

ACTIVITY-DEPENDENT INTERACTIONS BETWEEN NEURONS AND MYELINATING GLIA: AN IMMUNOCYTOCHEMICAL APPROACH

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Abstract

The current investigation examines the relationship between the purinergic signaling associated with impulse activity of developing axons and the role of those molecules in influencing the developmental stages and physiological responses of cultured oligodendrocytes. It was hypothesized that oligodendrocytes would demonstrate evidence for purinergic receptors under immunocytochemical analysis of the cell type across varying stages of development. Immunocytochemistry depicted the progressive stages of oligodendrocyte development through the OP, O4, O1, and MBP stages. A novel adenosine receptor immunocytochemical protocol was successfully developed to facilitate delineation of the developmental stages and cellular characteristics of myelinating glia that express the active purinergic receptors.

Introduction

The nervous system is composed of two separate types of cells, neurons and glial cells. In the vertebrate central nervous system, glial cells are known to significantly outnumber neurons in density **(1)**. Glia play a diverse variety of roles in both the function and development of the central and peripheral nervous systems **(1,2,3)**. These actions include but are not limited to glial function as support scaffolding for developing neurons and their synapses, influence on migration of neurons and outgrowth of axons, regulation of chemical composition and nourishment available to neurons, glial phagocytic removal of cellular debris following trauma and neuronal atrophy, integration of neuronal signaling and synaptic activity, and their role in insulating nerve axons through myelination to facilitate efficient signal conduction between neurons. Oligodendrocytes are the myelinating glia of the central nervous system and can insulate multiple axons in what is characterized as white matter. Schwann cells are the myelinating glia of the peripheral nervous system and are positioned at single periphery sensory axons to form compact myelination. The most abundant of the glial subtypes are astrocytes, which take action at the cell surfaces of neurons in the brain and spinal cord and are themselves non-myelinating glia. Glial cells facilitate developmental neuron progression and physiological neuron function. Various levels of specialized signaling molecules from neurons including neurotransmitters, ions, and cell adhesion molecules have been identified to influence glial action **(2)**. The purinergic molecules ATP and adenosine are broadly applicable signaling molecules throughout the mammalian body and have been

characterized to be both non-synaptically released from axons and subsequently available as a result of activity-dependent action potential firing (1,4,5).

The objective of this study was to examine how fetal and early post-natal neural impulse activity in developing sensory axons and their related signaling molecules influence the development of myelinating glial cells. The overall emphasis of this project encompasses activity-dependent signaling between neurons and glial cells in the central nervous system. Extracellular ATP and its breakdown product adenosine, have been recently identified as potent activity-dependent signals between neurons and developing oligodendrocytes (4). It was shown that adenosine, but not ATP, inhibited OP proliferation and significantly promoted oligodendrocyte differentiation and myelination. It was suggested that the electrical stimulation of DRG neurons and the subsequent availability of adenosine might serve as a means to activate oligodendrocyte adenosine receptors to promote myelination. RT-PCR revealed that OPCs indeed express mRNA for all four subtypes of adenosine receptors, yet it remains unknown 1.) when these subtypes are functional and 2.) how these receptors are regulated during oligodendrocyte development. In order to fundamentally address these questions immunocytochemical approaches were taken to characterize the purinergic receptors expressed at different stages of the oligodendrocyte lineage. Additionally, ATP receptors have been previously characterized for both oligodendrocytes of the CNS and Schwann cells of the PNS, and Schwann cells have been shown to arrest in development prior to myelin production in response to ATP (4,5,6).

Immunocytochemistry was used to characterize the different stages of oligodendrocyte development *in vitro*. A novel immunocytochemical protocol was developed to visually characterize the presence of adenosine receptors on glial cells. Immunocytochemical application successfully allowed for the *in vitro* determination of the developmental stages of oligodendrocytes and the successful protocol, using selective adenosine receptor antibodies (Chemicon), was developed using astrocytes as a positive control since they are known to express all four subtypes of adenosine receptors (7,8,9,10). Utilization of the novel adenosine immunocytochemical protocol displayed the presence of A1, A2a, A2b, and A3 adenosine receptors on oligodendrocytes, characterized here for the first time by means of *in vitro* immunocytochemistry. Future studies will determine how the expression of these receptors changes with development and at what stages specific receptor subtypes are functional and able to regulate oligodendrocyte proliferation, differentiation, and myelination.

Method

Oligodendrocyte Progenitor Dissections

Pregnant rats were sacrificed and embryos were surgically removed from the amniotic sac with sterile tools and technique. Surgical embryo isolation was carried out following approved animal study protocol. The brain of each 18 to 20-day old embryo (E18-E20) was removed after the pre-formed skull tissue was systematically peeled back starting with an incision in the region of the brain stem moving anteriorly, in order to expose both hemispheres of the cortex. Fine-tipped tweezers were used to hold the front of the head during isolation of the cortex. The cortical hemispheres were removed from the midbrain and hindbrain and the meninges stripped from both hemispheres. The

dissection was performed using a dissecting microscope and D1 saline solution as the dissection buffer. Cortices were dissociated mechanically by passing the cells through varying needle gauges. Dissociating passes were performed twice through 19-gauge, twice through 22-gauge, and once through 25-gauge. Cells were passed through a cell strainer and centrifuged at 5000 rpm for 5 minutes. Re-suspension of cells occurred in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Cells were transferred to non-coated 75mm² T-flasks and incubated for 4 days at 37°C in a CO₂-water jacketed incubator before the media could be changed once every two days.

Cellular Plating & Culturing

Flasks were pre-shaken for a 3-hour interval at 37°C and 198 rpm. The medium was changed and the cells were incubated for at least 30 minutes prior to the final shake of the prepared cells, which proceeded overnight. The flask medium was collected in 15 mL conical tubes and centrifuged at 1600 rpm for 5 minutes. The medium was removed and the cells were re-suspended with 1 mL of fresh DMEM medium. OPCs were purified by distributing the supernatant to a 100 mm Nunclon polystyrene dish with 5 mL of DMEM 10% FBS and incubating for 45 minutes. OP cells, which remained in suspension, were collected in 5 mL of 10% FBS DMEM growth medium in a 50 mL conical tube.

Cells were counted and accordingly plated with a density of 200,000 cells/mL onto poly-L-ornithine (Sigma P3655) coated glass coverslips. After an incubation period of 1.5 hours at 37°C the cells were switched into N1+0.5% FBS (oligodendrocyte and astrocyte) differentiating supplemental medium. Oligodendrocyte progenitor cells with bipolar morphology (OPCs) were maintained through application of PDGF 10 ng/mL (Upstate Biotechnology) when introduced into the N1 medium. O4+ cells (O4s) were established by allowing their development to continue 2 days in the N1 medium preparation without PDGF in N1+0.5% FBS. Developmental oligodendrocyte progression through the O1+ stage yields MBP+ cell characterization, obtainable after 4+ days in culture.

Oligodendrocyte Immunocytochemistry

Oligodendrocyte immunocytochemistry was used to distinguish the OP (NG2+), O4 (O4+), and O1 (O1+) differentiated developmental stages of CNS myelinating glia. Prepared cell dishes were washed with DMEM once at room temperature to change over the medium in the dish. Oligodendrocyte progenitor (OP) cells were incubated with monoclonal rabbit IgG NG2 primary antibody with a dilution of 1:1000 in DMEM. Live staining of (O4) and (O1) cells was achieved through respective incubation in monoclonal mouse IgM O4 and O1 primary antibody, each with a dilution of 1:10 in DMEM. Incubation was carried out at room temperature for one hour followed by consecutively dipping the coverslips 3 times per each of 3 one-ounce cups of DMEM washes for a total of nine.

Coverslips were returned to their respective cleaned dishes and detection of surface antigens was achieved through using FITC-conjugated goat anti-rabbit IgG secondary antibody for NG2 cells and FITC-conjugated goat anti-mouse IgG secondary antibody for O4 and O1 cells (Jackson Laboratories), each with a 1:100 dilution in

1xPBS. Incubation lasted 30 minutes at room temperature and was followed by dip rinses 3 times per each of 3 one-ounce cups of DMEM. Note the dishes of cells were covered with aluminum foil during the incubation because of the light sensitive nature of the secondary antibody. The incubation was followed by a 1xPBS rinse and a three-drop acclimation to 4°C 4% paraformaldehyde. Fixing of the cultures with 4% paraformaldehyde occurred at room temperature for 15 minutes after removal of the prior rinse. Cells were rinsed with 1xPBS and counterstained with Hoechst nuclear stain (Molecular Probes) prepared as a 1:2000 dilution in 1xPBS. FITC fluorescence was viewed under 40x oil immersion microscopy following mounting of the coverslip.

Adenosine Receptor Immunocytochemistry

Adenosine receptor immunocytochemistry was carried out for both astrocytes and oligodendrocytes. Astrocytes were rinsed 3 times with 37°C HBSS and dislodged from their 75mm² T-flasks with a 1:25 dilution of trypsin made up in HBSS. Trypsin was deactivated with 10 mL 10% FBS DMEM and the cell pellet was obtained by centrifugation for 5 minutes at 1,100 rpm. Pellets were suspended in 4 mL of growth medium, their count was averaged from six hemocytometer fields, and the samples were brought up to a density of 200,000 cells/mL to be plated. All astrocyte preparations were grown in 35mm Nunclon polystyrene dishes with sterile grease circles to conserve limited quantities of primary antibody. Cells were grown in 10% FBS DMEM at 37°C for 4 hours to allow for cell adherence to the dish after plating. Media was filled 2 mL per dish followed by either 24 or 48-hour 37°C CO₂ water-jacketed incubation. DMEM was removed and replaced with a single wash of 1xPBS following the incubation.

Cells were fixed in PFA dilutions from 1%-4% for 20 minutes at room temperature and washed twice with 1xPBS. Permeabilization was carried out with 0.1% Triton-X detergent made up in 1xPBS. Incubation for the permeability trials lasted for a duration of 5 minutes at room temperature. Cells were washed twice with 1xPBS and blocked from non-specific staining using 3% Normal Goat Serum (NGS) made up in 1xPBS, for a duration of 30 minutes at room temperature. The block was lightly suctioned out following its incubation.

The primary antibodies for A1, A2a, A2b, and A3 adenosine surface receptors were separately administered to specifically designated dishes, while controls for each lacked primary antibody. Incubation occurred overnight at 4°C. The primary antibody was prepared in concentrations of 50 µg/mL (1:10) and 25 µg/mL (1:20) with 1% NGS. After incubation, the cells were washed 3 times for 5 minutes each with 1% NGS. The secondary antibody was a FITC goat anti-rabbit IgG with a dilution of 1:100 in 1xPBS (Jackson Laboratories). The covered light-sensitive incubation lasted 30 minutes at room temperature after which the cells were washed in 1xPBS 3 times for 5 minutes each. Coverslips were mounted and FITC green fluorescence was observed under 40x oil immersion magnification. Bright field magnification was used to verify cell presence on the plate after the repeated washings.

The preliminary oligodendrocyte staining was carried out on cells 5 days post-plating on coverslips in 35mm Nunclon polystyrene dishes. The 10% FBS DMEM growth medium was removed and followed by a gentle wash with 1xPBS. The cells were not permeabilized and were fixed with 2% PFA for 20 minutes at room temperature. Following fixation, the oligodendrocytes were blocked against non-specific staining with

3% NGS for 30 minutes at room temperature. Each adenosine receptor subtype was separately stained for with the controls lacking primary antibody. The adenosine receptor primary antibodies were prepared as a 1:10 dilution in 1% NGS and their incubation went overnight at 4°C. The secondary antibody was a FITC goat anti-rabbit IgG with a dilution of 1:100 in 1xPBS. Incubation was 30 minutes at room temperature and followed by 3 washes with 1xPBS for 5 minutes each. Coverslips were mounted and analyzed under 40x oil immersion immunofluorescence microscopy.

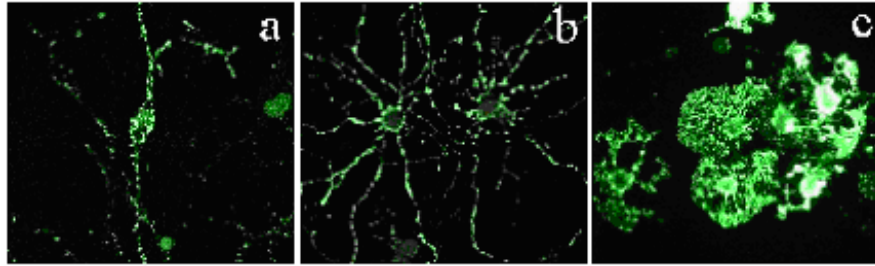
Results

Oligodendrocytes were immunocytochemically characterized to establish the stage of their cellular progression. Figure 1 illustrates the successful determination of OP, O4, O1, and supplemental MBP stages of oligodendrocyte development. The monoclonal surface antigen labeling of OP, O4+, O1+, and MBP+ glia determined the state of their antigen representation. Adenosine receptor characterization was examined using A1, A2a, A2b, and A3 primary antibodies marketed for immunohistochemical research (Chemicon). A protocol was adapted for immunocytochemical receptor labeling and successfully applied for both CNS astrocytes and oligodendrocytes as can be seen in Figures 2 and 3, respectively. The initial permeabilization of the astrocytes resulted in non-specific intra-nuclear staining compared to the non-permeabilized cells. The non-permeabilized astrocytes possessed an absence of nuclear staining. The adenosine receptors are surface receptors (**11**) and therefore permeabilization was discontinued for the succeeding immunocytochemical adenosine receptor classifications.

Adenosine receptor labeling in astrocytes was carried out at varying PFA fixing concentrations from 1%-4%. Morphological observation under 40x bright field microscopy confirmed the cellular identity of the astrocytes. Minor disruption of non-permeabilized nuclei was accredited to cellular lysis from inhospitable conditions during fixing or the repeated washes during antibody administration. Higher cell density yields were subsequently brought down to 150,000 cells/mL for the continued receptor analysis. The 1:10 adenosine receptor primary antibodies were much clearer overall under FITC immunofluorescence than the 1:20 dilutions. PFA fixes from 1%-4% were successful, although those fixes carried out at 2% were consistently bright and strongly defined under FITC fluorescence magnification.

