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Supplemental Data

Distinct Stages of Myelination Regulated by γ-Secretase and Astrocytes in a Rapidly Myelinating CNS Coculture System

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Figure S1

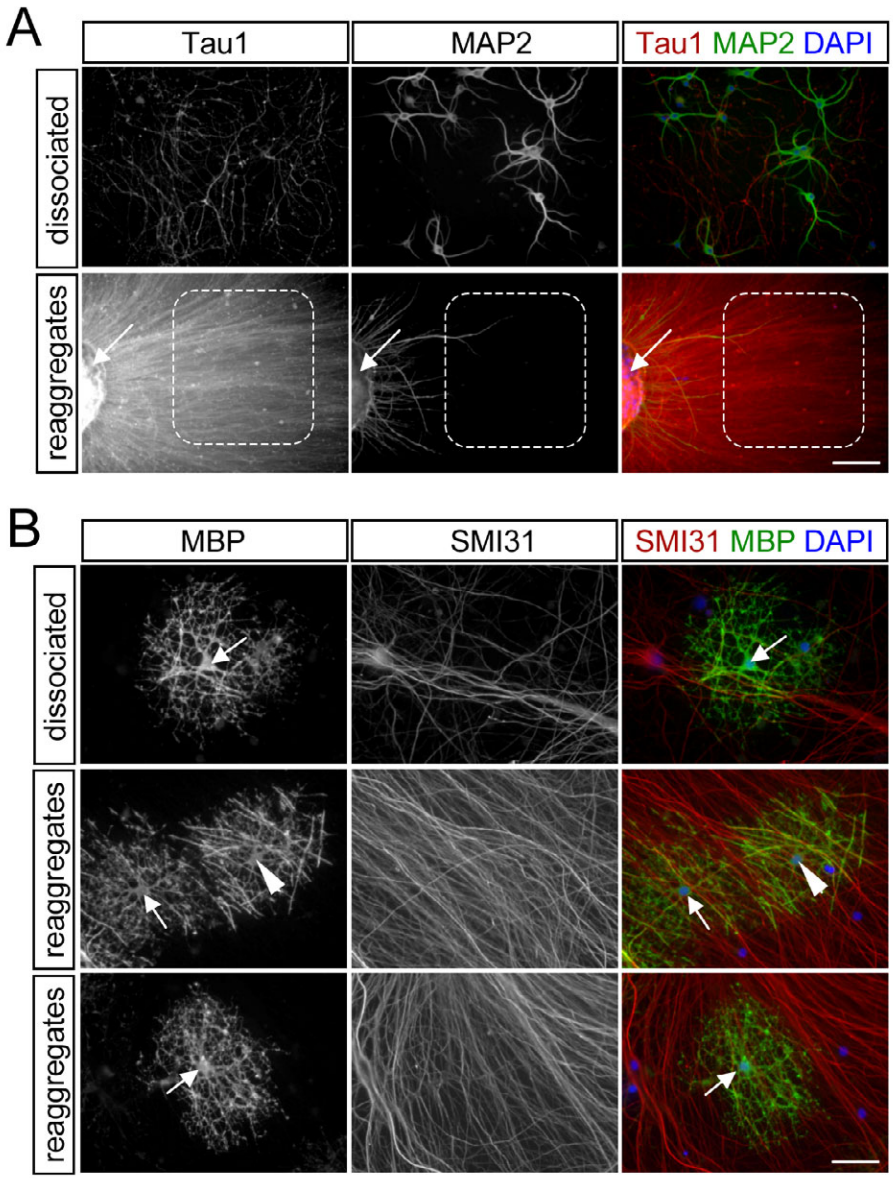

Figure S1. Reaggregates of RGCs extend dense bed of axons in culture that serve as a substrate for myelination by OLs.
(A) Immunostaining of 10-day cultures of dissociated RGCs or RGC reaggregates for markers of axons (Tau1) and dendrites (MAP2). Nuclei are counterstained with DAPI (*blue*). Cultures of dissociated RGCs have neuronal cell bodies and dendrites distributed throughout the assortment of neurites, whereas an RGC reaggregate (*arrow)* extends dendrites only a limited distance, leaving dense regions of axons running roughly in parallel (*dotted box*). Scale bar = 50 µm.
(B) Immunostaining of 6-day cocultures in ND-G of optic nerve OPCs and RGC reaggregates for MBP and neurofilament (SMI31). The increased axon density of RGC reaggregates is associated with the appearance of myelinating OLs (*arrowhead*), but the majority of MBP-expressing OLs in axon-dense regions still failed to form clear myelin segments around axons (*arrows*). Scale bar = 50 µm.

Figure S2

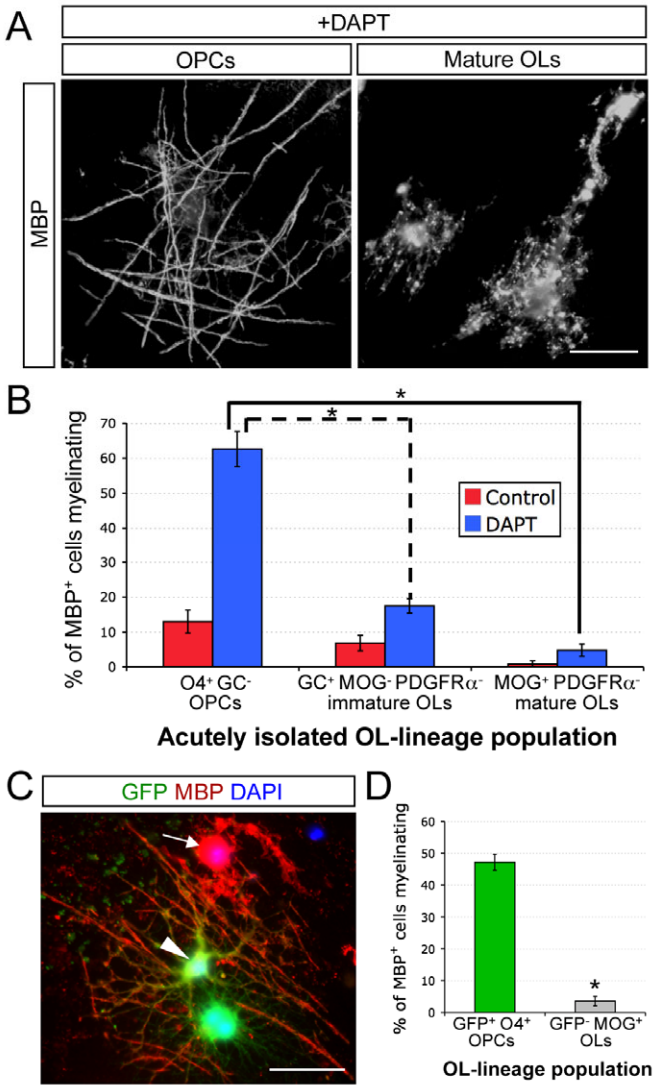

Figure S2. Acutely-isolated cortical oligodendrocytes largely fail to myelinate *in vitro*.
(A) Anti-MBP immunstaining of DAPT-treated cocultures of RGCs with either OPCs or mature OLs that were acutely purified in parallel from postnatal day 13 (P13) mouse cortices. Mature OLs extend MBP-rich processes but rarely generate the well-defined myelin sheaths that are often established by an OL that is newly formed from an OPC. Scale bar = 50 µm.
(B) OPC-depleted (PDGFRα$^-$) populations of GC$^+$ and MOG$^+$ OLs mostly fail to myelinate RGC axons over six days in ND-G with or without 1 µM DAPT (one-way ANOVA, $p<0.0001$, post-hoc Tukey-Kramer tests, *$p<0.001$, $n = 4$ per condition).
(C) A DAPT-treated triple coculture of RGCs with both wild-type mature cortical OLs and OPCs purified from the cortices of GFP transgenic mice to control for any potential environmental differences between the OPC-RGC and OL-RGC cocultures. Some GFP$^+$ OPCs (*green*) develop into myelinating OLs (*arrowhead*), whereas a neighboring GFP$^-$ OL (*arrow*) expresses MBP but does not myelinate. Scale bar = 50 µm.
(D) Mature OLs mostly fail to myelinate side-by-side with GFP$^+$ OPCs over six days in ND-G containing DAPT (unpaired *t*-test, *$p<0.0001$, $n = 4$ per condition).

Figure S3

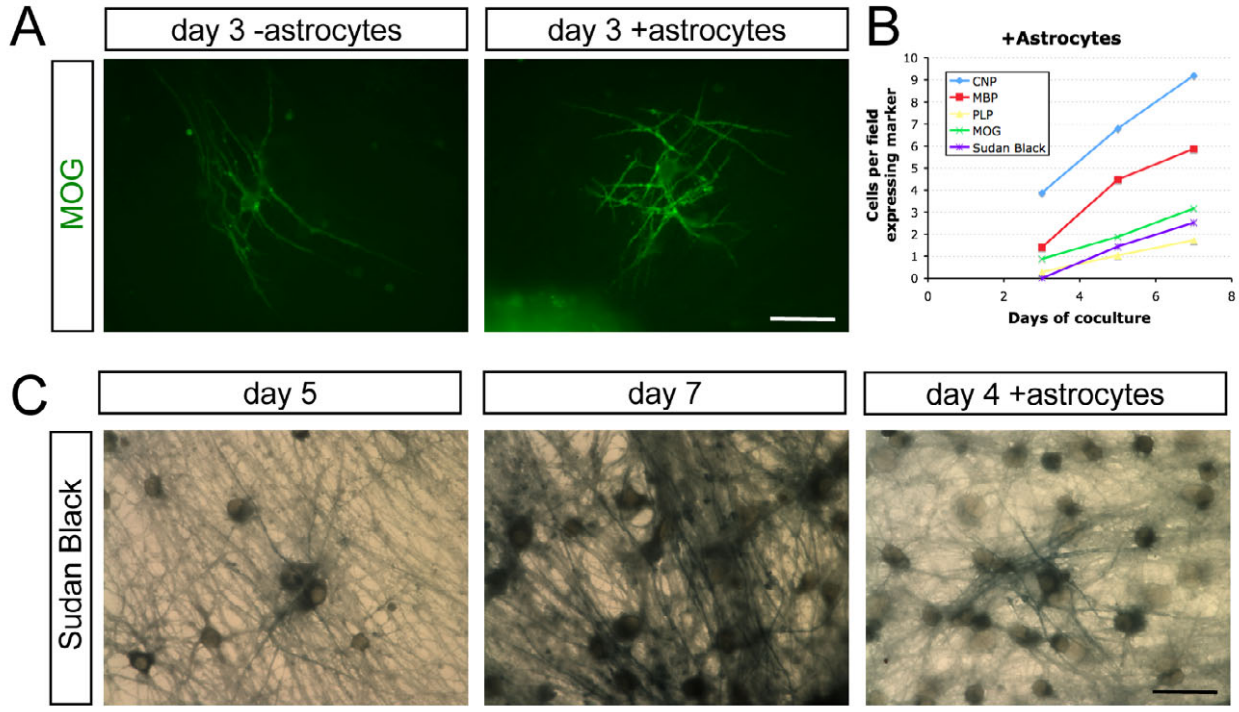

Figure S3. Time course of myelin markers in the presence of astrocytes.
(A) α-MOG immunostaining shows isolated ensheathing OLs in the presence or absence of optic nerve astrocytes in 3-day cocultures of rat RGCs and rat cortical OPCs maintained in MyM containing DAPT (1 µM). Scale bar = 50 µm.
(B) Time course of myelin markers in cocultures of rat cortical OPCs, optic nerve astrocytes, and rat RGCs over 7 days in MyM with DAPT, including sequential protein markers of OL maturation (CNP, MBP, PLP, and MOG) and a lipophilic dye reporting the degree of wrapping of the RGC axons with multiple layers of myelin membrane (Sudan Black). Compare with Figure 3F for time course in the absence of astrocytes.
(C) Sudan Black staining of cocultures reveals compact myelin by day 5 in the absence of optic nerve astrocytes and by day 4 in their presence. Note that extensive myelination can be observed by day 7 in axon-dense regions in the absence of added astrocytes. Scale bar = 50 µm.

PROTOCOL FOR MYELINATING COCULTURE OF RAT OPTIC NERVE OPCS AND RGC REAGGREGATES

Stocks to prepare:

1) BDNF (1000X): Thaw 200 µl of Human BDNF (Peprotech #450-02 ; 1 mg/ml) on ice. Prepare 10 mls of 0.2% BSA (Sigma A-8806) in D-PBS. Filter through 0.22-µm filter and discard the first 3 mls. Collect the rest, and cool 3.8 ml on ice. After chilled, add 200 µl aliquot of BDNF to BSA solution and mix well but gently to avoiding foaming. Make 20 µl aliquots, and flash freeze the aliquots in liquid nitrogen. Store at -80˚C.
2) CNTF (1000X): Dilute Human CNTF (Peprotech #450-13) to 10 µg/mL with sterile 0.2% BSA in D-PBS. Make 20 µl aliquots, and flash freeze the aliquots in liquid nitrogen. Store at -80˚C.
3) Sato (100X): Add the following to 40 ml Neurobasal medium: 400 mg transferrin (Sigma T-1147), 400 mg BSA (Sigma A-4161), 10 µl progesterone (Sigma P7505; from stock: 2.5 mg /100 µl EtOH), 64 mg putrescine (Sigma P5780), 400 µl sodium selenite (Sigma S5261; 4.0 mg/100 µl 0.1N NaOH, plus 10 ml Neurobasal). Mix well and filter through 0.22 µm filter. Make 400 µl aliquots; store -20˚C.
4) Hormone mix (200X): Dissolve 20 mg apotransferrin (Sigma T-1147) in 5 ml DMEM. Dissolve 64 mg putrescine (Sigma P5780) in 5 ml DMEM. Mix these two stocks with: 10 µl progesterone (Sigma P7505; from stock: 2.5 mg /100 µl EtOH), 51.9 µl sodium selenite (Sigma S5261; from stock: 4.0 mg+10 µl 1N NaOH in 10 ml DMEM), and an additional 10 ml DMEM giving final stock concentrations of 1 mg/ml apotransferrin, 20 mM putrescine, 4 µM progesterone, and 6 µM selenium (Thomson *et al.*, 2006). Mix well and filter through 0.22 µm filter. Make 200 µl aliquots; store -20˚C.
5) T3 (100X): Dissolve 3.2 mg triiodo-thyronine (Sigma T6397) in 400 µl 0.1 N NaOH. Add 10 µl to 20 ml DPBS. Filter 0.22 µm filter, discarding the first 10 ml. Make 200 µl aliquots; store at -20˚C.
6) 4% BSA (20X): Dissolve 8 g BSA (Sigma A4161) in 150 ml D-PBS (Gibco# 14287-080). Dissolve at 37˚C. Adjust pH to 7.4 (with approx. 1ml 1N NaOH). Filter through 0.22 µm filters. Make 1.0 ml aliquots; store at -20˚C.
7) Insulin (0.5 mg/ml; 100X): To 20 ml sterile water add: 10 mg insulin (Sigma I-6634) and 100 ul 1.0 N HCl. Mix well. Filter through 0.22 µm filter. Store at 4^{o}C for 4 to 6 weeks.
8) NAC (5 mg/ml; 1000X): Dissolve 50 mg N-acetyl cysteine (Sigma A8199) in 10 ml Neurobasal (will be yellowish). Make 40 µl aliquots; store at -20˚C.
9) Poly-D-Lysine (PDL) (100X): Add 5 ml water to a 5 mg bottle of PDL (Sigma P6407). Filter through 2 µm filter. Make 100 µl aliquots; store at -20˚C.
10) Forskolin (10 mM; 1000X): Add 2.4 ml DMSO to a 10 mg bottle (4.2 mg/ml). Make 20 ul aliquots; store -20˚C. Remainder can be left in the fridge for future use.
11) Biotin (5000X): 10 mg biotin (Sigma B4639) in 200 ml D-PBS with phenol red. Add 0.1N NaOH in 5 µl drops to aid in dissolving. Make 10 µl aliquots; store at -20˚C.
12) Vitamin B12 (5000X): 68 mg Vitamin B12 (Sigma V6629) in 50 ml D-PBS. Filter through 2-µm filter. Make 10 µl aliquots; store at -20˚C. Protect from light.
13) Ceruloplasmin (10,000X): Dissolve 1 mg Human Plasma Ceruloplasmin (Calbiochem 239799) with 1 ml sterile H_2O. Make 5 µl aliquots and store at -30˚C.

14) Low Ovomucoid (10X): To 150 ml D-PBS, add 3 g BSA (Sigma A8806). Mix well. Add 3 g Trypsin inhibitor (Worthington LS003086) and mix to dissolve. Adjust pH to 7.4; requires the addition of approx. 1ml of 1N NaOH. When completely dissolved bring to 200ml with DPBS filter through 0.22 µm filter. Make 1.0 ml aliquots and store at -20˚C.
15) High Ovomucoid (10X): To 150 ml D-PBS add 6g BSA (Sigma A8806). Add 6g Trypsin inhibitor (Worthington LS003083) and mix to dissolve. It is okay to leave at 4^{o}C overnight to allow everything to go into solution. Adjust pH to 7.4; requires the addition of at least 1.5ml of 1N NaOH. If necessary, add NaOH until solution no longer too acidic. Bring to 200ml with DPBS. When completely dissolved, filter through 0.22 µm filter. Make 1.0 ml aliquots and store at -20˚C.
16) DNAse (0.4%; 12,500units/ml): Add 1ml of EBSS (Ca, Mg free) per 12,500 units of DNase (Worthington #LS002007). Keep on ice. Filter sterilize, and make 200 ul aliquots. Store in -30˚C freezer.
17) Laminin: Thaw mouse laminin (Cultrex Cat. No. 3400-010-01; 1 mg/ml) at 4˚C. Make 10 µl aliquots, and store at -30˚C.

I. Establishing RGC reaggregate cultures

(i) RGC purification

Required Materials and Equipment:

1) 3 litters P5 Sprague-Dawley rats (30 pups)
    a. Note: P4-P10 pups may be used, but P5 is optimal for its combination of yield, viability, and purity.
2) 8-well chamber-slide (Nunc)
3) Papain (Worthington/Cooper, Cat. No. LS003126)
4) T11D7 α-Thy1 hybridoma supernatant
5) Rabbit α-macrophage antibodies (Accurate Chemical, AI A51240). Make 80 µl aliquots and store in -30˚C freezer.
6) 2.5% Trypsin: Dissolve trypsin (Sigma T9935) at a concentration of 30,000 U/ml in EBSS. Filter through 0.22 µm filter. Make 200 µl aliquots and store at -80˚C.
7) L-cysteine (Sigma)
8) Nitex filter, 3-20/14 from Tetko, Inc. Cut into 3 inch squares, wrap in small packets of foil, and autoclave.
9) dissection equipment: #11 scalpel blade with handle, forceps, sharp scissors, small spatula
10) Dulbecco's phosphate-buffered saline (D-PBS)

To prepare:

1) ND-Sato base medium: Mix 19.5 ml of Neurobasal medium with 19.5 ml DMEM-High Glucose medium. Add 400 µl each of 100X penicillan / streptomycin (Invitrogen), insulin stock, 100X sodium pyruvate (Invitrogen), 100X Sato stock, 100X T3, and 100X L-glutamine (Invitrogen). Add 40 µl each of 1000X NAC stock and 1000X Trace Elements B (Cellgro). Add 8 µl of 5000X biotin stock. Add 800 µl B27 Supplement (Invitrogen). Filter solution through a 0.22-µm filter. Store 4˚C; good for up to one week.
2) D-PBS/0.2%BSA: Mix 1 ml 4% BSA and 19 ml D-PBS.
3) Panning dishes: The day before purification, coat petri dishes with secondary antibodies overnight. To each of two 150-mm petri dishes (for anti-macrophage panning), add 20 ml Tris-HCl (50 mM, pH 9.5) and 50 µl affinity purified Goat anti-Rabbit IgG (H & L). To a 100 mm petri dish (for Thy 1/T11D7 panning) add 12 ml Tris-HCl (50 mM, pH 9.5) and 40 µl Goat anti-mouse IgM (µ chain specific). Mix well and make sure entire plate is covered. Incubate all three dishes at 4˚C on a flat surface overnight. On the day of purification, rinse large dishes 3X with PBS. Add 10 ml PBS/ 0.2% BSA to each and set aside at room temperature. Rinse small dish 3X with PBS. Add 8 ml T11D7 supernatant; incubate at room temperature for at least 2 hours.
4) Equilibrate Earles's Balanced Salt Solution (EBSS). Aliquot 4 ml EBSS into a small tube and 6 ml EBSS into another tube and let them equilibrate in the incubator by leaving their caps on loose.
5) Panning buffer: Mix 2 ml D-PBS/0.2% BSA with 18 ml D-PBS and add 200 µl insulin stock. Equilibrate to room temperature.
6) Ovomucoid solutions: Mix 1 ml low ovomucoid stock with 9 ml D-PBS and add 100 µl DNase stock. Mix 1.2 ml high ovomucoid stock with 5 ml D-PBS.

7) 30% FCS: Mix 4.5 ml fetal calf serum with 10.5 ml Neurobasal medium. Filter to sterilize. Equilibrate to room temperature.
8) 5 ml ND-Growth (ND-G) medium: Add 5 µl each of BDNF, CNTF, and forskolin stocks to 15 ml ND-Sato (see above). Equilibrate in incubator to 37˚C / 10% CO_2.

Procedure:

1) Dissect retinae from P5 rat pups. Decapitate a pup with sharpened scissors and remove the skin overlying the eyes with scissors. Pierce the cornea with a #11 scalpel balde and grasp each side of the slit with forceps. Pull eye open, and remove lens and vitreous humor with forceps. Gently lift retina out with a small spatula and transfer it to a 35-mm Petri dish with 1 ml D-PBS. Remove membranes from retinae with forceps under dissecting microscope.
2) Prepare papain solution: Add 165 units of papain to 10 ml D-PBS + 5 µl 1M NaOH. Place tube in 37˚C water bath until papain is dissolved. Add 2 mg L-cysteine and mix. Add ~5 µl 1M NaOH to bring pH to 7.4. Filter through a 0.22-µm filter to ensure sterility. Add 100 µl of 0.4% DNase stock.
3) Remove excess D-PBS from retinae with a Pasteur pipet. Transfer retinas to 10 ml round bottom Falcon tube by pouring about half of the papain solution into the retinae and then pouring the retinae into the target vessel. Repeat until all retinae have been transferred then add remaining papain solution to Falcon tube. Incubate tube in a 34˚C water bath for 30 minutes, agitating gently after 15 min.
4) Finish preparing low ovomucoid solution. Add 100 µl 0.4% DNase stock. Divide into 6 ml and 4 ml aliquots. To the 6 ml portion, add 80 µl rabbit α-macrophage antisera.
5) Dissociation of retinae: At end of digestion, aspirate papain solution from retinae. Wash tissue by adding 4 ml low ovomucoid (w/o antibody), allowing cells settle, and aspirate again. Add 1.5 ml low ovomucoid plus antimacrophage serum. Triturate very gently with P1000 Pipetman: on the first pass, suck up all the retinas and then slowly release them. Repeat. Allow remaining chunks to settle, and then transfer 1 ml of the supernatant to a separate 15 ml tube. Add another 1 ml of low ovomucoid plus antimacrophage serum and triturate again. Repeat this process until all cells are in suspension and have been transferred to the new tube. Trituration can be more vigorous with each cycle.
6) Incubate 10 min at room temperature to allow antibodies to bind macrophages. Spin cells at 200 x *g* (1000 rpm) in a clinical centrifuge at 25˚C for 11 min. Aspirate off supernatant and resuspend pellet in 1-2 mls of high ovomucoid solution. Add remaining high ovomucoid, mix gently, and spin again.
7) Negative selection against macrophages: Aspirate high ovomucoid off of cell pellet and resuspend in 1 ml panning buffer using P1000 Pipetman. Add 9 mls panning buffer to bring volume up to 10 ml. Filter the cell suspension through an autoclaved 20-µm nylon mesh. Form a cone with the filter on top of a 15-ml tube. Prewet the filter by adding 1 ml of panning buffer, and transfer the cell suspension through the filter. Rinse tube that was holding cells with 4 mls panning buffer and filter than through mesh as well. Remove DPBS/BSA from first antimacrophage dish. Add cell suspension to dish. Locate on flat surface at room temperature (but not in the hood). Incubate 20 min, shaking vigorously at the halfway point. At the end of this incubation, the plate should be nearly covered with stuck cells. Remove D-PBS/BSA from second antimacrophage

dish. Shake first dish vigorously on flat surface. Pour cells from the first dish to the 2nd antimacrophage dish, and then tilt plate up to drain. Use a P1000 to transfer the last bit. Incubate second plate 30 min., agitating every 10-15 min.

8) Positive selection for RGCs: Wash the small T11D7 plate 3X with D-PBS. Agitate the 2nd antimacrophage dish, and transfer the cell suspension this dish. Keep on a flat surface for 45 min, shaking gently at halfway point. At this point you should be able to see the large round RGCs sticking to the panning plate. Most of the cells from the suspension will still be floating around.
9) During the final 5 minutes of $Thy1^+$ panning, prepare the trypsin solution. To 4 ml pre-equilibrated EBSS add 100 µl 2.5% trypsin (from -80°C; Sigma T4665).
10) Release of cells from panning dish: Discard cell suspension from T11D7 dish. Wash 8X with D-PBS to remove all nonadherant cells. Check visually under the microscope that all nonadherant cells have been removed. Add 6 ml of pre-equilibrated EBSS to the dish; quickly swirl and discard. Add 4 ml Trypsin solution; incubate 4 min. in 37˚C incubator. Add 1 ml of 30% FCS to a 15 ml tube labeled "RGCs" to which the released cells will be transferred. When incubation is over, add 2 ml of 30% FCS buffer to cells, and then, using a P1000, squirt cells off the dish, pipetting toward the center of the dish, moving around the dish one time. Then squirt once around the dish pipetting along the edge. Transfer cells to the 15 ml tube containing 1 ml FCS buffer. Add another 5 ml 30% FCS to the plate. Pipette up and down as before, toward center and then around the edge. Check under the microscope that most of the cells have been dislodged from the plate; pay close attention to the edges of the plate. When all of the cells are dislodged, add these cells to the others. Rinse plate with remaining 30% FCS and add it to the others. Spin cells at 200 x *g* (1000 rpm) in a clinical centrifuge at 25˚C for 11 min.
11) Plating: Resuspend the purified RGCs in 2 ml ND-G. Divide the RGCs (typically ~2.4 million neurons) amongst 4 wells of an 8-well chamber-slide. Place in a 37˚C incubator (10% CO_2) for two days.

(ii) Plating of reaggregates

Required Materials and Equipment:

1) 24-well tissue culture plate
2) sterile distilled water
3) laminin stock
4) PDL stock

To prepare:

1) 8 ml ND-G medium: Add 8 µl each of BDNF, CNTF, and forskolin stocks to 8 ml ND-Sato (see above). Equilibrate in incubator to 37˚C / 10% CO_2.
2) Coverslips: Wash 6-mm glass coverslips in at least 3 washes of 70% ethanol over 7 days on a rotating platform. Store in 70% ethanol.

Procedure:

1) The day after the purification of the RGCs, gently resuspend the RGCs using a P200 Pipetman, gently pipeting the medium in the well up-and-down 8-12 times per well to redestribute the RGCs and promote reaggregation.

2) Preparation of coverslips: The day after the purification of the RGCs, rinse 24-32 glass coverslips four times with ~10 ml of sterile distilled water. Distribute the coverslips individually around a 150-cm Petri dish and aspirate any remaining water droplets. Dilute a 100 µl aliquot of PDL into 10 ml sterile distilled water. Add 100 µl of this diluted PDL directly to the surface of each coverslip. The surface tension will keep this large drop from spreading from the coverslip. Incubate at room temperature for 30 min. Rinse four times with sterile distilled water. Transfer the coverslips to the wells of 24 well culture plates. Aspirate the excess water form each well, using the tip of the pipet to dry the coverslip to the center each well. The following day, dilute a 10 µl aliquot of Cultrex laminin into 5 ml Neurobasal medium. Gently add 50 µl of this diluted laminin to each coverslip, allowing surface tension to keep this drop from expanding off the coverslip to the rest of the well. Gently transfer the plate(s) to a 37˚C incubator (10% CO_2) and incubate for 4-6 hours.
3) Collect and wash reaggregates: Two days after purification, equilibrate 6 ml of ND-G medium to 37˚C / 10% CO_2. Gently resuspend RGC reaggregates in each well with a P200 and transfer the entire volume, including reaggregates, to a 1.5-ml Eppendorf tube. Allow the reaggregates to settle to the bottom of the tube (approx. 30-100 seconds) and remove the supernatant containing debris and unaggregated RGCs. Wash the reaggregates by resuspending in 500 µl ND-G, allowing them to settle again, and removing supernatant. Repeat the washes 5-6 times until no further debris can be seen in the supernatant under the microscope.
4) While waiting for the reaggregates to settle during the washes, prepare the coverslips for plating by replacing the laminin solution with ND-G. Using a Pasteur pipet hooked to a vacuum line, gently aspirate the Neurobasal / laminin from each coverslip and immediately replace it with 30 µl ND-G. It is important not to directly contact the coverslips with the tip of the pipet. Return the plate to the incubator.
5) Plate reaggregates: After the final wash, resuspend RGC reaggregates in 600 µl ND-G (25 µl for each coverslip). The goal is to evenly distribute the reaggregates across the coverslips and across the surface of each coverslip. This requires gentle resuspension before plating of on each coverslip, as the reaggregates tend to rapidly settle. Resuspend the RGCs with a P20 Pipetman and add 20 µl containing reaggregates to each coverslip. Quickly distribute the 20 µl onto three spots near the edge of the coverslip. This approach helps to promote wide distribution and prevent collection of all of the reaggregates in the center of the coverslip.
6) Gently return the plate to the incubator. Excessive motion at this stage can result in spread of the liquid off of the coverslips or swirling of the reaggregates toward the centers of the coverslips, distrupting the even distribution.
7) The day after pre-plating, gently add 450 µl of ND-G to each well.
8) Maintain the cultures for 8-12 days, feeding every third day by removing 200 µl and adding 250 µl fresh equilibrated medium.

II. Purification and transfection of optic nerve OPCs

(iii) Isolation of optic nerve cells

Required Materials and Equipment:

1) 3 litters (30 animals) of P7 Sprague-Dawley rat pups
    a. Note: Pups of nearly any postnatal age will work, but P6-P8 gives the best yield.
2) dissection tools: sharp scissors, fine scissors, forceps
3) Dulbecco's phosphate-buffered saline (D-PBS)
4) Papain (Worthington/Cooper, Cat. No. LS003126)
5) L-cysteine (Sigma)
6) 2.5 ml syringe and #21 and #23 gauge needles
7) nylon mesh [Nitex mesh (HC3-20) can be obtained from Tetko Inc., 420 Saw Mill River Road, Elmsford, New York 10253, 914-592-5010]

To prepare:

1) D-PBS/0.2%BSA: Mix 1 ml 4% BSA and 19 ml D-PBS
2) Panning buffer: Mix 2 ml D-PBS/0.2% BSA with 18 ml D-PBS and add 200 µl insulin stock. Equilibrate to room temperature.
3) Ovomucoid solutions: Mix 1 ml low ovomucoid stock with 9 ml D-PBS and add 100 µl DNase stock. Mix 1.2 ml high ovomucoid stock with 5 ml D-PBS.

Procedure:

1) Dissect optic nerves and optic chiasm from P7 postnatal rats. Simply remove the head and skull, lift the brain out, and the optic nerves will be lying on the base of the skull. Cut the nerves behind the chiasm and also behind each eye. Grasp the chiasm with forceps and transfer the pair of nerves into a 35-mm Petri dish containing 2 ml of D-PBS.
2) Cut each nerve with a fine scissors into as many pieces as possible (at least 5 to 10 per nerve). Transfer the D-PBS and nerve pieces (excluding chiasm pieces) to a small tube.
3) Prepare a papain solution by adding 165 units of papain to 5 ml of D-PBS containing 100 µl DNase (0.004%). Dissolve the papain by placing this solution in a 37˚C water bath for a few minutes. Just before use, add L-cysteine (2 mg) to activate the papain solution, adjust the pH to 7.4 with 1M NaOH, and filter through an 0.22-µm filter.
4) Remove the D-PBS from the vial containing the tissue with a Pasteur pipet and add the papain solution. Incubate the tissue, with occasional agitation, for 75 minutes in a 37˚C water bath.
5) Remove the papain solution with a Pasteur pipet. Rinse excess papain by adding 3 ml of the low ovomucoid solution, let the tissue pieces settle, and remove the ovomucoid.
6) Serially triturate the nerve pieces in the ovomucoid inhibitor solution, 1 ml at a time, with a 2.5 ml syringe and #21 gauge needle (3 x 5 passes), followed by a #23 gauge needle (3 x 5 passes). That is, add 1 ml, pass the pieces through the needle one or two times, let the pieces settle, collect the suspension above the pieces with a Pasteur pipet into a 15 ml Falcon centrifuge tube. Add another 1 ml of inhibitor solution, triturate again, and so on. In this way, cells successfully removed from the nerve pieces are not repetitively passed through the needle.
7) Filter the cell suspension through an autoclaved 20-µm nylon mesh. Form a cone with the filter on top of a 15-ml tube. Prewet the filter by adding 0.5 ml of low ovomucoid,

and transfer the cell suspension through the filter. Add the remaining volume of low ovomucoid (~0.5 ml).

8) Spin down the dissociated cells at 200 x *g* (1000 rpm) in a clinical centrifuge for 13 min at 25˚C. Resuspend cells in 1 ml high ovomucoid inhibitor solution. Mix in remaining 5 ml high ovomucoid, and spin again for 13 min.
9) If transfection is required, proceed to the next section, "Nucleofection of optic nerve cells." If no transfection is needed, resuspend the optic nerve cells in 8.0 ml panning buffer, transfer the entire volume to a Ran-2 panning dish (see below), and proceed to section (v).

(iv) Nucleofection of optic nerve cells

Required Materials and Equipment:

1) Trypsin-EDTA (Gibco)
2) Clinical Centrifuge
3) Nucleofector I device (amaxa)
4) Rat oligodendrocyte Nucleofector kit (amaxa)

To prepare:

1) 5 ml ND-G media: Add 5 µl each of BDNF, CNTF, and forskolin stocks to 5 ml ND-Sato (see above). Equilibrate in incubator to 37˚C / 10% CO_2.
2) PDL-coated 10-cm dish: Dilute 100 µl PDL stock into 9.9 ml sterile distilled water. Incubate a tissue culture dish with 5 ml PDL solution for 30 min at room temperature. Rinse 3x with PBS.
3) 20% FCS: Mix 2 ml fetal calf serum with 8 ml D-PBS. Filter through 0.22-µm filter. Equilibrate to room temperature.
4) Panning buffer: Mix 2 ml D-PBS/0.2% BSA with 18 ml D-PBS and add 200 µl insulin stock. Equilibrate to room temperature.
5) Equilibrate 10 ml EBSS in incubator.

Procedure:

1) Resuspend the optic nerve cells in 500 µl ND-G medium. Seed all the cells on a PDL-coated 10-cm tissue culture dish containing 5 ml ND-G pre-incubated to 37˚C and 10% CO_2.
2) Recovery: Allow cells to recover 90 min in 37˚C incubator.
3) Release cells from the dish: Remove medium, rinse once with 6 ml pre-equilibrated EBSS, and incubate at 37˚C with 4 ml Trypsin-EDTA (1:10 in EBSS) for 8 min. Add 2 ml of 20% FCS, and use a P1000 Pipetman to gently squirt around the surface of the dish to release the cells. Collect the supernatant in a 15-ml Falcon centrifuge tube containing 1 ml 20% FCS. Add another 4 ml 20% FCS and repeat. Add a final 3 ml 20% FCS, and squirt around the surface of the dish the plate thoroughly to release any remaining cells.
4) Spin the cells at 200x*g* (1,000 rpm) for 11 min in the centrifuge.
5) Resuspend the cells in nucleofector solution (105 µl for each transfection). At least one litter of P7 rats is needed to provide enough cells for one transfection.
6) Nucleofection (for each of the desired transfections): Quickly transfer 100 µl of the cell suspension to a tube containing 1.5 µg plasmid or siRNA and mix gently. Transfer this volume to the provided cuvette, and electroporate the cells using program O-17. Add 500

µl ND-G, and use the provided plastic pipette to transfer the entire volume to a Ran-2 panning dish (see below) containing 8 ml panning buffer.

(v) Purification of optic nerve OPCs

Required Materials and Equipment:

1) Three 10-cm Petri dishes
2) A2B5 hybridoma supernatant or ascites
3) Ran-2 hybridoma supernatant
4) Galactocerebroside (GC) hybridoma supernatant
5) 500 ml bottle of Dulbecco's phosphate buffered saline (D-PBS)
6) Earle's balanced salt solution (EBSS)
7) Clinical centrifuge

To prepare:

1) MyM base medium: Measure 39 ml of DMEM-High Glucose medium. Add 400 µl each of insulin stock, 100X sodium pyruvate (Invitrogen), and 100X L-glutamine (Invitrogen). Add 200 µl each of 200X Hormone mix, and 40 µg/ml T3. Add 40 µl each of 50 µM hydrocrtisone (Sigma H6909), and 1000X Trace Elements B (Cellgro). Add 8 µl each of 5000X biotin stock and 5000X Vitamin B12 stock. Add 15 µl B27 Supplement (Invitrogen) and 4 µl ceruloplasmin stock. Filter solution through a 0.22-µm filter. Store 4˚C; good for up to one week.
2) Prepare D-PBS/0.2%BSA solution by mixing 19 ml of D-PBS with 1 ml 4% BSA stock.
3) Panning dishes: The day before OPC purification, incubate two Petri (100 mm, Falcon) dishes overnight at 4˚C in 10 ml of pH 9.5 Tris (50 mM) containing affinity-purified goat anti-mouse IgG (H+L) at 10 µg/ml and one Petri dish in affinity-purified goat anti-mouse IgM, u chain specific, also at 10 µg/ml. Wash each Petri dish with three washes of PBS. Add the primary antibody in an PBS/BSA (0.2%) solution for at least one hour at room temperature. Dish 1: RAN-2 supernatant 1 ml plus D-PBS/BSA 4 ml. Dish 2: GC supernatant 1 ml plus D-PBS/BSA 4 ml. Dish 3: A2B5 ascites 1:750 in D-PBS/BSA. (Note that A2B5 supernatant can also be used, at about 1:20). Immediately prior to adding cell suspension to each dish, rinse with 3 washes of PBS.
4) 2.5% Trypsin: Dissolve trypsin (Sigma T9935) at a concentration of 30,000 U/ml in EBSS. Filter through 0.22 µm filter. Make 200 µl aliquots and store at -80˚C.
5) Equilibrate 10 ml EBSS in incubator during panning.
6) 30% FCS: Mix 4.5 ml fetal calf serum with 10.5 ml D-PBS. Filter to sterilize. Equilibrate to room temperature.
7) 5 mM DAPT: Dilute 25 mM InSolution γ-Secretase Inhibitor IX (Calbiochem Cat. No. 565784) 1:5 with tissue-culture grade sterile DMSO (Sigma).
8) Medium (ND-G or MyM):
    a. Note: Differentiation and ensheathment occur well in either ND-G or MyM. MyM supports more reliable and robust wrapping and compaction over a short time course. MyM, however, does not support initial survival and recovery of acutely isolated cells as well as ND-G. Replacing ND-G (for purified RGC axon outgrowth) with MyM (for myelinating cocultures) is best achieved by beginning the switch with a ½ volume medium change in the final 1 or 2 RGC feedings prior

to OPC isolation. The plating of OPCs as described here further reduces ND-G, and subsequent ½ volume feedings are sufficient to complete the changeover.

i. 15 ml MyM: Dilute CNTF stock 1:100 by mixing 1 µl CNTF with 99 µl MyM. Add 15 µl each of BDNF and diluted CNTF, to 15 ml MyM stock (see above). Final CNTF concentration = 0.1 ng/ml. Equilibrate in incubator to 37˚C / 10% CO_2.

