Whole-exome sequencing of multiplex families identifies several rare coding variants in known and novel Late-Onset Alzheimer genes
AMPL3105	14.333	27.578	28.944			RNA-Edit	RNA Editing Genes Associated with Extreme Old Age in Humans and with Lifespan in C. elegans
AIM2	7.929	15.995	1.341	0.376	12	Immune	Activation of the inflammasome is a key function mediated by the innate immune system
AK5	5.211	7.063	1.341	2.631	3	X-Chr	X chromosome aneuploidy in the Alzheimer's disease brain
ALCAM	77.708	87.869	38.303	41.16	2	Immune	Vascular inflammation in central nervous system diseases: adhesion receptors controlling leukocyte-endothelial interactions
ALKBH3	22.655	26.797	8.81	7.894	3	Epigentic	Epigenetics of Aging and Alzheimer's Disease:
ANK2	77.255	70.628	18.96	15.787	5	Longevity	The Human Longevity Variants
ANK3	56.638	45.908	3.064	3.759	15	LAOD	Significant association with late-onset Alzheimer's disease for 4 SNPs
ANKS1B	12.687	10.386	4.213	2.631	5	APP	Interacts with amyloid beta protein precursor
APC2	28.687	37.599	13.981	6.014	4	Psychosis	Psychosis gene in Alzheimer's
ASPM	44.858	43.208	143.254	104.874	3	Microcep	Microcephaly genes and risk of late onset Alzheimers
ATP9A	33.757	28.043	12.832	14.284	2	g-Secretase	Proton myo-inositol cotransporter is a novel γ-secretase associated protein that regulates Aβ production without affecting Notch cleavage
AURKA	1.812	0.415	6.512	5.6382	3	DOWN	DOWN SYNDROME Gene Network Disruptions and Neurogenesis
CD36	22.429	14.126	0.383	0.94	18	Immune	Immune Function
CD3G	4.078	4.57	0.575	0	4	Immune	Immune Function
CD4	4.984	3.324	0.958	1.692	4	Immune	Immune Function

FIG. 21A

	AD	AD	WT	WT	CHANGE		COMMENT
CD52	0.453	0.831	4.596	7.894	6	Immune	Immune Function
EFHD2	48.709	74.574	206.646	209.747	4	Tau	EFhd2 is a novel amyloid protein associated with pathological tau in Alzheimer's disease.
LRRTM3	5.211	3.324	0.766	0.94		Tau	LRRTM3 Interacts with APP and BACE1 and Has Variants Associating with Late-Onset Alzheimer's disease. (LOAD)
AQP1	86.77	44.869	231.544	231.128	3	Water	AQP1 is expressed in the plasma membrane of choroid plexus epithelial cells
BET1	164.251	164.313	265.059	229.482	1.5	Ad	Genetic Determinants of Cognitive Function and Age-related Brain Changes
BMP6	2.719	3.116	1.149	0.564	3	Neurogenesis	Elevated Levels of BMP6 Impair Neurogenesis in Alzheimer's Disease
BRCA1	7.703	7.478	11.299	12.404	1.5	AD	Breast cancer gene BRCA1 may be involved in Alzheimer's Disease
C1QB	87.45	44.869	0	0	60	Immune	Complement (c) proteins, C1qB and C4 phagocytosis in the Alzheimer disease patogenesis
C1QC	38.967	15.787	0	0	26	Immune	Complement (C) proteins, C1qB and C4
C1QL3	2.492	1.246	0.383	0	2	Immune	Complement (C) proteins, C1qB and C4
CA2	89.262	262.776	27.004	24.621	12	PH	Plasma carbonic anhydrase II protein is elevated in Alzheimer's Disease
CD36	22.429	14.126	0.383	0.94	17	Immune	CD36, a class B scavenger receptor, is expressed on microglia in Alzheimer's disease brain and can mediate production of reactive oxygen species in response to beta-amyloid fibrils.
CD3G	4.078	4.57	0.575	0	4	Immune	
CD4	4.984	3.324	0.958	1.692	4	Immune	Women with the Alzheimer's risk marker ApoE4 lose Aβ-specific CD4+ T cells 10-20 years before men
CD52	0.453	0.831	4.596	7.894	6	Immune	The microglial sensome revealed by direct RNA sequencing
COL13A1	20.616	17.657	135.211	106.941	5	LAOD	A scan of chromosome 10 identifies a novel locus showing strong association with late-onset Alzheimer disease
DSCAM	12.234	23.266	2.107	0.752	15	Down	Possible compensatory events in adult Down syndrome brain prior to the development of Alzheimer disease neuropathology: targets for nonpharmacological intervention
KDM5D	68.646	56.71	0.383	0	60	Chromatin	UBE1Y and KDM5D are involved in DNA condensation
TLR4	7.929	4.155	0.766	0.94	12	Immune	Microglial activation is key feature in Alzheimer's Disease
NAV2	41.459	31.99	4.979	4.511	9	Memory	NAV2 was found to be significantly and consistently associated with all seven episodic memory scores
PCDHB16	14.499	14.333	1.724	4.511	5	AD	Linked to Alzheimer's Disease

