Title: Dual SMAD inhibition and Wnt inhibition enhances the differentiation of induced pluripotent stem cells into Retinal Ganglion cells (iPSC-RGCs)

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Running Title:

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Abstract:
Glaucoma is a group of progressive optic neuropathies that share common biological and clinical characteristics including irreversible changes to the optic nerve and visual field loss caused by death of retinal ganglion cells (RGCs). The loss of RGCs manifests as characteristic cupping or optic nerve degeneration, resulting in peripheral vision loss in patients with Glaucoma. Several initial studies carried out RGC differentiation from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), by modulating classical RGC signaling pathways to mimic in vivo retinal development. Recent studies used small molecules and peptide modulators at specific intervals to fine tune RGC differentiation, and also increase the yields to produce purified RGCs. In this study, we present a chemically defined and functionally novel *in vitro* methodology for cultivating unprecedented yields of RPC populations from iPSCs, that are then directed toward the RGC lineage. Using this method, we differentiated control iPSC lines into RGCs using in in a stepwise manner using small molecules and peptide modulator treatment to inhibit BMP and TGF-β (SMAD), and canonical Wnt pathways yielding a robust population of iPSC-RGCs.

Introduction:

Glaucoma encompasses a heterogenous group of optic neuropathies, tied together by their exhibition of permanent damage to the optic nerve.[1] This degeneration is due to the death of retinal ganglion cells (RGCs), with subsequent peripheral vision loss. [2]Primary open-angle glaucoma (POAG), the most common form of glaucoma, is characterized by chronic and progressive optic nerve degeneration and corresponding visual field deficits in the presence of an open and normal iridocorneal chamber angle.[3] This disease is the leading cause of irreversible blindness worldwide [4], with an estimated 11.1 million expected to become blind from POAG by 2020.[5] It is also the leading cause of vision loss among African Americans.[6] Despite the prevalence of POAG, its pathogenesis remains poorly understood. Elevated intraocular pressure (IOP) is a major risk factor [7], but degeneration of the optic nerve and loss of RGCs may still occur in those with normal eye pressure.[6] The complexity of glaucoma certainly makes it possible, if not probable, that RGCs become inherently susceptible to this disease process.

RGCs are found in the ganglion cell layer of the retina and serve as the projection neurons of the retina, utilizing long axons to effectively connect the eye to the brain. They transmit both image-forming and non-image-forming visual information, processed by retinal cells such as photoreceptors and horizontal cells, to higher visual centers in the lateral geniculate body through the optic nerve.[8] Currently, no promising treatment exists for glaucoma or RGC degeneration. Although shown to be effective in animal models of glaucoma, neuroprotective approaches have not proven practical in human settings.[9, 10] As mature mammalian RGCs are a terminally differentiated lineage, they do not regenerate after succumbing to disease[11], consequently leading to the irreparable blindness. Understandably, there is a great desire for an application devised to rejuvenate or replace injured RGCs. Hence, it is with good reason that translating

human stem cell technology to a viable regenerative therapy for use in degenerative conditions has become a worldwide priority.

Human pluripotent stem cells (hPSCs) offer a tantalizing value in medicine - they can be guided to differentiate into practically any type of cell within an organism. Owing to a variety of techniques that are established to derive every key retinal cell lineage, blinding disorders of the retina can be particularly well-modeled with hPSCs.[12, 13] Though the field was previously reliant on embryonic stem cells (ESCs), advances in stem cell research led to the discovery that overexpressing genes associated with “stemness” in somatic cells can reprogram them into induced pluripotent stem cells (iPSCs).[14, 15] The advent of iPSC manipulation has opened up opportunities for implementing this technology to cure diseases stemming from retinal cells.[16] Taking hPSCs and differentiating them toward RGC lineage commitment may generate enough healthy cells to compensate for the degenerative RGCs in glaucoma. As a result, stem-cell based therapy holds promise as a method to restore vision in retinal degeneration; however, success of these treatment strategies hinges on de novo synthesis of RGCs with stable phenotypes from hPSCs.[17, 18]

Several methods for differentiating ESCs and iPSCs into RGCs have been reported. Of the retinal lineages, RGC differentiation is perhaps the most complicated, historically involving three-dimensional (3D) culture of hPSCs.[19, 20] This enables the primordial eye to self-organize into 3D cell aggregates such as embryoid bodies (EBs), followed by optic vesicles (OVs) and later, optic cups, all of which are analogous to structures developed in vivo.[20, 21] The retinal progenitor cells (RPCs) within eventually mature into RGCs, characteristically the first neuronal subtype to develop, as well as other retinal cell types.[22, 23] While most of the lineages of the retina do arise in a properly organized manner, therein lies the problem with the earlier RGC generation protocols: instead of predominantly RGCs, majority of neural retinal cell types are present.[24-26] This heterogeneity and inefficient yield in culture restricts selection of specifically RGCs without an enrichment step, even if the 3D platform phase were to precede an adherent culture.[24, 27] To better specify the RGC cell lineage and ideally obviate 3D culture, latter studies modulated RGC signaling pathways, including Hedgehog and Notch, using various ligands and other factors.[26, 28] While this did enhance efficiency, there still resulted a suboptimal RGC yield and ambiguous picture of which pathways to best modulate, due to the ill-defined addition and administration of components. This shortcoming has been improved upon by administration of small molecules and peptide modulators at appropriate intervals in a calculated manner to more precisely modulate cellular signals and pathways such as bone morphogenetic protein (BMP), Wnt, IGF1, and TGF-β.[29] Canonical Wnt, BMP, and nodal are the three signaling pathways classically inhibited in vitro to spur pluripotent stem cells toward the neural retinal fate.[30-32] Once RPCs form the neural retina, the basic helix-loop-helix transcription factor (TF) ATOH7 plays a central role in RGC commitment and expression regulation of RGC-specific markers BRN3+ and ISL1.[27, 33, 34] Regardless of the protocol, adequate differentiation relies on emulating the pathways present in generating these cell lineages in vivo.

Developing a therapeutic approach employing stem cell technology requires an efficient, reproducible, and safe differentiation protocol for RGCs. To this end, we detail a chemically defined and functionally novel in vitro technique for cultivating unprecedented yields of RPC populations from iPSCs that are then directed toward the RGC lineage. True to their in vivo development, we directed unaffected control lines of iPSCs to differentiate in a stepwise manner using a temporal induction method centered on small molecule and peptide modulator treatment to inhibit BMP and TGF-β (SMAD), and canonical Wnt yielding a robust population of iPSC-RGCs after Day 30. Analysis at the molecular and physiologic levels using flow cytometry, immunolabeling, gene expression, and electrophysiology yielded results in accordance with the RGC lineage and allowed specific subtype identification.[26, 28, 35]

Methods:

Human induced pluripotent stem cell culture

Undifferentiated IPS cells (Control 1 (CHOPWT8). Control 2 (CHOPWT9), and Control 3 (CHOPWT10) were derived and characterized as previously published showing a complete analysis of iPSC characteristics. [36-38] All human samples were collected in accordance with institutional review board (IRB) requirements at the Children's Hospital of Philadelphia with informed consent. IPSc cells were maintained in IPSC medium ((Dulbecco's modified essential medium/ Ham's F12 nutrient media; DMEM/F12 (50:50; corning)) containing 1 x glutatmax (glut, invitrogen), 1 x penicillin/streptomycin (PS, invitrogen), 15% Knockout serum replacement (KSR; invitrogen), 1 x non-essential amino acids (NEAA; invitrogen), 0.1mM β-Mercaptoethanol (2-ME; invitrogen), and 10 ng/mL of basic fibroblast growth factor (bFGF; R&D Systems) on 0.1% gelatin coated dishes with irradiated mouse embryonic fibroblast (iMEFs).