Procedure:

1) Negative selection against astrocytes: Place the cell suspension on the Ran-2 antibody-coated Petri dish for 30 min at room temperature (agitate gently at 15 minutes to ensure access of all cells to the panning surface area).
2) Negative selection against oligodendrocytes: Transfer the non-adherent cells to the GC antibody-coated Petri dish for 20 to 30 minutes at room temperature (agitate at 10-15 minutes). This step depletes the suspension of virtually all oligodendrocytes.
3) Positive selection for OPCs: Transfer the non-adherent cells to the A2B5 antibody-coated Petri dish. Incubate the cells on the A2B5 dish for 30 minutes at room temperature. Remove the nonadherent cells, and wash the dish with D-PBS (about 10 ml per wash) about 5 times (follow progress under microscope), being careful not to let the adherent cells dry out. Several of these washes should be followed by gentle agitation of the dish.
4) Release cells from the dish: Add 200 ul (one aliquot) of 2.5% trypsin (stored in a -80º C freezer) to 4 ml of pre-equilibrated EBSS. Remove the D-PBS from the A2B5 dish and rinse once with 6 ml pre-equilibrated EBSS. Remove the EBSS wash and add the 4 ml of trypsin/EBSS to the cells. Place in the incubator for exactly 10 minutes. Add 2 ml of 30% FCS, and use a P1000 Pipetman to gently squirt around the surface of the dish to release the cells. Collect the supernatant in a 15-ml Falcon centrifuge tube containing 1 ml 30% FCS. Add another 4 ml 30% FCS and repeat. Add a final 3 ml 30% FCS, and squirt around the surface of the dish the plate thoroughly to release any remaining cells.
5) Spin the purified OPCs at 200x*g* (1,000 rpm) for 13 min in the centrifuge.
6) Plating of purified OPCs on RGC cultures: Aspirate the supernatant carefully but completely. Resuspend the cells in ND-G or MyM (20,000 OPCs in 0.5 ml for each well). For each well of RGC reaggregates, remove 300 µl and add 500 µl of OPCs in fresh ND-G or MyM. When appropriate, add the γ-secretase inhibitor DAPT to a final concentration of 1 µM. Cocultures may be maintained for up to six days with no further manipulation. For longer culture periods, one-half volume of medium (with fresh DAPT, if needed) may be changed every three days.

III. Analysis of glial cell fate and morphology

(vi) Immunostaining of cocultures

Required Materials and Equipment:

1) Forceps for handling coverslips
2) Phosphate-buffered saline (PBS)
3) Normal goat serum
4) 1˚ antibodies: rat α-MBP (Abcam), rabbit α-NG2 (Chemicon), and mouse α-GFAP (Sigma)
5) 2˚ antibodies (Molecular Probes): Alexa 488-conjugated goat anti-rat Ab, Alexa 594-conjugated goat highly cross-adsorbed anti-rabbit Ab , and Alexa 680-conjugated highly cross-adsorbed goat anti-mouse Ab
6) Vectashield with DAPI mounting medium

To prepare:

1) 20% Triton X-100 stock solution: Mix 10 ml Triton X-100 with 40 ml water.
2) 4% PFA: Dilute 16% PFA to 4% with phosphate buffer (93.3 mM Na_2HPO_4 and 40 mM NaH_2PO_4)
3) Ab buffer: 150 mM NaCl, 50 mM Tris base, 1% bovine serum albumin (Sigma A2153), 100 mM L-lysine, 0.04% azide, adjusted to pH 7.4 with 1 M HCl. Store @ 4˚C indefinitely.

Procedure:

1) Remove 24 well plate containing cocultures from the incubator. Gently aspirate the medium from each well and add 400 µl 4% paraformaldehyde. Incubate at room temperature for 10 min.
2) Gently remove paraformaldehyde and dispose of properly. Add 1 ml phosphate-buffered saline (PBS).
3) Gently but completely aspirate PBS. Allow the cocultures to air-dry for 30 min. This drying step is important for preventing the shifting and peeling of reaggregates and their axons from the coverslip during subsequent steps. Even with this precaution, it is important to take care with each manipulation and avoid changing and adding solutions to the cocultures with excessive force.
4) Gently rinse coverslips once with PBS.
5) Blocking: Add 50% normal goat serum with 0.4% Triton X-100 in Ab buffer. Incubate 30 min at room temperature.
6) 1˚ Ab: Prepare a 1˚ Ab staining solution containing 10% normal goat serum and 0.08% Triton X-100 in Ab buffer. Dilute 1˚ antibodies into this solution: rat α-MBP (1:200), rabbit α-NG2 (1:500), and mouse α-GFAP (1:2000). Spin this solution at 13,000 rpm for 10 min in a tabletop centrifuge to remove any precipitated antibodies. Remove blocking solution and add the 1˚ Ab. Incubate at room tempertaure for 90 min or at 4˚C overnight.
7) Gently rinse three times with PBS.
8) 2˚ Ab: Prepare a 2˚ Ab staining solution by diluting Alexa 488-conjugated goat anti-rat 2˚ Ab (1:1000), Alexa 594-conjugated goat anti-rabbit 2˚ Ab (1:1000), and Alexa 680-conjugated goat anti-mouse 2˚ Ab (1:1000), in Ab buffer. Spin this solution at 13,000

rpm for 10 min in a tabletop centrifuge to remove any precipitated antibodies. Incubate coverslips with this solution for 45 min at room temperature.

9) Gently rinse three times with PBS.
10) Mounting: For each coverslip, place a small drop of Vectashield with DAPI on a standard microscope slide. Invert the rinsed coverslip onto the drop of Vectashield, gently dab away excess Vectashield with a Kimwipe, and seal the coverslip to the slide with nail varnish around the edge.

(vii) Quantification

Required Materials and Equipment:

1) Nikon Eclipse E800 Epifluorescence Microscope (or equivalent) with a 20x oil objective
2) Spot CCD camera (or equivalent)
3) Spot Advanced Image Acquisition software (or equivalent)
4) Adobe Photoshop 7.0 (or equivalent)

Procedure:

1) Note: For unbiased quantification, make sure that the person acquiring the images is blinded to the condition of each coverslip.
2) For each coverslip, select 10 independent fields using the 20x objective. Each non-overlapping field of view is chosen in the blue (DAPI) channel, prior to observing the other channels, to include nuclei adjacent to or between RGC reaggregrates (easily identified by the tight collections of tens to hundreds of DAPI-labeled nuclei). This approach allows for bias toward fields that are likely to be dense in RGC axons, with no other bias for particular cell fates or morphologies, preventing the inclusion of irrelevant fields in which axons are not present to influence cell fate or allow for myelination.
3) For each field, use Spot software to acquire four-color images using defined exposure times for each channel. Begin by focusing the field in the green (MBP) channel, followed by images in the red (NG2), blue (DAPI), and infrared (GFAP) channels.
4) Merge the images of each field into a common Photoshop file, providing a layer for each channel so that it is easy to rapidly switch amongst the different channels during quantification.
5) To further blind the analysis, have an assistant randomize and encode the image identities prior to quantification.
6) For each image, quantify the number of non-RGC cells (*i.e.*, DAPI-labeled nuclei) that express each of the three markers (NG2, GFAP, and MBP), including a distinction as to whether each MBP-expressing cell is myelinating. A myelinating oligodendrocyte is one that is extending at least two clear, smooth tubes of MBP^+ membrane ensheathing axons.
7) For each coverslip, calculate the total number of cells and the total numbers of cells in each category summed across the ten fields for that coverslip. At least three independent coverslips is analyzed per condition. Data may be expressed as the percentage of cells with each cell fate or the percentage of total oligodendrocytes that have formed myelin.