FIG. 21B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013231

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/12; A61L 27/00; A61L 27/40; A61P 19/00; A61P 25/00; A61P 43/00 (2017.01)

CPC - A61K 35/30; A61K 48/00; A61L 27/18; A61L 27/34; A61L 27/38/34; C12N 5/0619; C12N 2501/115 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/93.21; 424/491; 435/366 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2015/069736 A1 (THE MCLEAN HOSPITAL CORPORATION) 14 May 2015 (14.05.2015) entire document	1-3, 5, 7, 9-11, 13 --- 4, 6, 8, 12, 14, 15, 17, 21-24
Y	US 2006/0171935 A1 (ABELIOVICH et al) 03 August 2006 (03.08.2006) entire document	4, 8
Y	KAEWKHAW et al. "Transcriptome Dynamics of Developing Photoreceptors in Three-Dimensional Retina Cultures Recapitulates Temporal Sequence of Human Cone and Rod Differentiation Revealing Cell Surface Markers and Gene Networks," Stems Cells, 31 July 2015 (31.07.2015), Vol. 33, Pgs. 3504-3518. entire document	6
Y	LIN et al. "Heat Shock Alters the Expression of Schizophrenia and Autism Candidate Genes in an Induced Pluripotent Stem Cell Model of the Human Telencephalon," PLoS ONE, 15 April 2014 (15.04.2014), Vol. 9, Pgs. 1-11. entire document	12
Y	WO 2013/130769 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 06 September 2013 (06.09.2013) entire document	14
Y	VAN DE LEEMPUT et al. "A Temporal Transcriptome Analysis of In Vitro Human Cerebral Cortex Development from Human Embryonic Stem Cells," Neuron, 02 July 2014 (02.07.2014), Vol. 83, Pgs. 51-68. entire document	14
Y	WO 2014/168585 A1 (AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH) 16 October 2014 (16.10.2014) entire document	15, 17, 21-24

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 April 2017	25 APR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013231

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LANCASTER et al. "Generation of Cerebral Organoids from Human Pluripotent Stem Cells," Nature Protocols, 04 September 2014 (04.09.2014), Vol. 9, Pgs. 2329-2340. entire document	16, 18-20
Y	US 2014/0030805 A1 (KASUTO et al) 30 January 2014 (30.01.2014) entire document	16, 18-20
P, X	ANAND et al. "Human Neural Organoids 'NexGen' Platforms for CNS Disease, Therapeutic Target, & Drug Discovery Research," The World CNS Summit 2016, 23 February 2016 (23.02.2016), Pgs. 1-70. Retrieved from the Internet: <http://world-cns.com/wp-content/uploads/sites/113/2015/11/Day-1-1605-Rene-Anan-Yes.pdf> on 05 April 2017 (05.04.2017). entire document	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013231

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: