

Research report

# *N*-Cadherin expression and function in cultured oligodendrocytes

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## Abstract

*N*-Cadherin is a major cell adhesion molecule that is expressed in the developing nervous system where it has been implicated in neural migration and axon growth. Recently, a role for *N*-cadherin in oligodendrocyte differentiation has been identified [23]. Oligodendrocyte precursors adhere to *N*-cadherin and mature rapidly to produce myelin sheets. Since this implies that oligodendrocytes express *N*-cadherin, we examined the expression of *N*-cadherin by oligodendrocytes in culture. *N*-Cadherin was expressed by O-2A progenitors, immature oligodendrocytes and mature oligodendrocytes, but at a lower level than in type 1 astrocytes in the same cultures. On mature oligodendrocytes, the *N*-cadherin was concentrated on the major processes emerging from the soma. The ability of *N*-cadherin and merosin to promote oligodendrocyte precursor migration was also studied. Average migration rates were significantly higher on merosin (11.2  $\mu\text{m}/\text{h}$ ) than on *N*-cadherin (5.6  $\mu\text{m}/\text{h}$ ). These results suggest that *N*-cadherin is not likely to function predominantly as a substrate that stimulates migration of O-2A progenitors, but may be more important in initiating early oligodendrocyte–axon interactions that promote the process of myelination.

**Keywords:** Cell migration; Merosin; Myelination; Immunofluorescence; O-2A progenitor

## 1. Introduction

Analysis of the molecular basis of interactions between a neural cell and its environment is one of the central problems in developmental neurobiology. Cell adhesion molecules that regulate cellular differentiation and control cell migration have been found in many systems. Prominent examples of this influence are found in neural crest cell migration [6] and axon guidance [18]. *N*-Cadherin, a  $\text{Ca}^{2+}$ -dependent cell adhesion molecule [12] that acts via a homophilic binding mechanism, has been implicated in the regulation of the intercellular recognition that leads to myelination of axons in the PNS [19]. *N*-Cadherin expression in mammals has been difficult to examine directly until quite recently, and little is known about cadherin expression by oligodendrocytes or their precursors. Mammalian *N*-cadherin was cloned [27], and it is now possible to produce monoclonal and polyclonal antibodies to *N*-cadherin that can be used in immunohistochemical studies and Western blot analyses of the central nervous system (CNS). It has been found that neuroepithelial cells express

*N*-cadherin at very early ages and that neurons continue to express *N*-cadherin. Recently, we found that *N*-cadherin is an excellent adhesive substrate for oligodendrocytes and their precursors [23]. We observed that *N*-cadherin induces extensive plasma membrane formation by oligodendrocytes. These phenomena imply that oligodendrocytes also must express *N*-cadherin because it functions as a homophilic receptor.

The rat optic nerve system provides a useful model for studies of antigen expression by different developmental stages of glia [25]. The morphological development of oligodendrocytes in vitro and the sequence of stage-specific antigen expression have been reviewed recently [24]. We used this culture system to study *N*-cadherin expression in the oligodendrocyte lineage.

We conducted this study to determine if *N*-cadherin is expressed by oligodendrocytes during development. We found that *N*-cadherin is expressed by oligodendrocyte precursors, and by both young and 'mature' oligodendrocytes. 'Mature' oligodendrocytes have a more complex pattern of *N*-cadherin distribution than do earlier stages. We also found that *N*-cadherin is a relatively poor substrate for promoting migration of oligodendrocyte precursor cells (O-2A progenitors), despite their expression of

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*N*-cadherin and their excellent ability to bind to substrate-bound *N*-cadherin. These results may have important implications for understanding oligodendrocyte development and differentiation and also oligodendrocyte behavior in pathological conditions of the CNS.

## 2. Materials and methods

### 2.1. Preparation of culture substrates

*N*-Cadherin from chick brains was purified with the monoclonal antibody NCD-2 (obtained from M. Takeichi and G. Grunwald) on affinity columns as described previously [23]. This procedure produces purified *N*-cadherin that supports cell attachment on nitrocellulose [4]. Fractionated samples were tested for purity by polyacrylamide gel electrophoresis. *N*-Cadherin, merosin (human: Telios Pharmaceuticals, Inc.) and bovine serum albumin (BSA, Sigma, Fraction V) were attached to plastic tissue culture dishes with a nitrocellulose coating [16].

### 2.2. Oligodendrocyte cultures for immunofluorescence

Cultures of rat optic nerve oligodendrocytes were grown as previously described [23] using procedures developed by Raff et al. [25]. Briefly, optic nerves of rat pups at postnatal day 7 were minced then incubated in 0.2% collagenase followed by 0.1% trypsin. The tissue was triturated in 0.04 mg/ml DNase then single cells were harvested by centrifugation and resuspended in DME/Hams F-12 (1:1) supplemented with 10% fetal calf serum (FCS medium). The cells were plated at a density of 20000 cells per cm<sup>2</sup> in 28 mm<sup>2</sup> wells in plastic tissue culture dishes that had been coated with nitrocellulose [16] and treated with 0.1% poly-L-lysine overnight at 37°C. After incubation at 37°C for 2 h to allow cell attachment, the cultures were grown in a serum-free, chemically defined medium (N2) [5,20,25] to promote oligodendrocyte development. Approximately half of the medium was changed every third day of cultivation. Cultures incubated for more than 1 week were treated with the anti-mitotic drug 5-fluoro-2'-deoxyuridine (10 mM, Sigma No. F-0503) in combination with uridine (10 mM, Sigma No. U-3750) for a 6-h period on every third day of cultivation to minimize the accumulation of dividing cells [15]. At the end of the anti-mitotic treatment, the cultures were rinsed once with N2 medium then fed with fresh medium.

### 2.3. Antibody characterization

The previously characterized antibodies that were used included monoclonal antibody A2B5 [8] which specifically labels O-2A lineage cells in these cultures [25], monoclonal anti-galactocerebroside (GalC) antibody [26] which specifically labels oligodendrocytes, and rabbit anti-

CNPase which binds 2',3'-cyclic nucleotide 3'-phosphohydrolase, a myelin marker enzyme that is detectable in 'mature' oligodendrocytes in culture [2,24]. The two anti-*N*-cadherin antibodies used were a mouse monoclonal antibody (NCAD2) and a rabbit (5835) antibody. They were prepared by immunization of appropriate animals with a synthetic peptide from the extracellular domain of *N*-cadherin coupled to KLH using standard procedures. The anti-*N*-cadherin antibodies were characterized by immunoblots of rat brain extracts prepared by PAGE. Extracts were prepared from adult rat brain by homogenization in 0.32 M sucrose, 1 mM sodium bicarbonate, and 1 mM MgCl<sub>2</sub> with 20 strokes of a Dounce homogenizer followed by centrifugation at 250 × *g* for 15 min to remove nuclei and debris. The collected supernatant was centrifuged at 10000 × *g* for 20 min, and the pellet was resuspended in PBS to make a crude membrane fraction. A portion of this membrane fraction was adjusted to 1% NP40 detergent and kept on ice for 1 h. The detergent extract was microcentrifuged for 15 min at 4°C. The detergent soluble fraction (supernatant), and the detergent insoluble fraction (pellet) were restored to the original volume with PBS. The extracts were electrophoresed in an 8% polyacrylamide gel, electrophoretically transferred to an Immobilon-P membrane and reacted with either monoclonal (NCAD2, 1:200 dilution of mouse ascites) or rabbit (5835 antisera, 1:300 dilution) anti-*N*-cadherin for 1 h. Bound antibody was detected by incubating the blots with alkaline phosphatase-conjugated anti-mouse IgG (for NCAD2) or anti-rabbit IgG (for 5835) and development in NBT/BCIP.

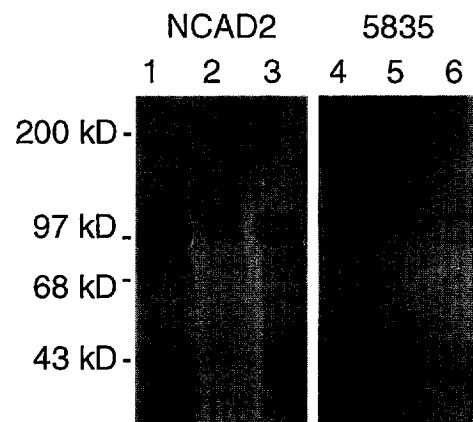


Fig. 1. Immunoblots of rat brain fractions with anti-*N*-cadherin. The gels were loaded with the total membrane extract (lanes 1 and 3), the NP40 insoluble fraction (lanes 2 and 5), and the NP40 soluble fractions (lanes 3 and 6) of rat brain. The gels were probed with either monoclonal anti-*N*-cadherin (NCAD2, lanes 1–3) or rabbit anti-*N*-cadherin (5835, lanes 4–6). Monoclonal anti-*N*-cadherin labeled two prominent bands of the total brain extract (lane 1). The 127-kDa band is much more prominent in the particulate fraction (lane 2), whereas the 97-kDa band is the more prominent in the soluble fraction (lane 3). Rabbit anti-*N*-cadherin labels only a 127-kDa band in the total membrane fraction (lane 4). This band is also labeled in the particulate fraction (lane 5), but no bands are labeled by rabbit anti-*N*-cadherin in the soluble fraction (lane 6).

#### 2.4. Immunostaining of cell cultures

Antigen expression in O-2A progenitors and oligodendrocytes was characterized by indirect immunofluorescence. Cultures for staining were fixed for 30–60 min at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer. *N*-Cadherin expression was identified in double stains with either monoclonal anti-*N*-cadherin or anti-*N*-cadherin raised in rabbit. For *N*-cadherin/A2B5 and *N*-cadherin/GalC double stains, fixed cultures were incubated for 1 h with a mixture of rabbit anti-*N*-cadherin and the appropriate monoclonal antibodies diluted in phosphate-buffered saline with 10% horse serum. For *N*-cadherin/CNPase double stains, cultures were first labeled by incubation with monoclonal anti-*N*-cadherin for 1 h, then were permeabilized by 0.1% saponin during a second 1-h incubation with rabbit anti-CNPase antibody. Live oligodendrocytes were also stained in some experiments by incubation for 1 h at 4°C with the anti-*N*-cadherin before fixation to test the effects of formaldehyde treatment on the stain intensity and cellular distribution. The antibody-labeled cells were then incubated with the appropriate fluorescein- or rhodamine-conjugated secondary antibodies for 1 h. The specificity of each immunostain was controlled by: (1) omission of the primary antibody; and (2) incubation of cells labeled by each primary antibody with the non-corresponding secondary antibody system. The stained cultures were exam-

ined with a Zeiss Axiovert fluorescence microscope equipped with epi-illumination and selective filters for fluorescein and rhodamine.

#### 2.5. Time-lapse studies

For time-lapse studies, O-2A progenitors were cultured from rat forebrains which provided larger numbers of cells for study than optic nerves. Forebrain cultures were prepared by a modification [1] of the method of McCarthy and de Vellis [22]. Newborn (P1) rat cerebral hemispheres and brainstems were forced gently through a 230- $\mu$ m nylon mesh. The resulting cell suspension was sedimented by centrifugation at  $200 \times g$  for 5 min. The cell pellet was resuspended in DME/Hams F-12 (1:1) supplemented with 10% FCS medium, then plated in poly-L-lysine-coated tissue culture flasks and cultivated for 7 days at 37°C. The flasks were shaken at 225 rpm for 3 h to remove loosely adherent cells which were discarded. The cells were covered with fresh medium then shaken again at 260 rpm for 18 h to dislodge O-2A progenitors from the cell monolayer. Contaminating microglia and astrocytes were removed from this cell suspension by a 5 h incubation at 37°C in plastic culture dishes. The remaining cells, enriched to more than 95% O-2A cells, were suspended in FCS medium and plated on prepared substrates at a density of 10000 cells/cm<sup>2</sup>. The cultures were incubated at 37°C

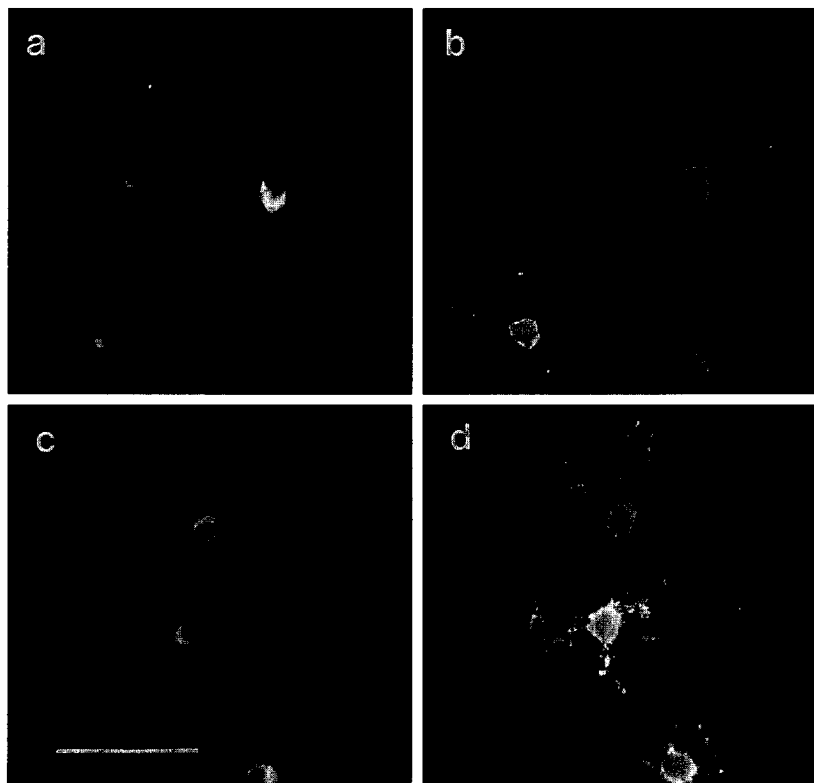


Fig. 2. Expression of *N*-cadherin by O-2A progenitor fixed at 2 days in vitro. The cells were double stained with anti-*N*-cadherin (a, c) and anti-A2B5 (b, d). Cell processes typically lack *N*-cadherin at this stage while cell bodies of A2B5<sup>+</sup> cells are intensely stained by anti-*N*-cadherin. The scale bar in all figures equals 50  $\mu$ m.

with 5% CO<sub>2</sub> for 18 h then transferred to a microscope stage incubator. Time-lapse images were recorded at 5-min intervals for at least 6 h for each substrate with a Zeiss Axiovert 405M microscope, a Hamamatsu silicon-intensified video camera and an Image1 computer analysis system. Sequences were analyzed by tracking the current position of the nucleus of each cell in the field at every tenth image.

### 3. Results

Immunoblots of rat brain extracts demonstrated that both monoclonal (NCAD2) and rabbit (5835) are specific for the *N*-cadherin molecule. Both anti-*N*-cadherin antibodies prominently stained a band at about 127 kDa (Fig. 1, lanes 1 and 4). The location of this band, which also was present in the detergent insoluble fraction, is consistent with the electrophoretic mobility of *N*-cadherin [11]. NCAD2 also stained a band at 97 kDa which was stained

in the soluble fraction as well (lane 3) and very likely to be a proteolytic fragment of *N*-cadherin [11].

Rat optic nerve cultures fixed at 2 days in vitro were composed predominantly of A2B5<sup>+</sup> O-2A progenitor cells. More than 90% of the A2B5<sup>+</sup> cells, when double stained with anti-*N*-cadherin, were found to express *N*-cadherin (Fig. 2). *N*-Cadherin staining was limited to the cell body and little or no staining was seen in the relatively simple processes of the O-2A progenitors at this stage of development.

Optic nerve cultures fixed after 3 and 4 days of incubation contained mostly GalC<sup>+</sup> oligodendrocytes with an immature type of morphology characterized by a fibrous interconnecting network of cell processes with few areas of flattened lamellar regions. The cells typically have 3–5 large primary processes that extend for a short distance from the cell body before branching out into numerous smaller diameter processes. *N*-Cadherin was expressed by virtually all of the GalC<sup>+</sup> cells after 3 and 4 days in vitro. *N*-Cadherin staining in these cells was seen both at the cell

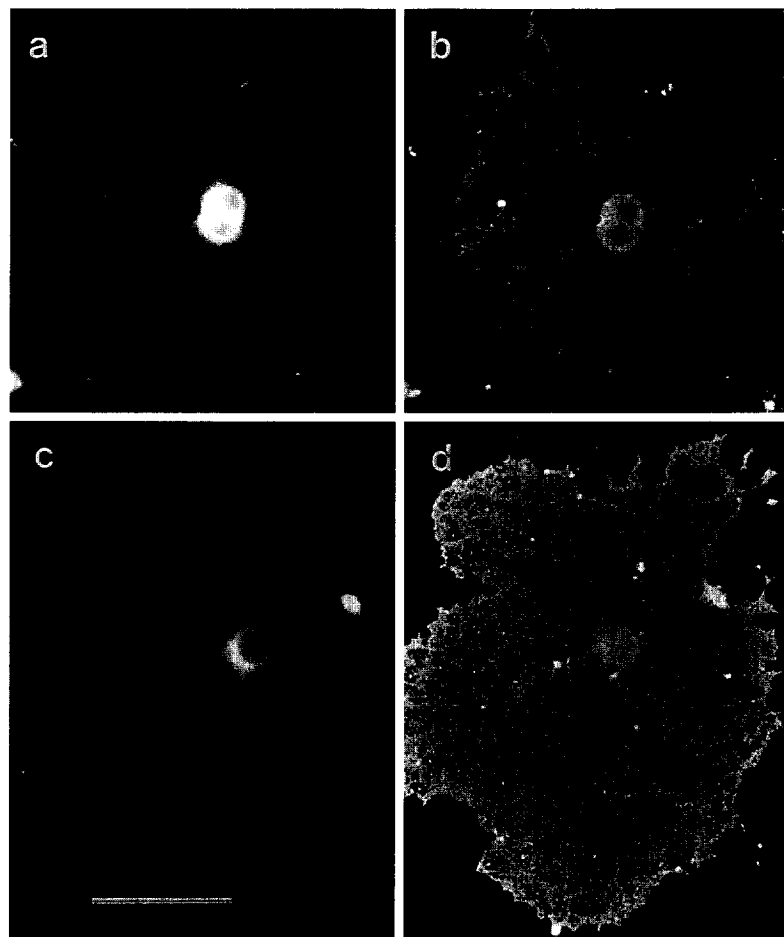


Fig. 3. Expression of *N*-cadherin by immature oligodendrocytes fixed at 4 days (a, b) or 7 days (c, d) in vitro. The cells were double stained with anti-*N*-cadherin (a, c) and anti-GalC (b, d). Anti-*N*-cadherin staining of oligodendrocytes is evident in the cell body and in proximal portions of the major processes.

