Neuronal cadherin (NCAD) increases sensory neurite formation and outgrowth on astrocytes

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HIGHLIGHTS

► Genetic deletion of neuronal cadherin (NCAD) from astrocytes decreases neurite growth.
► Deletion of NCAD does not cause increased expression of other classical cadherins.
► NCAD may support axon outgrowth in circumstances of axon–astrocyte contact after CNS injury.

ARTICLE INFO

ABSTRACT

We examined the neurite outgrowth of sensory neurons on astrocytes following the genetic deletion of N-cadherin (NCAD). Deletion abolished immunostaining for NCAD and the other classical cadherins, indicating that NCAD is likely the only classical cadherin expressed by astrocytes. Only 38% of neurons grown on NCAD-deficient astrocytes for 24 h produced neurites, as compared to 74% of neurons grown on NCAD-expressing astrocytes. Of the neurons that produced neurites, those grown on NCAD-deficient astrocytes had a mean total length of 378 μm, as compared to 1093 μm for neurons grown on NCAD-expressing astrocytes. Thus, the loss of NCAD greatly impairs the formation and extension neurites on astrocytes.

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1. Introduction

After central nervous system injury axonal regeneration is poor and often only limited recovery is possible. David and Aguayo demonstrated that peripheral nerve grafts support robust growth of some central axon populations suggesting the injured CNS is a growth-inhibitory environment [9]. Subsequent work has described multiple myelin and proteoglycan growth-inhibiting components found in the CNS [61]. Chondroitin sulfate proteoglycans are produced by reactive astrocytes, associate with the glial scar, and contribute to regenerative failure after CNS injury [10,11,37,48]. However, the role of reactive astrocytes is not solely detrimental. Indeed, selective ablation of astrocytes after spinal cord injury increases the volume of tissue loss and worsens functional outcomes [16] and cytokine activated astrocytes appear to support recovery after nervous system injury [30]. Furthermore, in vivo astrocytes serve as substrate for axon growth under conditions in which CNS axon growth after injury is enhanced [1,22].

In order to determine what molecules found on astrocyte cell membranes may be important for axon growth in these discrete circumstances, we have developed an in vitro neurite outgrowth assay with which genes of interest may be genetically deleted from astrocytes. Early in vitro work has suggested candidate molecules. Neurons grow well on cultured astrocytes [15,21,42], and the ability of blocking antibodies to inhibit neurite outgrowth suggests that NCAD is a key molecular mediator of axon growth [21,39,55]. Antibodies against the extracellular domain of NCAD, or lowering extracellular Ca^{2+}, also inhibit neurite outgrowth on purified NCAD [5,27,34]. Conversely, NCAD is also sufficient to promote neurite outgrowth [13,36]. These data suggest that NCAD mediates, in part, the growth of neurites on astrocytes.

Function blocking antibodies, however, may have unintended effects, including binding to other molecules and/or steric hindrance. Genetic deletion of the corresponding molecule transcends

these limitations, and has both confirmed and repudiated prior blocking antibody studies that implicated NCAD in various cellular interactions [8,32,51]. Because mice lacking NCAD have many developmental defects and die embryonically [45], we have developed an in vitro neurite outgrowth assay after deleting NCAD from astrocytes cultured from $Ncad^{flox/flox}$ mice with an adeno-associated virus (AAV) that expresses Cre recombinase. We found that NCAD can be eliminated from astrocytes, without apparent compensatory expression of other classical cadherins, and that this severely diminishes neurite formation and neurite outgrowth. These observations suggest that NCAD may be important for axon formation and growth on astrocytes.

2. Materials and methods

All chemicals used in our experiments were obtained from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA) unless otherwise stated.