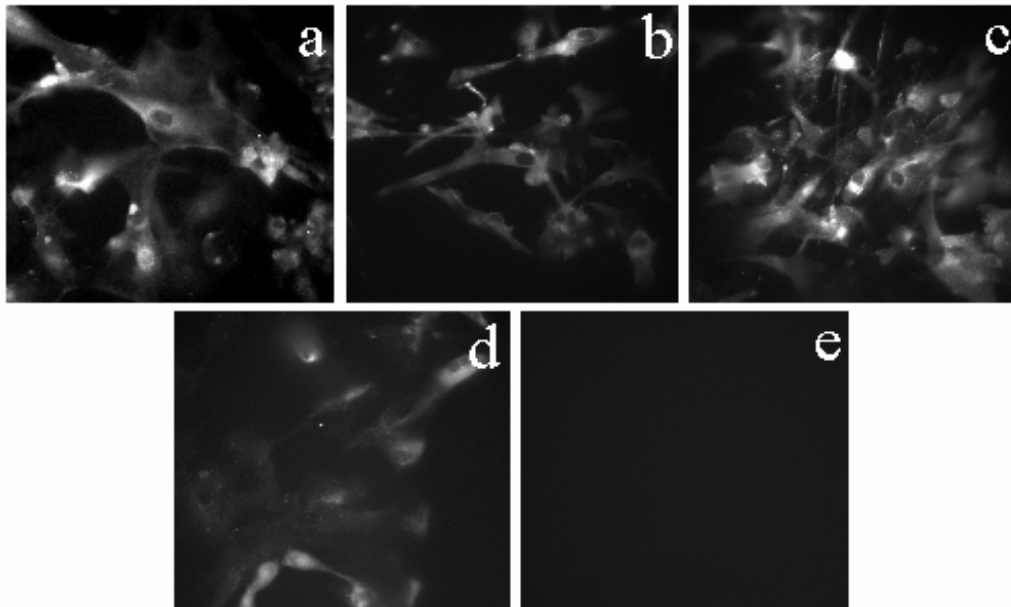
Oligodendrocytes were immunocytochemically labeled with adenosine receptor antibodies for A1, A2a, A2b, and A3 subtypes at a dilution of 1:10. The fix concentration was selected to be 2% based on the successful findings from the astrocyte immunocytochemistry. Prior to this research project, immunocytochemistry had not been used to identify adenosine receptors on *in vitro* nervous system glia. The refined protocol developed for application on both CNS and PNS glia, has here been established as an effective tool for visually verifying the expression of adenosine receptor subtypes.

Figure 1.



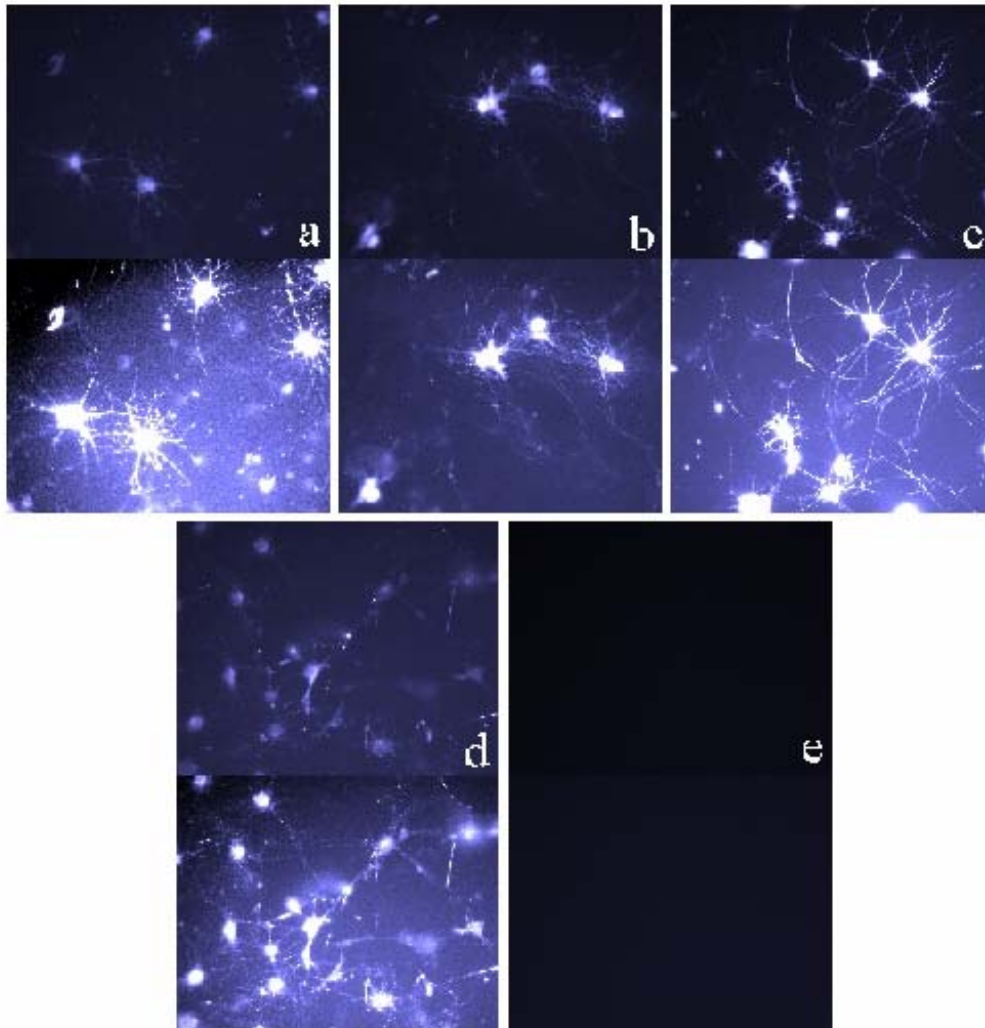
Morphological progression of the oligodendrocyte progenitor cell as shown through immunocytochemistry. **a)** The oligodendrocyte progenitor is bipolar in morphology and only stains positive for NG2+. **b)** The immature oligodendrocyte stains positive for O4+ and like the NG2+ OPC, experiences active proliferation, migration, and differentiation. **c)** The pre-myelinating oligodendrocyte will cease migration yet still actively proceed through proliferation and differentiation. It stains positive for O4+, O1+, and MBP+ but the focus of its morphology lies in the cell body and associated processes. The final mature myelinating oligodendrocyte (not depicted) stains positive for O4+, O1+, and MBP+ yet its morphological distinction is in its highly expansive and developed processes.