Retinal Progenitor Cells (RPCs) generation

iPSCs were cultured on 0.1% gelatinized plates containing iMEFs in 37°C 5% O_2 and 5% CO_2 conditions. Cells were maintained until approximately 70-75% confluence, then feeder cells were depleted and approximately 1.5 - 1.8 x 10^6 iPSCs were seeded one well of 6-well (9.8 cm^2) tissue culture dish (Corning) coated with 1:100 diluted growth factor reduced (GFR) Matrigel in iPSC media containing Thiazovivin (2 mM, Tocris). iPSCs were maintained in iPSC: MEF-conditioned medium (80:20) + 20 ng/mL of bFGF. iPSCs were maintained in 37C 5% O_2 and 5% CO_2 until reaching 100% confluence then transferred to in 37°C, 5% CO_2 overnight prior to induction. All induction media changes were performed on a daily basis. On the first day, iPSC cells were changed to RPC induction media 1 (RPC1) (DMEM/F12 (50:50), 1 x P/S, 1 x Glut, 2% B27 supplement (w/o vitamin A), 1% N2 supplement containing 2 μM XAV939 (X) (Wnt inhibitor; SelleckChem, 10 mM SB431542 (SB) (TGF-Beta inhibitor; SelleckChem), 100 nM LDN193189 (L) (BMP inhibitor; Stemgent), 10 mM nicotinamide (Sigma-Aldrich), 10 ng/mL IGF1 (R&D Systems), cultures were fed for 4 days. On day 5, cultures were fed with retinal progenitor media 2 (RPC2) (DMEM/F12 (50:50), 1 x P/S, 1 x Glut, 2 x B27 supplement (w/o vitamin A), 1 x N2 supplement containing 2 μM XAV939 (Wnt inhibitor,

SelleckChem, 10 mM SB431542 (TGF-Beta inhibitor, SelleckChem), 100 nM LDN193189 (BMP inhibitor, Stemgent), 10 ng/mL IGF1 (R&D Systems), and 10 ng/mL bFGF (R&D Systems) with media changes daily for 12 days.

Retinal ganglion cell (RGC) differentiation conditions.

On day 16 of differentiation, four conditions were evaluated for RGC generation. The base media included DMEM/F12 (50:50),1 x P/S, 1 x Glut, 2 x B27 supplement (w/o vitamin A) and 1 x N2 supplement. For experimental conditions 1) LSBX, 2) LSB, 3) LX, and 4) LSB-CHIR (CHIR, Wnt agonist) were evaluated. Cultures were treated for 5 days prior to RGC patterning using SHH (100 ng/mL; R&D systems) or SAG (100 nM; Tocris), SHH was replaced with SAG after establishing equal competence, FGF8 (100 ng/mL, R&D systems), DAPT (3-10 mM Tocris), Y27632 (10 mM, Tocris), Follistatin 300 (100 ng/mL; R&D systems), cyclopamine (0.5 mM; Tocris) BDNF (50 ng/mL; R&D systems), NT4 (5 ng/mL; R&D systems), CNTF (5 ng/mL; R&D systems), forskolin (25 mM; SelleckChem), and cAMP (400 mM; Sigma-Aldrich) for RGC patterning. see figure 1A and 2A for kinetics. Cells were replated at a density of 1.0 x 10^5 cells/well of 6-well using the crosshatching technique as previously described (Espuny-Camacho et al. 2013) in RPCs base media containing Follistatin 300, Cyclo, DAPT, and 10 mM Rock inhibitor (Y27632, Tocris) on day 23 and culture media changed 24h post-plating with RPC2 media containing Follistatin 300 and DAPT. After maturation, medium was exchanged every 3-4 days with neural basal media supplemented with 2% B27 without vitamin-A and 1 x Glutamax.

Flow cytometry analysis of RGCs

Culture of RGCs were collected using TrypLE (Invitrogen) and run through a 100 □m filter to ensure single cell suspension. A portion of cells were incubated with antibodies anti-CD90-PE-cy7 (Thy1; Biolegend 1:100) and anti-SSEA4-APC (Biolegend 1:100) in 1 x PBS supplemented with 0.5% bovine serum albumin (BSA) +0.1% Na Azide (FACS buffer) and incubated at RT for 30 minutes on ice as previously described.[41] We analyzed stained cells AccuriC6 on and data analyzed using FlowJo Version 10.0.8 software (TreeStar).

Immunohistochemistry

Immunohistochemistry analysis was performed as previously published (Duong et al. 2018). For immunolocalization of antigens, RGCs on coverslips were fixed and thoroughly washed with phosphate-buffered saline, cryoprotected in 30% sucrose and embedded in OCT tissue freezing medium before sectioning. For characterizing RGCs immunohistochemistry was performed using SOX2, PAX6, RAX, Tuj1, Thy1/CD90 antibodies and dilution as described earlier.[38-41] Sections were mounted in mounting media containing DAPI (4',6-diamidino-2-phenylindole), observed under an Olympus FV1000 Confocal microscope, and images were captured with the use of appropriate filters and lasers.

Voltage recordings

The circular cover glass coated with 1:100 matrigel containing adherent cells was transferred to the recording chamber filled with iNS maintenance medium. The camber was placed on the microscope stage (Olympus BX-61 upright microscope) and perfused with the oxygenated Ames's solution (Sigma-Aldrich) resulting in gradual replacement of the growth medium with the Ames medium. The temperature of the solution inside the chamber was gradually increased to 37°C prior to the beginning of the imaging and recording session using Warner Instruments TC 344B temperature controller. Confocal images of the cells were acquired with Olympus Fluoview 1000 MPE system and cells demonstrating strong GFP expression were selected for patch-clamp recording. Whole cell configuration was achieved in the voltage-clamp mode at -60 mV holding potential. After achieving whole-cell configuration cells were either maintained in the current clamp mode at zero holding current and depolarized with calibrated current steps, or in the voltage-clamp mode at -60 mV holding potential from which level they were depolarized with calibrated voltage steps. Warner Instruments PC 505B amplifier and Molecular Devices Digidata 1440 digitizer under the control of the Clampex software were used for controlling and recording of the membrane voltages and currents. Patch pipettes were pulled from 1.2/0.69 mm OD/ID borosilicate glass on Sutter Instruments P-87 puller. Pipette filling solution included (in mM) 110 K-gluconate, 12 NaCl, 10 HEPES, 1 EGTA. Pipette were mounted on Sutter Instruments MPC-200 micromanipulators, MTI CCD 72 camera system was used to provide video control over pipette and cell positioning in the chamber.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using Purelink RNA isolation kit (invitrogen), with DNAse treatment. For each sample, 1 ug of total RNA was reverse transcribed using the high-capacity cDNA reverse transcription kit using random primers (ThermoFisher). Amplified material was detected using Taqman probes (target-FAM and housekeeping-TET) and AmpliTaq Gold Master mix (ThermoFisher) on a 7500 Fast Real-time PCR system (Applied Biosystems). All results were normalized to a B2M control (housekeeping) and are from 3 technical replicated of 3 independent biological samples for each time-point and experimental condition.

Statistical Analysis

Quantitative data were obtained from three independent experiments per cell line in triplicate. Statistical analysis was performed with Student T-test in Prism. *p-values of <0.05 were considered statistically significant.

RESULTS:

Combined inhibition of WNT, BMP and TGF-β and nicotinamide contribute to efficient generation of early retinal progenitor cells.

The hiPSC retinal differentiation methods differ between two- and three-dimensional culture conditions, and the use of proteins, and small molecules to recapitulate processes responsible for vertebrate retina development [28, 42]. These methods require a mid-differentiation enrichment step that involved the establishment of retinal rosettes followed by manual isolation and maturation to generate retinal subtypes. We have developed and characterized a modified two-stage protocol that differentiates hiPSCs into an enriched population of retinal progenitor cell (RPC) cultures followed by targeted patterning to retinal ganglion cells (RGCs) [28]. In order to accomplish this, hiPSCs were grown to confluence and subsequently treated with a retinal induction media containing, DMEM/F12 plus N2 and B27, XAV939 (WNT inhibitor), SB431542, (TGF-β inhibitor), LDN193189, (BMP inhibitor), nicotinamide, and IGF1 for 4 day (Fig. 1A). The inhibition of Wnt and BMP signaling has been documented to enhanced the expression of eye field transcription factors (EFTFs) during retinal differentiations of hPSCs [29]. We observed that addition of TGF-beta inhibition induced greater EFTFs expression during early retinal patterning. Nicotinamide was added to the differentiation media (d0-4) to promote the expression of early eye field markers LHX2 and RAX, as previously published [43]. Nicotinamide has been shown to promote cell expansion and adaptation to a radial/rosette morphology.[44] Patterning factors such as insulin like growth factor-1 (IGF-1) and basic growth factor-2 (bFGF2) aid in the specification of eye field identity to differentiating retinal progenitors.[29] From day 5-10, nicotinamide was removed and bFGF was added to retinal induction media. Analysis at day 7, showed an enriched population of SOX2, RAX and PAX6 positive cells (Fig. 1B). The expression of early retinal progenitor markers, LHX2 and RAX, were identified in over 97% of day 7 cultures (Fig.1 C) indicating and efficient generation of RPCs. Quantification of EFTFs, LHX2, RAX, PAX6, SIX6, SIX3, and VSX2, showed significant expression relative to pluripotent stem cells in all three individuals.

Extended exposures of BMP, WNT, and TGF-β inhibition leads to efficient generation of retinal ganglion cell neurons.

The efficient induction of retinal progenitors is largely dependent on the inhibition of pathways responsible for patterning of the diencephalon; however, how modification on of these inhibitory pathways play a role on retinal ganglion cells generation is unclear. Here, we tested whether pharmacological inhibition of WNT, TGF-β, and BMP can improve RGC induction and subsequently enable the controlled generation of mature iPSC-RGC neurons. We evaluated four different treatment modalities to determine pathways that enhance or restrict RGC generation from hiPSCs. Among the conditions tested: 1) BMP (L), TGB-B (SB), and Wnt inhibition (X) (LSBX), 2) LSB, 3) LX, and 4) LSB-CHIR (CHIR, Wnt agonist) (Fig. 1A). The progression of the differentiation proceeded through three phases; uniform RPC cultures (DIV7), rosette forming cultures (D16), then late stages neuronal cultures (D35) (Fig 2B). At day 21, cells were treated with RGC induction conditions which included the activation of sonic hedgehog, by augmenting with SHH (100 ug/mL). Comparative studies showed the use of SAG (100nM, smoothened agonist) could replace SHH with no difference in RGC induction changes. Fibroblast growth factor 8 (FGF8) and notch inhibitor (DAPT) were added along with SHH or SAG. At day 23, we SHH, TGF-β, and notch signaling were inhibited

to promote RGC maturation using cyclopamine, follistatin, and DAPT. RGC cultures expanded using a cross-hatching technique known separate cells into small clusters in RPC base media supplemented with rho-kinase inhibitor (Y-27632), follistatin, and DAPT to improve survival of RGCs during the expansion [45]. Neurotrophins, BDNF, neurotrophin 4 (NT4) and CNTF, were added to the media to promote the development and survival of cells with neuronal fate.[46] Forskolin and Y-27632 were included as a potent promoter of RGC neurite growth, survival and lineage commitment.[42] These conditions were established by combining previously described conditions used by Sluch et al., 2015) and Teotia et al., 2017 for hiPSC-RGCs.
After 35 days in vitro, we dissociated cultures and performed flow cytometry analysis of the single cell suspension obtained from three independent iPSC lines, and data showed that using the LSBX condition resulted in the highest proportion of RGC, with a range between 34-45% Thy-1 (CD90) positive iPSC-RGCs in our study (Fig. 2C-E). The LX, CHIR, and LSB conditions resulted in 26.7% (±3.2), 22.5% (±4.7), and 27.2% (±6.2), respectively. We observed consistent results across individuals and experiments (Fig. 2D). All the RGCs generated displayed small soma and elongated axonal processes, with Immunocytochemical analysis showing the expression of Thy-1/Tuj1, Brn3a and SNCG (Fig. 2A, 5).

Gene expression profiles during differentiation of iPSC to RPCs and RGCs:

During eye formation in vertebrates, cell intrinsic and extrinsic signals and/or transcription factors control the differentiation and fate determination of retinal cells. We evaluated the gene expression profile of three eye specific transcription factors (RAX, PAX6 and Six3) called eye field transcription factors (EFTFs) that are known to play a role in the anterior neural plate. The expression of Rx (encoded by RAX gene) is maximum in the RPC inhibited with L and SB when compared to the other conditions at DIV23. The detection of RAX expression at DIV35 RGCs was minimal suggesting a commitment to a more differentiated retinal fate in at the consequence of retinal progenitor cell expansion. PAX6 is expressed in the cornea, lens, ciliary body, and retina through development and plays a role in determining their cell fate. The expression of PAX6 gene transcript is expressed in all experimental conditions in RPCs and RGCs, however PAX6 is predominantly expressed at DIV 23 and 35 in the CHIR condition, which stimulated the expression of canonical Wnt signaling. This indicates that a prolonged stimulation of RPCs with Wnt restricts the differentiation potential and maintains more cells as multipotent progenitors.

Among the other gene expression transcripts analyzed, we observed an increase in SIX3 gene expression in both LX and LSBX conditions DIV23 days when compared to RPCs stimulated with LSB or CHIR. The expression of Six3 decreased in RGCs at DIV35 indicating that neurospecification is reaching completion at this stage.

The SOX11 transcript is heavily expressed in developing retina during embryonic stages [47]. It is required for the maintenance of hedgehog signaling and is critical for axonal growth, extension and driving adult neurogenesis [48-50]. The expression of SOX11 increases nearly two-fold in LSBX and LX condition when compared to LSB and

CHIR in RPCs at DIV23. The expression of SOX11 is significantly reduced in RGCs at DIV35. Gli3 has a dual function as a transcriptional activator and a repressor of the sonic hedgehog (Shh) pathway. GLI1 is a simple transcriptional activator encoded by a target gene of Shh signaling. We observed increased expression of Gli3 in RPCs at DIV23 when compared to RGCs at DIV35. Expression of both Gli3 and Gli1 genes show that sonic hedgehog signaling is important for development of RPCs.

Direction selective RGC (DS-RGC), that respond to motion of light in different directions can be identified by expression of specific molecular markers, such as CART, CDH6, and FSTL4, among other genes. [51-54] CDH6 mediates axon-target matching and promotes wiring specificity that does not lead to image formation. These data provide some of the first evidence that a specific classical cadherin can promote wiring specificity in the mammalian visual system. Cadherin mediated cell-cell adhesion ensures precise connectivity of neurons in the eye to target nuclei in the brain. Increased expression of CDH6 was observed in LSBX condition in DIV35 RGCs demonstrating their maturation towards increased specificity for axonal wiring between RGCs. The CARTPT transcript (cocaine- and amphetamine-regulated transcript) is expressed by a major subtype of RGCs, ooDSGCs. In our study, the expression of *CARTPT* is seen in RGCs matured only in LSB, CHIR and LSBX conditions at DIV35 indicating that these conditions develop specific subtypes of mature RGCs that are known to be markers for ON-OFF direction-selective RGCs.[55] FSTL4 is a gene expressed in ON DS-RGCs and is colocalized with BRN3B in few RGCs [56]. We have seen increased expression of FSTL4 gene transcript in CHIR induced RGCs when compared with other conditions at DIV35 indicating that development of various subtypes of mature RGCs in our culture conditions. We did not see any expression of CARTPT or FSTL4 transcripts in RPCs at DIV23.

The RGCs at DIV35 expressed Brn3a transcript predominantly in LSBX growth condition when compared to other conditions. Interestingly we did see ATOH7 expression early in RPC differentiation at DIV23 and lower expression in DIV35 RGCs. We detected low expression of CRX in DIV35 RGC cultures across all culture conditions implying that the differentiated RGCs using these conditions restrict the photoreceptor precursor cell population. We observed low expression of RCVRN (expressed by photoreceptors) and MITF in our cultures at DIV23 and 35 indicating that our cultures are differentiated predominantly towards RGC fate, with minimal retinal pigmented epithelium cell identity. We observed the development of few interneurons in mature RPCs (amacrine or horizontal cells) as evidenced by expression of CALB2 especially in RGCs with Wnt activation at DIV35.

Functional Analysis of iPSC-Retinal Ganglion Cells (iPSC-RGCs)

Confocal imaging of iPSC-RGC cultures at DIV35 cell show the presence of the GFP-expressing neurons with neurite projections, and an increase in the density and complexity of the projections over the period of one month (Fig. 5). Two out of the six cells tested demonstrated action potential firing in the current clamp mode in response to the depolarizing currents from patch pipettes. The responses of one of these cells

(*cell a*) are illustrated in Fig. 6. Although cells are not expected to fire action potentials under ideal voltage-clamp conditions, we believe that imperfect clamping in distal processes can lead to such firing we observed as illustrated on the Fig. 6 for the same *cell a* from Fig. 5. Interestingly, all but one cell (6 out of 6 and 4 out of 5 DIV35 old cells) demonstrated reliable firing when depolarized with voltage steps in the voltage-clamp mode. On Fig. 7 *cell b* illustrates firing of the older cells and *cell c* illustrates firing of the younger cells in response to the depolarizing voltage steps. As expected, later stage iPSC-RGCs cultures (DXX) produced higher frequency, sustained firing and generated larger spikes when compared to early born iPSC-RGCs.

Cells firing under current-clamp conditions had reasonably strong resting potential around -50 mV while all cells that fired under voltage-clamp but not current-clamp conditions had rather weak resting potentials around and above -30 mV. Maintaining these cells at zero-current level before and between depolarizing current steps in the current-clamp mode keeps membrane potential at its weak resting level, likely resulting in the weaker sodium/potassium gradient precluding cells from firing upon depolarization. Alternatively, in the voltage clamp mode membrane potential was maintained at -60 mV before and between depolarizing voltage steps likely resulting in recharging of ion gradients which enabled action potential firing in response to the depolarization. This may partly be the reason why some cells fired under voltage-clamp but not current-clamp conditions.

Discussion:

In the current study, we employed a two-step/stage differentiation to induce RGC differentiation from iPSCs by modification of existing methodologies.[28, 29, 56] The first stage involves differentiation of iPSCs to RPCs. The RPCs were matured in a stage specific manner using small molecules to modulate sonic hedgehog pathway, Wnt pathway and Notch signaling to produce abundant RGCs reliability, that stain positive for RGC markers and emulate action potential.

Historically generation of RGCs from hiPSCs used matrigel as an adherent support to culture cells obtained from Embryoid bodies (EB) or ESCs. A cocktail of factors supplemented with FGF2 and proneural supplements was later used to differentiation.[57] RGCs resulting from such differentiations were characterized for their expression of Brn3b, Neurofilament-200. Matrigel was successfully replaced as a matrix for RGC differentiations using several additive factors like Noggin, DKK-1, IGF-1 and FGF in follow-up protocols. [58] Teotia *et al*, 2017 successfully recapitulated the “Lamba protocol” by differentiating hiPSCs initially into neural rosettes and then to functional RGCs by recapitulating retinal developmental pathways using chemical regulators.[28] Generation of a CRISPR engineered iPSC line with a fluorescent RFP tag reporter in the Brn3B locus, greatly assisted in evaluation of pathways necessary for RGC differentiation and characterization. [59]

We developed a quick and efficient RGC generation protocol involve embryoid bodies or need retinal rosette production or enrichment to initiate RGC differentiation. In our

method, the entire pluripotent iPSC culture is differentiated into RPCs in 21 days using a chemically defined medium. We employ crosshatching technique to generate clumps of cells that undergo stage specific differentiation to produce functionally mature RGCs by DIV28. This is a must faster timeline considering other published methods to date. [24, 28, 59-61]. We used the a modified chemically defined media composition standardized by Teotia *et al*, 2017 [28] to take advantage of 15-day RGC differentiation and paired it with our novel RPC generation protocol to generate RGCs making our method unique. The RPCs thus generated showed immunoreactivity to RAX and LHX2 in the retinal precursor lines for over 97% of cells in iPSC culture indicating that our protocol committed cells towards RPC lineage. Using our method, we reliability differentiated six control iPSC lines and multiple clones from those lines (data not shown) to generate mature RGCs that stain positive for Thy1/Tuj1, Brn3a and SNCG markers. Using different chemical conditions, we were able to produce Thy1 positive cells upto/ in over 58% of the differentiated RGCs. We also generated other retinal cell types in addition to mature RGCs that express pan-retinal markers and appear to be amacrine and/or bipolar cells. We also did not find any gene transcripts produced by any cells that relates to RPE and photoreceptors.

Several published methods to generate RGCs from iPSC and ESCs in 2D cultures used stepwise differentiation protocols generated different types of RGCs based on morphological and functional characteristics [56]. The RGC subtypes were characterized by their distinct single-cell RNA sequencing profiles, morphological features and distinct expression profiles. [56] Based on the nature and type of physiological responses, several RGC subtypes like ON-, OFF- and alpha-RGCs were observed. Evaluation of the gene expression profile of transcripts relevant several RGC markers at DIV18 and DIV28 time point of RGC differentiation confirming the validity of our method. Futher, gene expression profiles revealed that in addition to the known RGC subtypes that express ATOH7, Brn3a, SNCG, we produced several specialized RGCs like direction selective RGCs (DS-RGCs) that demonstrate gene expression profiles for *CART*, *CDH6*, and *FSTL4*, among other genes.

A strategy to bypass embryoid body (EB) formation by adding exogenous factors and in presence of proneural supplements like B27 and N2 was developed by Reichman *et al*., 2014.[62] The long-term cultures of retinal organoids (RO) when supplemented with B27 allowed the differentiation of all retinal cell types including RGCs.[63] The RGC survival in these ROs derived retinal cultures decreased in vitro as the culture time was extended as floating cultures were a hinderance to the normal RGC growth and induced apoptosis. Adherent cultures obtained by enzymatic dissociation of ROs greatly improved the survival of these RGC [64] Several protocols following this study, differentiated iPSCs to neural rosettes and embryoid bodies that were further differentiated into RGCs using chemically defined media. [28, 56, 64, 65] These methods have several shortcomings for large scale growth and culture of RGCs as, they not only introduce bias during manual selection of desired RPCs, but also introduce variability in the total numbers or RGCs produced resulting from RPC numbers used for differentiation. Hence to reduce variability and increase the efficiency of the RGC generation, we differentiate the entire iPSC culture vessel to RPCs, and use a

crosshatching technique that does not need manual selection/screening to produce abundant RPC clumps that can be readily differentiated into RGCs.

Methods to isolate and purify RGCs have improved significantly with protocols using magnetic-activated cell sorting (MACS) with magnetic beads coupled to CD90 antibody, fluorescent cell sorting (FACSs), or immunopanning demonstrated enrichment of Thy1 positive RGCs cells.[65, 66] Using CD90 Thy1 magnetic beads, we were able to purify our Thy1 positive RGCs to over 90% to generate enriched cultures with defined characteristics.

The maintenance of later stage RGC cultures were cultured initially in NeuralBasal Media containing 1 x N2, and 1 x B27; however, this resulted in significant overgrowth of proliferative retinal progenitors cells. The removal of N2 supplement reduced the number of dividing cells in our cultures, providing a culture with predominantly neurons. We have also used 1 x CultureOne supplement within the RGC maturation media, which also reduced the number of non-RGC cell growth in day 45 in culture and beyond. After day 35 in culture, the mature RGCs produce appropriate morphological and physiological features. We observed extensive neurite outgrowth, and migration of RGCs to form cell clusters with elongated axonal projections forming a compartmentalized space by pushing away other cell aggregates may be by secreting unknown paracrine secretions, which warrants further investigation (Supplementary Fig).[26] Furthermore, the RGCs differentiated using the LSBX condition exhibited a electrphysiological function, with the ability to conduct sodium and potassium through voltage-dependent channels and fire action potentials. When comparing action potentials obtained from RGCs at day 35, day 70 and day 100, we observed that RGCs were able to fire continuously producing larger spikes with higher frequency, as they mature and age in culture when compared to younger cells.

We were unable to estimate the proportions of different RGC subtypes generated using our protocol. A systematic analysis of RGCs by single-cell RNA sequencing at different maturation time points is currently underway in our laboratory to identify RGC subtype markers and any maturation markers in the RGCs. Our validated methodology can reliably harness iPSC technology as a renewable source of RPCs to efficiently produce highly pure populations of RGCs for in vitro studies of glaucoma and potential therapeutic modalities for incurable RGC-related diseases.

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Author contributions:
VRMC proposed the project and developed the concept with JAM. VRMC, NH, SN, TTD, SR, JAM conceived the method. VRMC, NH, SR, SN, RN, KG, JH, TTD, JAM conducted the experiments. DM generated the SNCG-GFP construct. VRMC, JAM, TTD, SR, NH, and SN performed the data analysis. TA, VRMC and JAM wrote the manuscript. VRMC, JAM, TTD, SR, NH, SN, RN, JMO, JH and TA revised the manuscript. VRMC and JAM supervised this work. All authors read and approved the final manuscript.

Additional information:

Competing financial interests: The authors declare no competing financial interests.

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Figure Legends:

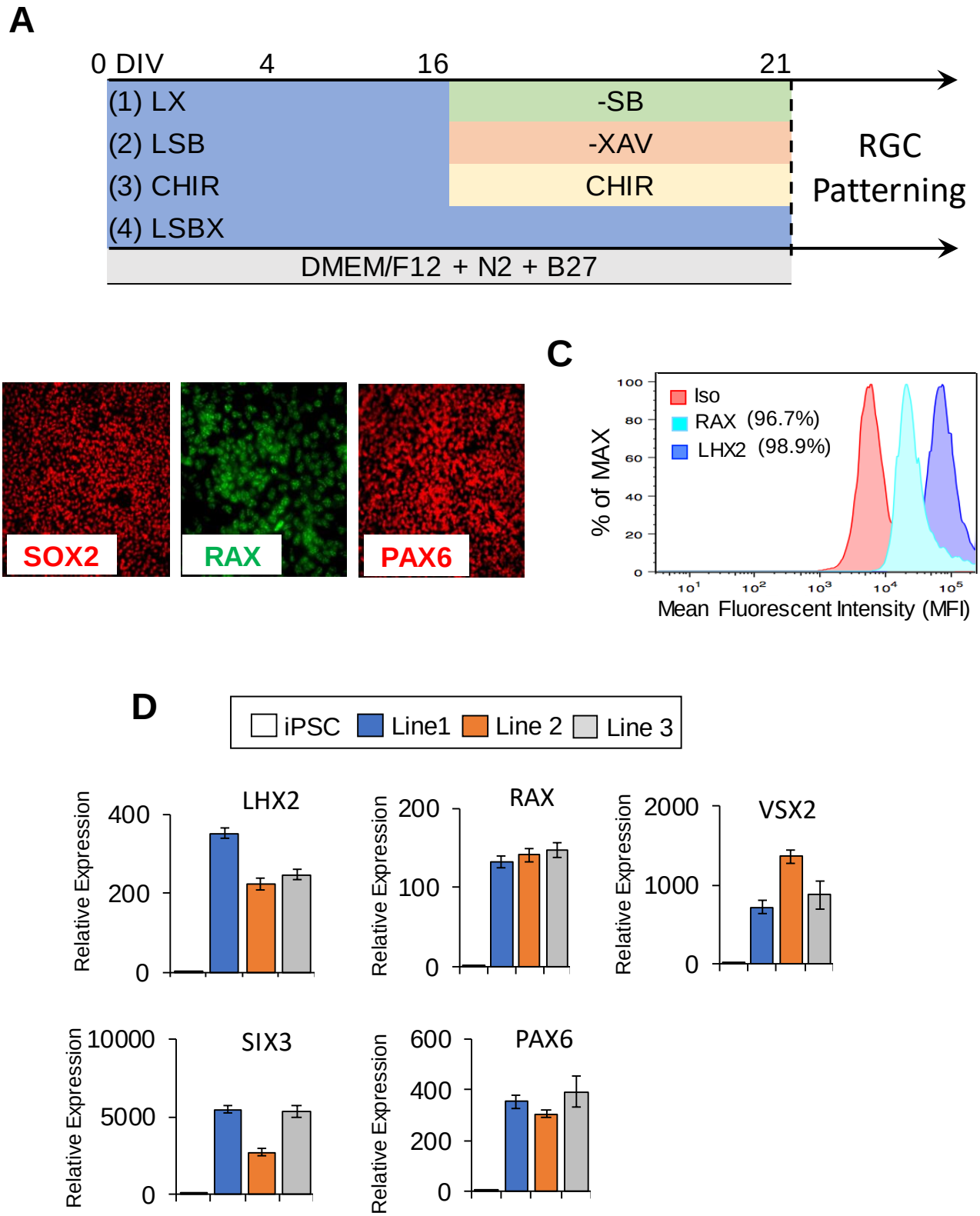
Figure 1: Differentiation of iPSCs into neural retina and retinal ganglion cells (RGCs). A) Schematic showing iPSCs differentiation in neural retinal differentiation media (DMEM/F12 + 2% B27 + 1% N2 + 10 ng/mL IGF1), including inhibitors of BMP, WNT and TGF- beta for 15 days, bFGF2 was included in media from day 4-14. At 16 days *in vitro*, cells were treated with RGC induction and maturation media. Differentiation using four different conditions, LX, LSB, CHIR and LSBX are shown. B) Characterization of RPCs using immunocytochemistry with SOX2, RAX and PAX6 of early retinal progenitor markers. C) Percentage of RPCs expressing retinal progenitor markers (RAX and LHX2) by flow cytometry D) Gene expression profiles showing RPC markers in three different iPSC lines, showing a high enrichment. Mean ± SEM represented from at least 3 independent experiments per individual.

Figure 2: Efficient generation of iPSC-RGCs require inhibition of TGF-beta, Wnt, and BMP signaling. A) Phase contract images of different stages of iPSC-RGC differentiation at DIV7, DIV16 and DIV35 is shown. B) Differentiation of retinal progenitor cells (RPCs) to retinal ganglion cells (RGCs) using defined inhibition or activation of developmental pathways, C) Percentage of Thy-1 (CD90) positive iPSC-RGCs in our studies in different differentiation conditions, D) Proportions of CD90 positive RGC cells across four differentiation conditions is shown. Each symbol represents an individual experiment. E) Percentage of CD90 positive cells obtained across three control iPSC lines across each differentiation condition. Mean ± SEM represented from at least 3 independent experiments per individual.

Figure 3: Transcriptional Profiling of iPSC-RPCs. Quantitative-RT-PCR profiles showing relative expression of retinal ganglion cell and retinal subtypes

(photoreceptors, amacrine, horizontal, and RPE) at Day 23 and Day 35 stages of development from iPSCs to RGCs.

Figure 4: Functional Characteristics of Induced Retinal Ganglion Cells. A) RGCs were transduced with AAV7m8-SNCG-eGFP and immunofluorescence RGC markers (BRN3a, BRN3b, THY1, TUBB3). B) Density of RGCs expressing GFP and other axonal processes increases between DIV35 (4B, cell c) and maturation D72 (4B, cells a and b). Blue arrows point to cells selected for patch clamp recordings and that fired action potential upon depolarization. C) Action potential firing in response to depolarizing voltage step recorded from cell a and cell b (D72) and from cell c (DIV35). D) This trace demonstrates action potential firing in response to the two different depolarizing steps of current recorded from *cell a* from Fig. 4B. We also observe a spontaneous spike firing at the start of the trace. Same scale bar applies to all images.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0 DIV
4
16
21
(1) LX
(2) LSB
(3) CHIR
(4) LSBX
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-XAV
CHIR
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DMEM/F12 + N2 + B27
B
SOX2
RAX
PAX6
C
Iso
RAX (96.7%)
LHX2 (98.9%)
% of MAX
Mean Fluorescent Intensity (MFI)
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iPSC
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Line 2
Line 3
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SIX3
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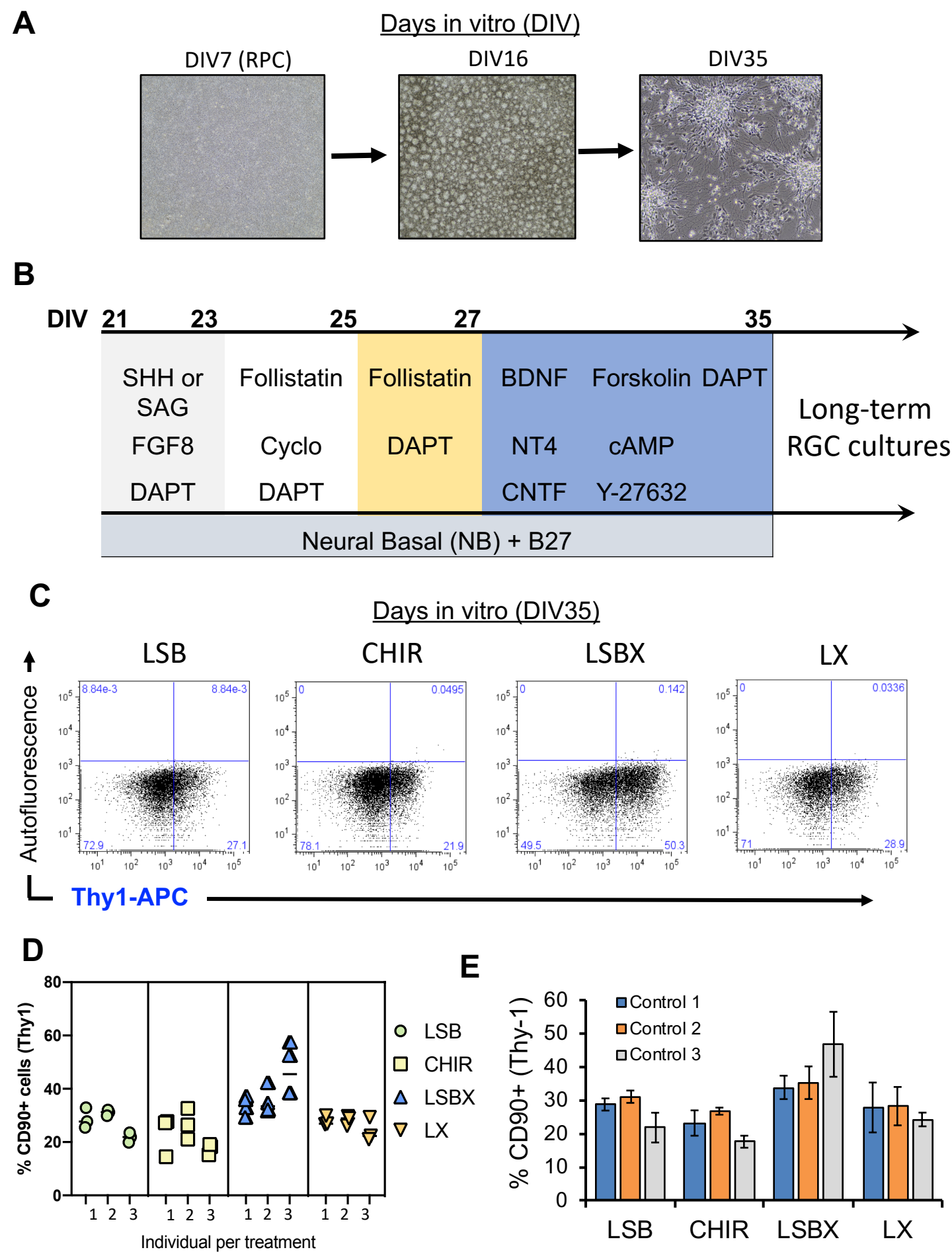
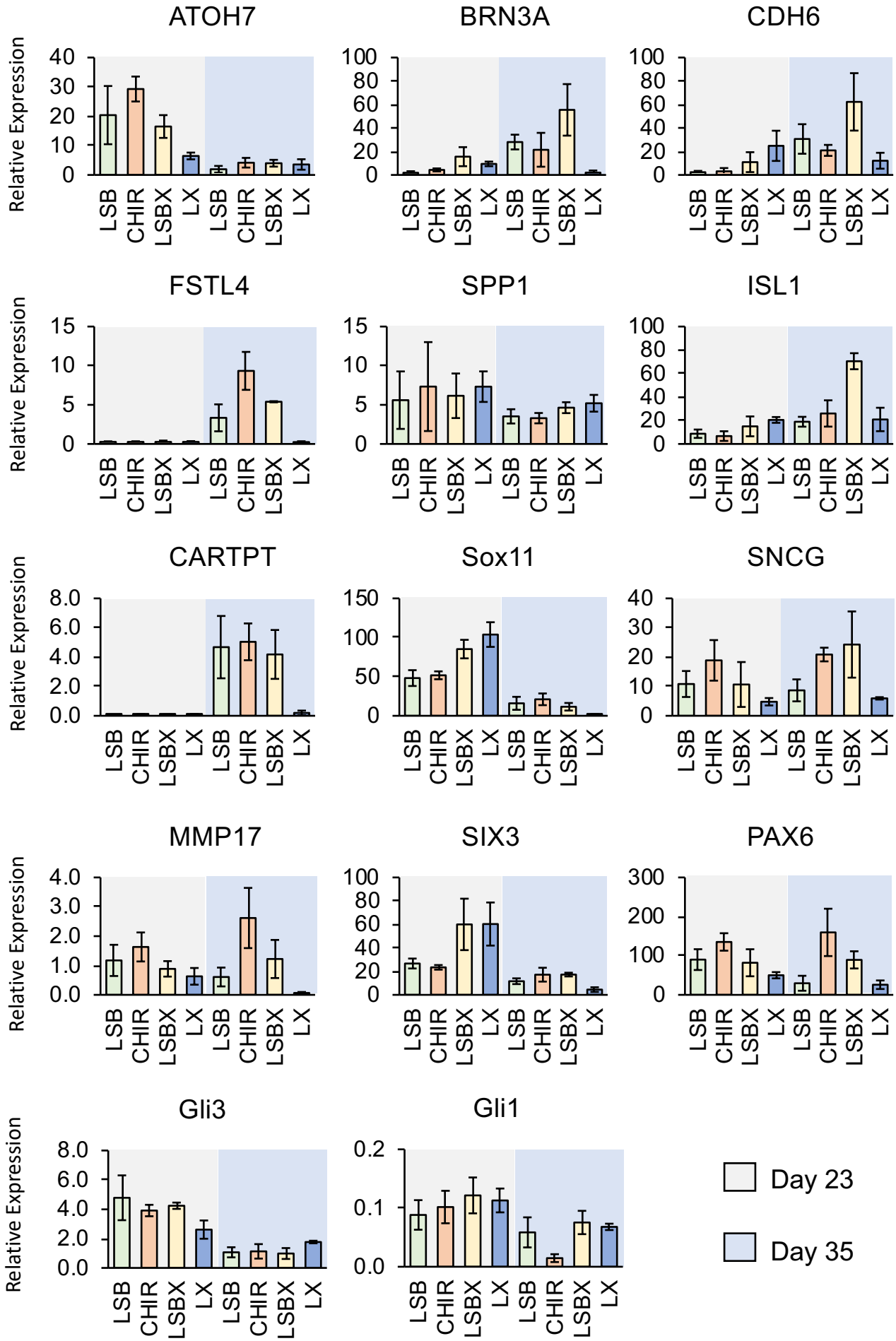
Relative Expression


A
Days in vitro (DIV)
DIV7 (RPC)
DIV16
DIV35
B
DIV 21 23 25 27 35
SHH or SAG
FGF8
DAPT
Follistatin
Cyclo
DAPT
Follistatin
DAPT
BDNF
NT4
CNTF
Forskolin DAPT
cAMP
Y-27632
Long-term RGC cultures
Neural Basal (NB) + B27
C
Days in vitro (DIV35)
LSB
CHIR
LSBX
LX
Autofluorescence
Thy1-APC
8.84e-3
8.84e-3
72.9
27.1
0
0.0495
78.1
21.9
0
0.142
49.5
50.3
0
0.0336
71
28.9
D
% CD90+ cells (Thy1)
Individual per treatment
LSB
CHIR
LSBX
LX
E
% CD90+ (Thy-1)
Control 1
Control 2
Control 3
LSB
CHIR
LSBX
LX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Relative Expression
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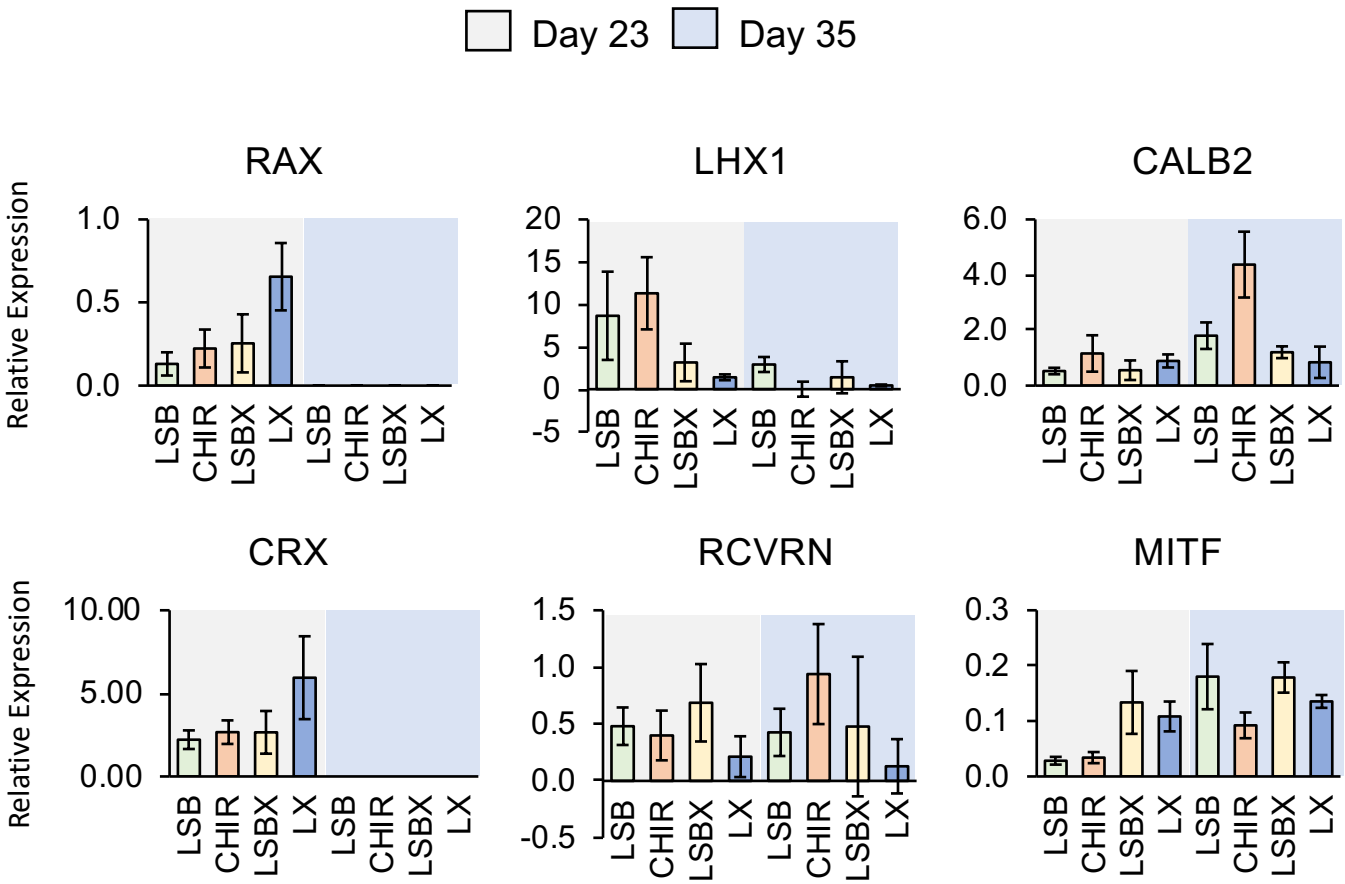
CHIR
LSBX
LX
Day 23
Day 35


Day 23
Day 35
RAX
LHX1
CALB2
CRX
RCVRN
MITF
Relative Expression
Relative Expression
1.0
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0.0
20
15
10
5
0
-5
6.0
4.0
2.0
0.0
10.00
5.00
0.00
1.5
1.0
0.5
0.0
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0.2
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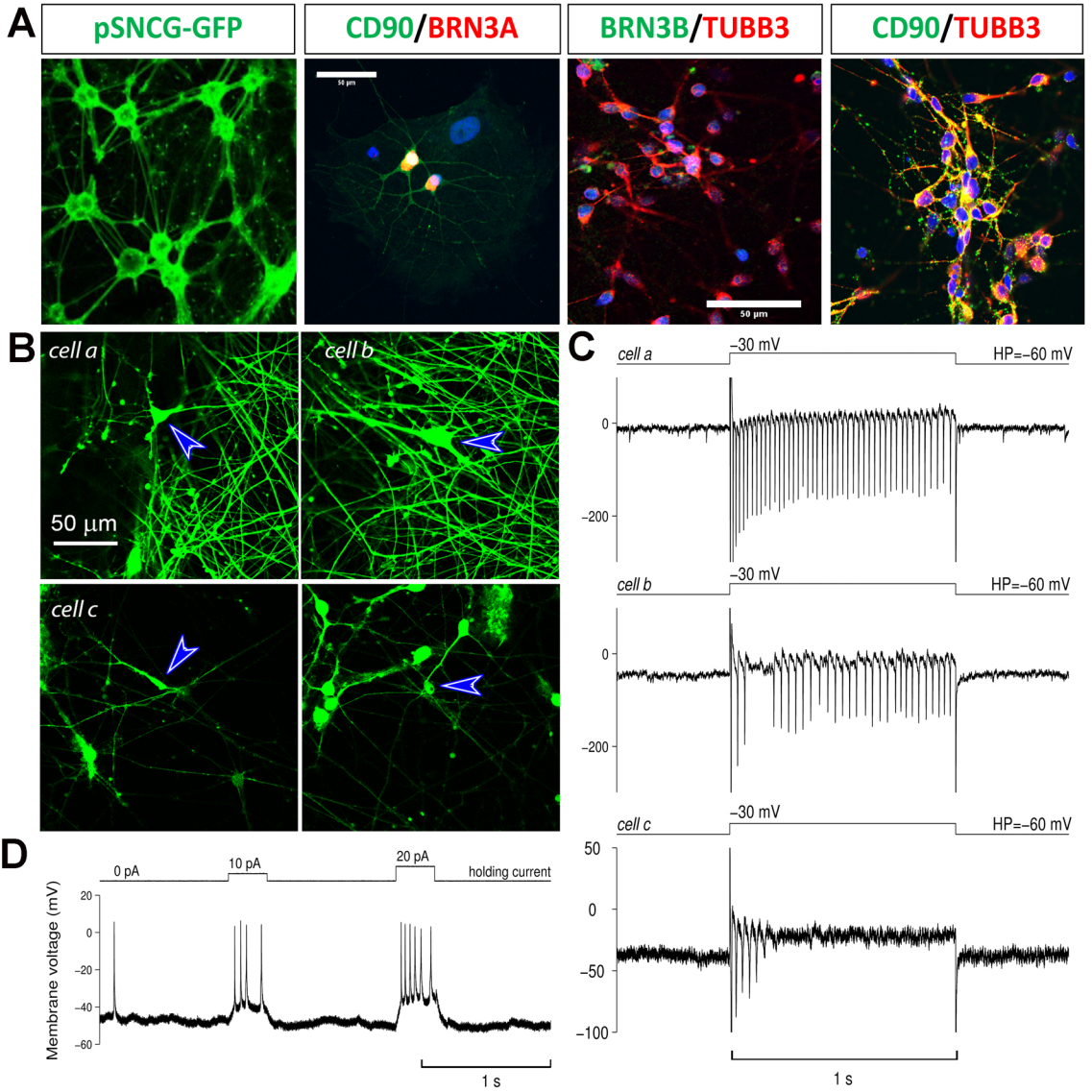
CHIR
LSBX
LX
LSB
CHIR
LSBX
LX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pSNCG-GFP
CD90/BRN3A
BRN3B/TUBB3
CD90/TUBB3
50 µm
B
cell a
cell b
cell c
50 µm
C
cell a
–30 mV
HP=–60 mV
0
–200
cell b
–30 mV
HP=–60 mV
0
–200
cell c
–30 mV
HP=–60 mV
50
0
–50
–100
1 s
D
0 pA
10 pA
20 pA
holding current
Membrane voltage (mV)
20
0
–20
–40
–60
1 s