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Homophilic NCAM interactions interfere with L1 stimulated neurite outgrowth

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Abstract The cell adhesion molecules NCAM and L1 are considered to play key roles in neuronal development and plasticity. L1 has been shown to interact with NCAM, possibly through NCAM binding to oligomannosidic glycans present in L1. We investigated the effect of recombinant immunoglobulin (Ig) modules of NCAM involved in homophilic NCAM binding, on L1 induced neurite outgrowth from PC12-E2 cells and found a complete inhibition of L1 induced neurite outgrowth after addition of Ig-modules 1, 2 and 3 of NCAM, suggesting that the ligation state of NCAM is crucial for normal L1 signaling.

Key words: Cell adhesion; Neurite outgrowth; L1; NCAM; Interaction; Ig-module

1. Introduction

Neural development in the embryonic and postnatal period is known to involve a complex pattern of changes in cellular behavior including cell migration, axonal outgrowth, fasciculation and synapse formation. In the mature nervous system, similar processes are known to be involved in the structural remodeling associated with neuroplasticity.

Cell adhesion molecules (CAMs) have been demonstrated to play a pivotal role in normal neural development, during neuronal regeneration and in connection with learning and memory consolidation [1–3]. Two structurally related CAMs, the neural cell adhesion molecule (NCAM) and L1, are prominent members of the immunoglobulin superfamily (IgSF) and are both believed to be important mediators of neuronal differentiation [4–8]. NCAM and L1 expression is both spatially and temporally regulated during development [9]. Whereas NCAM is widely expressed in the developing and mature nervous system, L1 expression is restricted mainly to postmitotic neurons [10–12].

L1 mediates a strong, calcium-independent, homophilic *trans*-binding between neuronal cells [13] and has been shown to stimulate neurite outgrowth in vitro [14,15]. Furthermore, L1 and its homologues have been demonstrated to be involved in proper axonal growth and guidance in intact systems in vivo [16–18].

The neuritogenic effect of the homophilic L1-L1 binding has been suggested to be supported by a *cis*-interaction with NCAM in the neuronal membrane, a so-called 'assisted homophilic binding' [19]. The interaction between L1 and NCAM is thought to rely on a specific recognition of oligomannosidic glycans present in L1, by a lectin-like site in the fourth Ig-module of NCAM [20,21]. NCAM-L1 interactions involving carbohydrates have previously been suggested to be involved in long-term potentiation [22]. Recently, phosphorylation of the short, very conserved cytoplasmic domain of L1 was reported to depend on a *cis*-interaction with NCAM in connection with L1 stimulated neurite outgrowth [23] indicating a physiological importance of the interaction between L1 and NCAM [24–27].

Like L1, NCAM engages in a calcium-independent, homophilic binding [28] which has been suggested to depend on a reciprocal interaction involving the third Ig-module [29], or on all five Ig-modules of two opposing NCAM molecules [30]. However, recently, it has been shown that the first and the second Ig-modules of NCAM bind to each other in a so-called double reciprocal interaction [31]. Using NMR spectroscopy the three-dimensional structure of the first and second Ig-module of NCAM was recently solved [32,33], and the putative reciprocal binding sites were identified, providing a structural model of an anti-parallel binding between the two Ig-modules [34].

In order to elucidate the role of NCAM in L1 stimulated neuritogenesis we investigated the effect of individual NCAM Ig-modules, presumed to be involved in homophilic NCAM-NCAM binding, on neurite outgrowth induced by homophilic L1-L1 binding. Our results indicate that homophilic ligation of NCAM perturbs L1-L1 induced neurite outgrowth.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), bovine fibronectin and saponin were from Sigma-Aldrich (Vallensbaek Strand, Denmark A/S). Thy-1 monoclonal antibody was from Caltag-Laboratories (California, USA), biotinylated goat anti-mouse antibody and fluorescein isothiocyanate (FITC) conjugated streptavidin was from Dako A/S (Glostrup, Denmark). All other reagents for cell culture experiments were from Life Technologies A/S (Roskilde, Denmark) and cell culture plasticware was from Nunc (Roskilde, Denmark).

2.2. Cell culture

The rat glioma cell line BT4Cn, which is an L1 and NCAM negative subline of BT4C [35], was grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS), 2 mM GlutaMAX (L-alanyl-L-glutamine), 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone at 37°C, 5% CO_2. Stably transfected BT4Cn cells were grown in the presence of 0.8 mg/ml G418. PC12-E2 rat adrenal pheochromocytoma

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cells [36] were grown in DMEM containing 10% heat inactivated horse serum (HS), 5% FBS, 2 mM GlutaMAX, 100 U/ml penicillin and 100 μg/ml streptomycin.