2.1. Cell culture

The Institutional Animal Care and Use Committee at the University of Pennsylvania approved animal procedures. Postnatal day 1-2 (P1-2) $Ncad^{flox/flox}$ mice were genotyped by analysis of tail DNA by PCR as described by Kostetskii [23]. Cortical astrocytes were prepared from postnatal day 2-3 (P2-3) $Ncad^{flox/flox}$ mice [29] as previously described [2]. Briefly, cortices were isolated, stripped of meninges, digested with trypsin, and plated. After cultures were confluent, astrocytes were purified by shaking (yielding ~98% GFAP-positive cells by immunostaining), replated on poly-lysine coated coverslips in DMEM/10% FBS, and penicillin/streptomycin. One day after re-plating cells were infected with AAV or AAV-Cre (AAV2/8 obtained from the Vector Core at the University of Pennsylvania); 3×10^{11} genome copies/ml infected almost all astrocytes in initial experiments; this dose was used for subsequent infections. Two weeks after infection, the cells were fixed and double-labeled with a rabbit antiserum against NCAD (Calbiochem 205606) and a mouse antibody against Cre (Millipore, MAB3120). To determine the efficacy of Cre-mediated NCAD deletion, astrocytes were scored for both NCAD and Cre immunoreactivity. NCAD immunostaining was considered positive if any border of the cell exhibited staining. Cre immunostaining was considered positive if clear nuclear Cre stain was present on comparison with DAPI. Other primary antibodies used in this study to stain astrocytes included rat anti-NCAM (Abcam 19782), mouse anti-Cx43 (Chemicon MAB3067), and goat anti-CHL1 (R&D Systems AF2147), and rat anti-L1 (Millipore MAB5272). Appropriate secondary antibodies were purchased from Jackson Labs (West Grove, PA).

2.2. Neurite outgrowth assay

Sensory neuron cultures were prepared from P2-3 $Ncad^{flox/flox}$ or wild type mice as described by Maline et al. [33]. About 20 dorsal root ganglia (DRG) were collected in Hank's Balanced salt solution, digested with papain followed by dispase/collagenase, gently triturated through a fire-polished pipette, and plated onto astrocyte-coated coverslips; ~10,000 neurons/coverslip. DRG neurons were cultured in DMEM/10% FCS and penicillin and streptomycin. After 24 h, the coverslips were fixed (4% parformaldehyde for 5 min), blocked (5% fish skin gelatin in PBS and 0.1% TritonX-100) for 1 h, and immunostained with mouse anti-βIII tubulin (Sigma T5076) and a rabbit antiserum against NCAD (Calbiochem 205606) at 4 °C overnight, and visualized with FITC- and TRITC-donkey anti-mouse and anti-rabbit secondary antibodies (Jackson Labs, West Grove, PA). In one neurite outgrowth assay, both neurons and astrocytes were also stained with DAPI and neuronal nuclei examined

for nuclear hallmarks of apoptosis including small, condensed, and fragmented nuclei [14]. In this experiment, at least 50 neurons were scored for apoptosis and a Fisher's test was used to determine if the proportion of apoptosis was different between neurons grown on NCAD-expressing and NCAD-deficient astrocytes.

Neurons were also scored for the presence or absence of neurites. To measure neurite length, individual coverslips were scanned systematically and neurites meeting the following criteria were measured: (1) the astrocyte substrate had to be clearly NCAD-positive (Fig. 2A and B) or NCAD-negative (Fig. 2C and D); (2) the neuronal cell bodies had to be clearly NCAD-positive, but the NCAD-immunoreactivity of axons was not used as a criterion because it was less intense than that of either astrocytes or neuron cell bodies; (3) the neurite had to be at least 10 μm in length; (4) the neuronal cell bodies had to be at least 50 μm apart, and individual neurites had to clearly distinguishable from those of nearby neurons. Using these criteria, at least 150 neurons were scored per condition per experiment; and the results of 3 independent experiments (both newly generated astrocytes and neurons) were grouped.

3. Results

3.1. AAV-Cre efficiently deletes NCAD from cultured $Ncad^{flox/flox}$ astrocytes

Like reactive astrocytes, cultured astrocytes express GFAP. Furthermore, their gene expression patterns are partially consistent with reactive astrocytes [6]. Therefore, we used AAV-Cre to delete NCAD from cultured astrocytes. However, it should be noted that it has recently become possible to culture acutely isolated astrocytes from much older rodent brain using a combination of immunopanning and heparin-binding epidermal growth factor supplementation [17], which will be an important method to understand astrocyte function in the future. Astrocytes were obtained from $Ncad^{flox/flox}$ mice, treated with AAV-Cre, AAV alone, or vehicle, and immunostained for NCAD and Cre after 2 weeks. As shown in Fig. 1, astrocytes infected with AAV-Cre lacked NCAD-immunoreactivity (C) and had Cre-positive nuclei (D). The lack of NCAD-immunoreactivity was highly correlated with expression of Cre in the nucleus – 97% of astrocytes were NCAD-negative and Cre-positive, 2% were NCAD-negative and Cre-negative (suggesting some astrocytes expressed Cre at levels not detectable by our immunostaining protocol), and 0.3% were NCAD-positive and Cre-negative; NCAD-positive and Cre-positive astrocytes were not observed. Therefore, as estimated by Cre-immunoreactivity, at least 97% of astrocytes were infected with AAV-Cre. In concurrently prepared control cultures (vehicle-treated astrocytes), there was robust NCAD expression, particularly at cell borders, and no Cre-immunoreactivity (Fig. 1A and B). In pilot experiments, neurite outgrowth and NCAD expression were compared between AAV only and vehicle-treated astrocytes. In these experiments mean neurite outgrowth was 532 μm on AAV-treated cultures and 583 μm on vehicle-treated cultures ($p = 0.49$). In both cases NCAD expression was markedly similar to that of Fig. 1A (data not shown). Because no difference was found in the ability of AAV alone or vehicle-treated astrocytes to support neurite outgrowth on astrocytes, vehicle was used as the control condition for neurite outgrowth assays.

3.2. Diminished neurite formation and outgrowth on NCAD-deficient astrocytes

We then compared neurite outgrowth of isolated sensory neurons on NCAD-deficient and NCAD-expressing astrocytes. Sensory neurons were acutely isolated from neonatal DRG, grown on
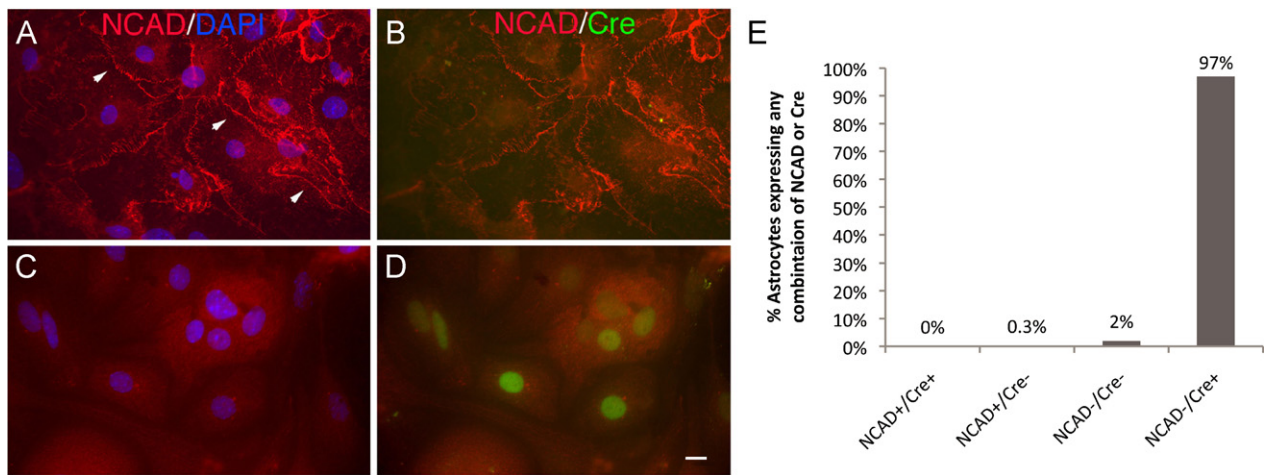
Fig. 1. AAV-Cre-mediated loss of NCAD from *Ncad^flox/flox* astrocytes. These are digital images of astrocytes purified from *Ncad^flox/flox* mice, immunostained for NCAD (red) and Cre recombinase (green), and counterstained with DAPI (blue), 14 days after treatment with AAV-Cre (C and D) or vehicle (A and B). The loss of NCAD from astrocytes in two experiments is quantified (E). Panels A and B and panels C and D are the same field of astrocytes. Arrowheads denote NCAD enrichment at cell borders. Note the lack of cell border NCAD-immunoreactivity that correlates with Cre-positive nuclei (scale bar = 10 μm).

astrocytes for 24 h, then immunostained for βIII-tubulin (to visualize neurites) and NCAD; examples are shown in Fig. 2. The percentage of neurons with neurites was 74% and 38% on NCAD-expressing and NCAD-deficient astrocytes, respectively ($p < 0.01$). Even after excluding neurons with neurites shorter than 10 μm, the total neurite length of neurons grown on NCAD-deficient astrocytes was shorter, as shown by the mean (378 μm vs. 1093 μm; Fig. 2E, top panel) and by the cumulative distribution of neurite lengths (Fig. 2E, bottom panel). Immunostaining confirmed that all DRG neurons expressed NCAD and that NCAD was successfully removed from the astrocyte substrate. We also considered the possibility that NCAD deletion increased neuronal death thereby indirectly affecting growth. Therefore, in our neurite outgrowth assay we also examined DAPI-stained neuronal nuclei for hallmarks of apoptosis, as has been done previously [14,25]. We found evidence of neuronal apoptosis in 18% of DRG neurons grown on NCAD-expressing astrocytes and 11% of NCAD-deficient astrocytes ($p = 0.32$), suggesting deletion of NCAD did not affect DRG neuron death in these cultures.

Taken together, these data demonstrate that NCAD is important for both neurite formation and extension on astrocytes.

3.3. Does the absence of NCAD result in the altered expression of other molecules?

Astrocytes may express multiple cadherins [20,46,62], therefore we considered whether other classical cadherins might be upregulated after deleting NCAD. We immunostained astrocytes with a pan-cadherin monoclonal antibody that recognizes an intracellular epitope common to classical cadherins [18], combined with an antiserum against NCAD. In contrast to the robust staining of both NCAD and pan-cadherin of wild type astrocytes, the pan-cadherin antibody did not label NCAD-deficient astrocytes (Suppl. Fig. 1), demonstrating that NCAD-deficient astrocytes do not significantly express other classical cadherins in culture.

We also examined the expression of other growth-promoting cell adhesion molecules in NCAD-deficient astrocytes (Suppl. Fig.
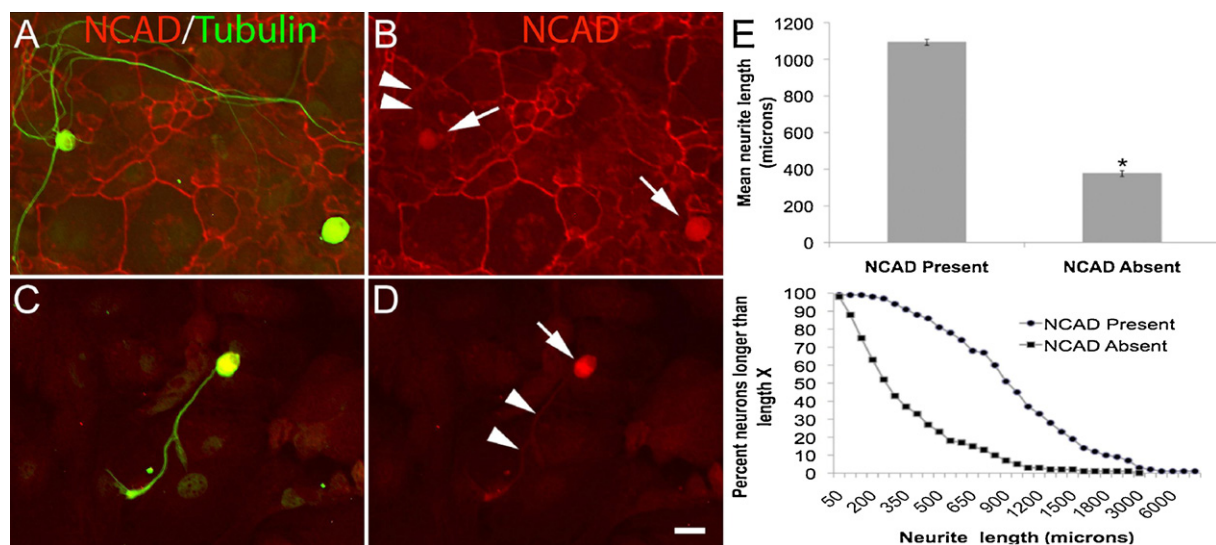

Fig. 2. NCAD facilitates neurite outgrowth on astrocytes. These are digital images of DRG neurons grown on NCAD-expressing astrocytes (A and B, top panels) or NCAD-deficient astrocytes (C and D, bottom panels) for 24 h, and then immunostained for both NCAD (red) and βIII tubulin (green). As before (Fig. 1) NCAD has been effectively deleted. The mean total neurite length on NCAD-deficient astrocytes was significantly shorter than on NCAD expressing astrocytes (*t*-test, $P < 0.01$) (E, top graph). The cumulative distribution of neurite lengths on NCAD-deficient astrocytes was also significantly shorter than on NCAD expressing astrocytes ($P < 0.01$, Kolmogorov–Smirnov test) (E, bottom graph) (scale bar = 20 μm).