Figure 2.



Adenosine receptor immunocytochemistry of astrocytes depicting receptor expression on the cell surface. **a)** A1 receptor staining at 1:10 dilution and 2% PFA fix. **b)** A2a receptor staining at 1:10 dilution and 4% PFA fix. **c)** A2b receptor staining at 1:10 dilution and 2% PFA fix. **d)** A3 receptor staining at 1:10 dilution and 2% PFA fix. **e)** Representative astrocyte control under FITC immunofluorescence lacking primary antibody. Note lack of non-specific labeling of the adenosine surface receptors.

Figure 3.



Preliminary adenosine receptor immunocytochemistry of O1-stage oligodendrocytes successfully depicting receptor presence. **a)** A1 receptor staining. **b)** A2a receptor staining. **c)** A2b receptor staining. **d)** A3 receptor staining. **e)** Representative oligodendrocyte control under FITC immunofluorescence lacking primary antibody. Note lack of non-specific labeling of the adenosine surface receptors. All cells receiving primary antibody were fixed with 2% PFA and treated with a 1:10 dilution of their respective antibody. Second image for each receptor stain is included under different contrast to facilitate observation of the receptor distribution.

Discussion

The successful oligodendrocyte developmental immunocytochemistry supported the methodology currently used for characterization of the OP, O4, O1 and MBP stages. The most practical application of immunocytochemistry of the oligodendrocyte developmental stages is in calcium imaging with the incorporation of live staining and

imaging of the cells present in the field of view on the confocal microscope itself. This would allow for direct observation of the relationship between morphological and immunocytochemical cellular identity, and the activation of particular cells or lack thereof under agonist treatment. Calcium imaging remains a highly reliable research tool for interpreting the oligodendrocyte response to purinergic agonist administration (12, 13). The future use of mutant glial strains lacking certain purinergic pathways could facilitate the identification of pathways being activated during calcium imaging through selective agonists.

As the immunocytochemistry protocol is being applied currently, it is possible to suggest that the cells being stained are at comparable stages to cells being treated under functional confocal calcium imaging. However, direct immunocytochemical staining of the cells being treated with the agonists would work to yield more of a quantitative means for examining the relationship between receptor expression and activation throughout glial development. In addition to live staining on the confocal, western blot and PCR analysis could be implemented as quantitative methods to observe receptor expression at varying oligodendrocyte developmental points (4). The OP and O4 stages of oligodendrocyte development are of particular interest in examining intercellular signaling because their associated functional activation would be taking place during the developmental stages where proliferation and differentiation are on the verge of turning over to myelination.