IV. Modifications to immunopanning for various cells types

Immunopanning is conceptually and technically similar across a range of cell types. The detailed protocol described above for rat RGCs and rat optic nerve OPCs can be adapted for a variety of cell types by simply modifying the antibodies used for positive and negative selection (being certain to use the species-appropriate secondary antibodies for initial coating of the Petri dishes). Times for trypsinization from the final dish must be determined empirically for each batch of trypsin and Ab, but range from 3 min for protein antigens (*e.g.*, PDGFRα) to 10 min for O4. Below are the modifications necessary for isolating neurons and glia from various sources:

(i) Mouse RGCs

Source: 3-6 litters of P5 mice (C57Bl6)

(Papain incubation time is 45 min for mouse RGCs, instead of 30 min for rat RGCs)

Panning dishes (one set for every 2 litters):

1) Negative selection: 2x 15-cm dishes: 20 µl aliquot of **BSL I** (Vector Labs L-1100 Bandeiraea simplicifolia lectin I; frozen stock: 5 mg in 2.5 ml D-PBS) in 20 ml D-PBS.
    a. Note: No 2˚ Ab is needed for lectins, but dishes should be blocked for 1 hr with D-PBS / 0.2% BSA after BSL I coating.
2) Positive selection: 1x 10-cm dish: 10 µl aliquot of **Thy1.2** (Mouse α-mouse CD90 Serotec MCA02R; 1 mg/ml) in 10 ml D-PBS / 0.2% BSA
    a. Note: 1˚ Ab contains azide, so rinse with PBS thoroughly before adding cells.

(ii) Mouse optic nerve OPCs

Source: 3-6 litters of P5 mice (C57Bl6)

Panning dishes (one set for 3-6 litters):

1) Negative selection: 1 x 10-cm dish: 10 µl aliquot of **BSL I** (Vector Labs L-1100 Bandeiraea simplicifolia lectin I; frozen stock: 5 mg in 2.5 ml D-PBS) in 10 ml D-PBS.
    a. Note: No 2˚ Ab is needed for lectins, but dishes should be blocked for 1 hr with D-PBS / 0.2% BSA after BSL I coating.
2) Positive selection: 1x 10-cm dish: 20 µl rat α-mouse **PDGFRα** (BD Pharmingen 558774 rat α-mouse CD140A, 0.5 mg/ml) in 5 ml D-PBS / 0.2% BSA

(iii) Rat optic nerve astrocytes (Mi and Barres, 1999)

Source: 3-6 litters of P2-P8 Sprague-Dawley rats

(Papain incubation time 50 min for P2 up to 75 min for P8)

Panning dishes (one set for 3-6 litters):

1) Negative selection: 1x 10-cm dish: 1 ml **OX7** α-Thy1 hybridoma supernatant + 4 ml D-PBS / 0.2% BSA
2) Negative selection: 1x 10-cm dish: 10 µl **A2B5** ascites + 5 ml D-PBS / 0.2% BSA
3) Positive selection: 1x 10-cm dish: 2 ml **C5** hybridoma supernatant + 4 ml D-PBS / 0.2% BSA

(iv) Rat cortical OPCs (Dugas *et al*., 2006)
Source: One or two P5-P9 Sprague-Dawley rat pups
Panning dishes (one set for 1-2 pups):

1) Negative selection: 1x 15-cm dish: 4 ml **Ran-2** hybridoma supernatant + 8 ml D-PBS / 0.2% BSA
2) Negative selection: 1x 15-cm dish: 4 ml **GC** (Ranscht *et al.*, 1982) hybridoma supernatant + 8 ml D-PBS / 0.2% BSA
3) Positive selection: 1x 10-cm dish: 1 ml **O4** hybridoma supernatant + 4 ml D-PBS / 0.2% BSA

(v) Mouse cortical OPCs (Wang *et al.*, 2001)
Source: 1-4 P5-P9 mice (C57Bl6)
Panning dishes (one set for 2-3 pups):

1) Negative selection: 1x 15-cm dish: 20 µl aliquot of **Thy1.2** (Mouse α-mouse CD90 Serotec MCA02R; 1 mg/ml) in 10 ml D-PBS / 0.2% BSA
    a. Note: 1˚ Ab contains azide, so rinse with PBS thoroughly before adding cells.
2) Negative selection: 1x 15-cm dish: 4 ml **GC** (Ranscht *et al.*, 1982) hybridoma supernatant + 8 ml D-PBS / 0.2% BSA
3) Positive selection: 1x 10-cm dish: 1 ml **O4** hybridoma supernatant + 4 ml D-PBS / 0.2% BSA

(vi) Rat optic nerve OLs
Source: 3 litters of P13 Sprague-Dawley rats
Panning dishes (one set):

1) Negative selection: 1x 10-cm dish: 1 ml **Ran-2** hybridoma supernatant + 4 ml D-PBS / 0.2% BSA
2) Negative selection: 1x 10-cm dish: 10 µl **A2B5** ascites + 5 ml D-PBS / 0.2% BSA
3) Positive selection for mature OL: 1x 10-cm dish: 4 ml **MOG** (clone 8-18C5) in 1 ml D-PBS / 0.2% BSA
4) Positive selection for immature OL: 1x 10-cm dish: 2 ml **GC** (Ranscht *et al.*, 1982) hybridoma supernatant + 3 ml D-PBS / 0.2% BSA

(vii) Adult optic nerve OPCs (Shi *et al.*, 1998)
Source: Ten P30 Sprague-Dawley rats
Panning dishes (one set):

1) Negative selection: 1x 10-cm dish: 1 ml **Ran-2** hybridoma supernatant + 4 ml D-PBS / 0.2% BSA
2) Positive selection: 1x 10-cm dish: 5 µl **A2B5** ascites + 5 ml D-PBS / 0.2% BSA

(viii) Mouse cortical OLs (Cahoy *et al.*, 2007)
Source: Five P13 mice (C57Bl6)
Panning dishes (two sets):

1) Negative selection: 2x 15-cm dishes: 20 µl aliquot of **BSL I** (Vector Labs L-1100 Bandeiraea simplicifolia lectin I; frozen stock: 5 mg in 2.5 ml D-PBS) in 20 ml D-PBS.
    a. Note: No 2˚ Ab is needed for lectins, but dishes should be blocked for 1 hr with D-PBS / 0.2% BSA after BSL I coating.
2) Negative selection: 1x 15-cm dish: 20 µl rat α-mouse **PDGFRα** (BD Pharmingen 558774 rat α-mouse CD140A, 0.5 mg/ml) in 12 ml D-PBS / 0.2% BSA
3) Negative selection: 1x 10-cm dish: 6 µl **A2B5** ascites + 12 ml D-PBS / 0.2% BSA
4) Positive selection for mature OL: 1x 15-cm dish: 10 ml **MOG** (clone 8-18C5) in 2 ml D-PBS / 0.2% BSA
5) Negative selection: 1x 15-cm dish: 10 ml **MOG** (clone 8-18C5) in 2 ml D-PBS / 0.2% BSA
6) Positive selection for immature OL: 1x 15-cm dish: 4 ml **GC** (Ranscht *et al.*, 1982) hybridoma supernatant + 8 ml D-PBS / 0.2% BSA