2). The distribution and intensity of NCAM immunostaining [21,42] was not different between vehicle treated and NCAD-deficient astrocytes. We similarly examined L1 and close homologue of L1 (CHL1), which both can support neurite outgrowth [19,24,26]. We did not observe any L1 staining in astrocytes (data not shown; [19]), and CHL1 was diffusely found on the surface of both NCAD-positive and -negative astrocytes. These observations were consistent with other's observations that NCAD deletion from astrocytes does not alter the expression of both axon growth-promoting and axon growth-inhibitory cell surface molecules, including fibronectin and CSPGs [21]. As a more general assay for cell surface molecules, we also examined Cx43-immunoreactivity, which is associated with NCAD in cardiac junctions [29]. Both NCAD-expressing and NCAD-deficient astrocytes had numerous Cx43-positive puncta at cell borders, as has been previously seen in mouse heart [29] and 3T3 cells [58]. Together, these observations suggest that NCAD deletion from astrocytes does not substantially alter the expression of other cell surface molecules in astrocytes, including those known to either promote or inhibit neurite growth.

4. Discussion

Genetic deletion of NCAD in astrocytes provides the most direct demonstration that NCAD is required for proper neurite formation and extension on astrocytes. Our observations extend prior studies showing blocking antibodies against NCAD diminish neurite outgrowth of several kinds of neurons on astrocytes [21,39,55]. These results are likely true for other cell types, as at least three types of neurons (retinal, ciliary, and sensory) also utilize NCAD for neurite growth on Schwann cells [3,28,34] and myotubes [4]. Other experimental approaches also indicate that NCAD plays a central role in axon growth in diverse species. In *Xenopus*, dominant negative inhibition of cadherin function impairs axon extension of developing retinal ganglion cells [47], zebrafish homozygous for recessive *Ncad* mutations have severe developmental abnormalities of the eye, including misrouted optic axons [35], and deletion of an *Ncad* orthologue in individual R cells in *Drosophila* impairs both axon extension and target selection [43]. Recent experiments have also suggested NCAD is important for dendrite growth in mouse [52].

The effect of NCAD deletion on both neurite formation and extension was substantial but incomplete. Because the reduction of NCAD-immunostaining was profound, it is unlikely that the remaining neurite growth results from incomplete loss of NCAD; sensory neurites probably use additional cell surface receptors for growth. Blocking antibody experiments implicate neuronal β1-integrin-mediated attachment to ECM molecules on the surface of astrocytes including fibronectin [39,53–55], so it will be important to determine whether sensory neurons can extend axons if both astrocyte NCAD and neuronal β1-integrin are deleted using the techniques developed for this study. On developing or immature astrocytes NCAM may also be important for axon growth [39,49].

Though reactive astrocytes are largely thought to impede axon growth after nervous system injury, especially near an injury site [48] and the potency of astrocyte neurite growth-promotion declines with age [49], reactive astrocytes may support axon growth and survival in discrete circumstances after injury and during disease [38,50,60]. Adult DRG neurons transplanted into corpus callosum or spinal cord grow in close association with reactive astrocytes beyond the initial transplant site [10,11]. After transplantation of NGF-producing fibroblasts into rat striatum, cholinergic axons grow almost exclusively on astrocytes [22] and after optic nerve crush the occasionally regenerating retinal ganglion axon grows on astrocytes [7]. These observations suggest that both central and peripheral populations of neurons may use astrocytes as growth substrate. Furthermore, if the inherent growth capacity of a neuron is increased such as through deletion of PTEN, by pre-conditioning injury, or through local injection of cAMP analogues, regenerating axons grow through strongly GFAP-positive cells, some of which are likely astrocytes [31,40,41,44]. These experiments suggest that reactive astrocytes may be critically important for axonal regeneration, especially under conditions in which the regenerating axon is capable of robust growth. Finally, increasing the axon-supportive phenotype of endogenous or transplanted astrocytes may promote recovery after CNS injury [59,12].

In summary, we have demonstrated, by genetic deletion, that DRG sensory neurons utilize NCAD for both neurite formation and extension on astrocytes. As NCAD is expressed by astrocytes after stretch injury vitro [56] and stab injury in vivo [57], we hypothesize that NCAD may support CNS axon growth in circumstances of axon–astrocyte contact after injury.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.06.013.

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