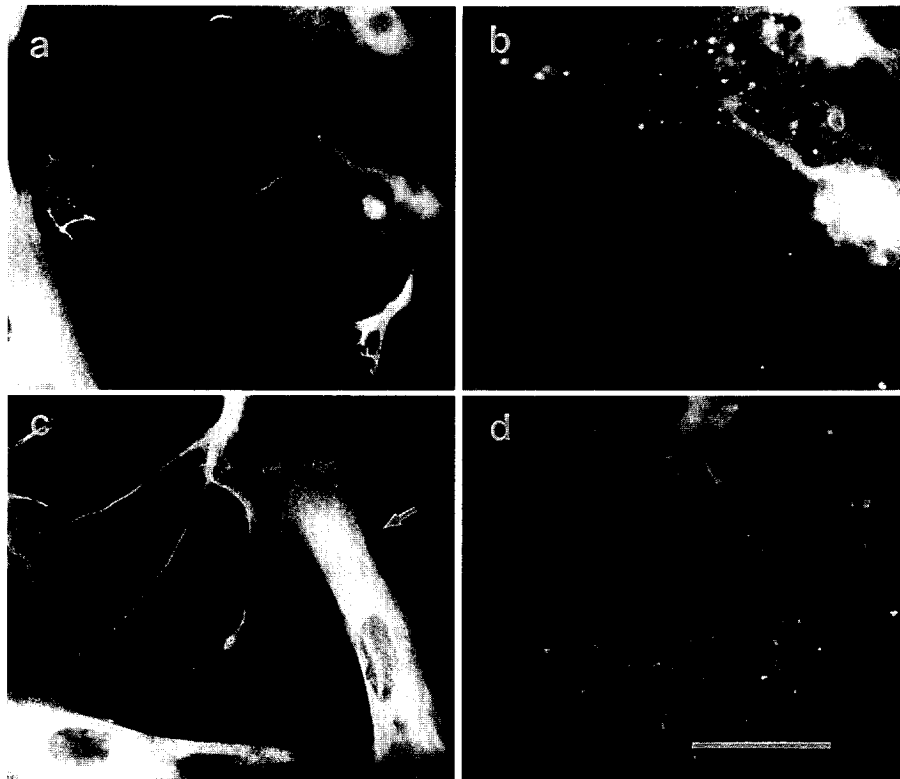


Fig. 4. Expression of *N*-cadherin by mature oligodendrocytes fixed at 11 days in vitro. The cells were double stained with anti-*N*-cadherin (a, c) and anti-CNPase (b, d). Anti-*N*-cadherin staining of mature oligodendrocytes is evident in small distal portions of the process network, but not in lamellar regions of the network. The arrowhead in c points to a type 1 astrocyte that is stained with anti-*N*-cadherin.

body and also along the primary cell processes (Fig. 3) although staining of these processes was diminished with increasing distance from the cell body. The fine processes distal to the cell body were not stained for *N*-cadherin above background levels. These distal elements were found to have a low level of surface stain in preparations of oligodendrocytes that were stained with anti-*N*-cadherin before fixation. Otherwise, the cellular distribution of the fluorescent stain was similar to that of fixed cells. (data not shown). This pattern of staining was observed in oligodendrocytes from days 3 to 7. Rat forebrain cultures stained with A2B5, anti-*N*-cadherin, or anti-GalC at 2–4 days after incubation in SFM contained O-2A progenitors and oligodendrocytes with morphological features and staining patterns similar to cells in optic nerve cultures (data not shown).

By incubation day 11, differentiated oligodendrocytes in optic nerve cultures were overgrown by the type 1 astrocytes and contaminating fibroblasts which had continued to proliferate. Anti-mitotic treatment on day 4 reduced this overgrowth and permitted oligodendrocytes to survive without overcrowding. These cultures were fixed, stained with anti-*N*-cadherin, then stained with anti-CNPase (Fig. 4). *N*-Cadherin stained the cell body and primary processes of most CNPase<sup>+</sup> cells. In addition, finer elements of the process network distal to the cell body were discontinuously stained for *N*-cadherin. However, lamellar expansions of the processes seemed to have relatively low

levels of *N*-cadherin immunoreactivity. A2B5<sup>+</sup> cells in the culture morphologically similar to type 1 astrocytes were stained intensely by anti-*N*-cadherin (for example, see Fig. 4).

Time-lapse studies on migration of O-2A progenitors on uniform fields of *N*-cadherin, merosin and BSA revealed significant differences between merosin and *N*-cadherin

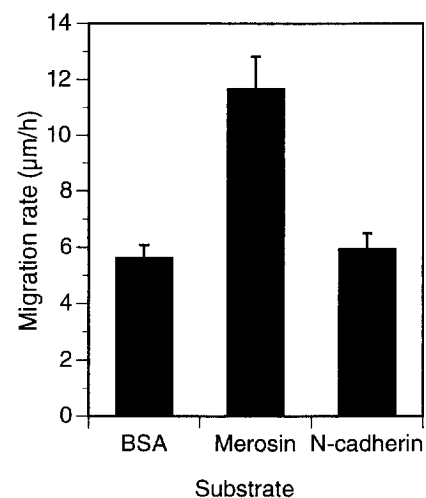


Fig. 5. Average migration rates of O-2A progenitors on *N*-cadherin, merosin, and BSA. Error bars represent the S.E.M. for 30 cells on each substrate tracked over a 6-h period.

(Fig. 5). O-2A progenitors on merosin were very actively motile, constantly extending and retracting cell processes in all directions. In bipolar cells, the predominant cell shape, the cell body typically moved along the axis defined by the two major cell processes. O-2A progenitors on merosin moved at an average rate of 11.2  $\mu\text{m}/\text{h}$ , although rates as fast as 24.5  $\mu\text{m}/\text{h}$  were measured. In contrast, O-2A progenitors on *N*-cadherin and BSA, a control substrate presumed to be a non-physiological substrate for adhesion and migration, were relatively inactive (average rates of migration were 5.6 and 5.8  $\mu\text{m}/\text{h}$ , respectively). Most of the O-2A progenitors on these two substrates did not migrate during the observation periods.