The successful characterization of adenosine receptors on astrocytes allows for their use as a positive control for the analysis of receptor expression on other glia. Thus far, the novel immunocytochemical adenosine receptor protocol has been able to show all four of the possible receptor subtypes expressed in O1+ oligodendrocytes. Preliminary immunocytochemical results show adenosine receptor presence in bipolar OP cells, as well as expression in MBP+ cells. Obviously with the morphological differences between these developmental stages comes change in the physical distribution of the cell surface adenosine receptors. Application of the novel immunocytochemical protocol has become an integral aspect of the qualitative evaluation of receptor expression and distribution for the glial developmental stages.

The A2a and A2b receptor subtypes were of particular interest in development of an immunocytochemical technique of verification because these subtypes cannot be demonstrated using calcium imaging. Their activation is cAMP-linked and cells activated through these receptors will therefore not show a response in calcium imaging even if they are present and functional. A2a and A2b expression shown through immunocytochemistry was therefore highly relevant to the project because calcium imaging could not be used for their identification. A possible tool for future study of the activation of A2a and A2b adenosine receptors could be a cAMP enzymeimmunoassay (Amersham). Using this method, lysis reagents would be used to facilitate the rapid extraction of cAMP from stimulated cell cultures. This in turn allows for the quantification of cellular responses due to the activation of these two adenosine receptor subtypes. Future application of the immunocytochemical adenosine receptor protocol could be expanded to apply to different developmental stages of glia in the PNS as well as potentially other cell types altogether.

Co-culturing experiments are an aspect of this research project that incorporate developmental assessment of myelination in response to electrical stimulation of dorsal

root ganglion axons (14, 15). Sensory neurons send their signals to dorsal root ganglia associated with the vertebrae. Ascending neural branches are responsible for the conveyance of the signal to the CNS for processing (1). DRG neurons are a useful tool because the neurons themselves associate with both oligodendrocytes of the CNS and Schwann cells of the PNS. Therefore, co-culturing experimentation looking at glial myelination associated with electrophysiological input used to activate DRG axons can be applied to the assessment of myelination in both nervous system divisions. Since we are interested in investigating the signaling associated with the origins of myelination by developing glia, co-cultures represent an essential method for the evaluation of developing axons, which have been established to be associated with the signaling of oligodendrocyte maturation and myelination (16, for review). The signals inducing myelination in both the CNS and PNS remain uncharacterized and co-culturing experiments provide an opportunity to look directly at the physiological relationship between stimulated DRG axons and their associated glia. An ideal clinical application of the activity-dependent neuron-glia research would be the successful transplantation of developing pre-oligodendrocyte stem cells treated with myelination-inducing adenosine (4), as a potentially therapeutic treatment of damaged spinal cord tissue.

In vivo experiments can be foreseen as a necessary part of this project. Isolated slices of the rat sciatic nerve could be a successful means for the evaluation of Schwann cells of the PNS. Slice isolation of rat cortex, spinal cord, or optic nerve could additionally prove to be ideal means for oligodendrocyte *in vivo* receptor expression characterization. The immediate future of the current *in vitro* work on the expression of activity-dependent purinergic receptors lies in the complete classification of what oligodendrocyte developmental stages show both expression and functionality of the different adenosine receptor subtypes. It is possible that the different receptor subtypes may be up or down-regulated based on the developmental stage of the glial cells. It is imperative that the current and future study of purinergic receptor expression in myelinating glia includes not only immunocytochemistry, quantitative RT-PCR, western blot analysis, and gene array study, but also the incorporation of calcium imaging and cAMP assay to address the functionality of the varying levels of receptor expression for the developing glial cell types of the CNS and PNS.

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