#### 4. Discussion

The basic findings from this study are that cells in the oligodendrocyte lineage, from oligodendrocyte precursors to mature oligodendrocytes express *N*-cadherin in vitro, albeit at lower levels than astrocytes and neurons. Also, *N*-cadherin, when used as a substrate, does not promote migration of O-2A progenitors to any significant degree, when compared with BSA and merosin.

*N*-Cadherin is a major cell adhesion molecule in the developing nervous system, functioning in cell migration and axon growth and guidance. Cadherins typically function in a homophilic binding mechanism, with like cadherins on the surfaces of two opposing cells binding to each other. The fact that cells of the oligodendrocyte lineage bind to *N*-cadherin when it is used as a substrate implies these cells express *N*-cadherin. Therefore, the observation that oligodendrocytes express *N*-cadherin was expected. However, the pattern of expression was not anticipated. *N*-Cadherin was found on somata and major processes, but at a much lower level on the minor processes or lamellar sheets of membrane produced by oligodendrocytes. Based on our earlier studies, we had predicted that the lamellar sheets would also express *N*-cadherin since they appear to be firmly attached to *N*-cadherin substrates. If *N*-cadherin on the lamella was localized to the membrane surface adjacent to the substrate, it is possible that there might be a problem of antibody accessibility which would minimize the signal. However, we used permeabilizing conditions with monoclonal and polyclonal antibodies directed to different parts of the *N*-cadherin molecule, including the cytoplasmic domain, yet the staining we observed in these regions was minimal. Since we found little staining of lamella on either poly-lysine substrates, which would not be expected to cause a redistribution of *N*-cadherin to a particular membrane surface, or on *N*-cadherin we conclude that *N*-cadherin is not expressed to a significant degree by lamellae.

The restricted distribution of *N*-cadherin is consistent with a recent confocal study of cadherin on Schwann cells

in vivo [9]. They observed that an antibody that recognizes the cytoplasmic domain of all cadherins binds to the Schmidt–Lanterman clefts of Schwann cells and was not uniformly distributed in the lamellae of the Schwann cells forming myelin sheets around axons in the PNS. While it is not proven, it is likely their antibody was binding to *N*-cadherin, because there is a body of evidence showing that Schwann cells express *N*-cadherin [3,19]. Therefore, *N*-cadherin expression in lamellae is probably restricted by some mechanism. It could either result from the expression on a cadherin binding protein in the cytoplasm of the soma and major processes, such as a catenin, or there could be a process that excludes *N*-cadherin from myelin sheets.

Our earlier study [23] found that O-2A progenitors avidly bound to *N*-cadherin. Since axons in the developing CNS express *N*-cadherin this suggested two possible functions for *N*-cadherin in O-2A progenitor–axon interactions. The first is that *N*-cadherin might serve as a substrate for O-2A progenitor migration. Since O-2A progenitors are known to migrate along axon fascicles in different regions of the CNS [10] in order to populate these areas with oligodendrocytes, this seemed like a plausible function. The second is that *N*-cadherin might be an important early player in the interaction between axons and O-2A progenitors that leads to formation of stable, long-term interactions between axons and oligodendrocytes and may function in the initiation of myelin formation. Our studies on the ability of O-2A progenitors to migrate on *N*-cadherin suggest that *N*-cadherin does not promote migration, certainly when compared with merosin, another candidate substrate for O-2A progenitor migration [7,14]. Since, in our previous studies, we found that *N*-cadherin rapidly induces the formation of lamellar structures when compared with other substrates, such as L1 or poly-lysine, it seems likely that the principal role of *N*-cadherin in O-2A progenitor development is the promotion of axonal interactions that lead to myelin formation.

The expression of *N*-cadherin in the mature nervous system of mammals has been not studied extensively. However there is much evidence that *N*-cadherin expression and post-translational modifications are under precise spatial and temporal control during developmental processes [13,17,21]. Thus, if axons do not express appropriate levels of *N*-cadherin, then it is possible that this could interfere with the establishment or re-formation of myelin around axons in the adult CNS that have experienced demyelination.

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