Dissociated cells were prepared from the hippocampal formation of rat embryos at gestational day 17–19 (E17–E19) as described previously [37,38]. The cells were diluted to 3.3×10^6 cells/ml in neurobasal medium supplemented with 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4% w/v BSA, 2 mM GlutaMAX and B-27 supplement (50×).

2.3. Aggregation assay

For aggregation assays, 50 000 cells were seeded in a 60-well microtiter plate in a final volume of 15 μl per well. Recombinant Ig-domains were added to the medium prior to plating of the cells. The cells were maintained at 37°C and 5% CO_2. Twenty-four hour after seeding, the number of aggregates in a circular microscopic field ($\approx$0,25 mm^2) was counted using a 10×Nikon objective (Yokohama, Japan). Aggregates were defined as clusters of more than 20 cells.

2.4. Neurite outgrowth from single hippocampal neurons

For analysis of neurite outgrowth, 5000 neuronal cells/well were seeded in 8-well plastic LabTek cell culture chamber slides in a final volume of 200 μl/well. The growth surface was precoated with 1.2 μg/cm^2 bovine fibronectin. Recombinant Ig-modules were added to the appropriate wells at the time of plating. After 21 h of incubation at 37°C and 5% CO_2, images of neurons were recorded and the pictures were analyzed using computer assisted microscopy.

2.5. Neurite outgrowth from neuronal cells grown on a monolayer of cells with or without L1 expression

All neurite outgrowth experiments were performed in 8-well plastic LabTek cell culture chambers. PC12-E2 cells were seeded on top of monolayers of transiently or stably transfected glioma cells (see below). PC12-E2 cells diluted in low serum medium (DMEM containing 1% HS, (100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM GlutaMAX) were plated at a density of 1000 PC12-E2 cells/well on top of the substrate monolayers. At the same time, 100 μg/ml (10 μM) of recombinant protein was mixed into the appropriate wells and medium was added to a final volume of 200 μl/well. Twenty-four hour after plating of the PC12-E2 cells, the cells were fixed with 4% w/v paraformaldehyde in PBS. After washing, the cells were incubated with monoclonal mouse anti-Thy-1 (CD90) diluted 1:100 in PBS. After 1 h, the cells were washed and incubated with biotinylated goat anti-mouse antibody diluted 1:100 in PBS and subsequently with FITC conjugated streptavidin diluted 1:100 in PBS. Images were recorded by computer assisted microscopy. Neurite outgrowth was measured using the computer program 'Process Length', developed at the Protein Laboratory (Copenhagen, Denmark).

2.6. Transfection of BT4Cn glioma cells

For transient transfection BT4Cn cells (35 000/well on fibronectin) were transfected with the eukaryotic expression vector pcDNA3,1+ (Invitrogen, The Netherlands) containing full-length cDNA encoding the murine form of L1. Control cells were transfected with the expression vector alone. The cDNA encoding L1 was a generous gift from Dr. M. Schachner, Zentrum für molekulare Neurobiologie, Universität Hamburg, Germany. Transfection was performed in serum free OptiMEM medium by means of 'LipofectAMINE PLUS' according to the manufacturer's recommendations. Specific protein expression was ensured by immunoblotting of crude cell lysates.

For stable transfections, 150 000 glioma cells were transfected using the same constructs as those employed for transient transfection, and at 48 h after transfection the culture medium was exchanged with standard medium containing 0.8 mg/ml G418. Selected clones were tested for specific L1 expression by Western blotting and by immunofluorescence. To establish L1 expressing monolayers, 50 000 cells/well were seeded on fibronectin and grown overnight.

2.7. Preparation of recombinant immunoglobulin (Ig) domains

Recombinant NCAM Ig-modules Ig1, Ig2 and Ig3 were prepared in an eukaryotic expression system in the methylotrophic yeast *Pichia pastoris*. The preparation of Ig1 and Ig2 was done in accordance with the published protocols and proper folding of the protein was confirmed by NMR spectroscopy [32,34]. Ig3 was prepared from rat cDNA corresponding to amino acids 213–308 of the NCAM sequence, essentially as described for the modules Ig1 and Ig2. Recombinant titin from the M5 muscle specific protein was prepared from the pET3d vector containing the cDNA encoding the protein (a generous gift from A. Pastore, Heidelberg, Germany). It was purified on a Ni-resin column from Novagen (Bie and Berntsen, Denmark). Protein from this construct was confirmed to be folded correctly by NMR when an identical protocol for purification was followed.

2.8. Statistical evaluation of the results

Observations were evaluated using the paired *t*-test. All data points represent the mean of 4–6 independent experiments.

3. Results

Initially, we examined the effects of the Ig-modules 1,2 and 3 of NCAM, on aggregation of primary rat hippocampal neurons. These experiments clearly established that after 24 h of incubation, aggregation of hippocampal cells was reduced in the presence of any of the three Ig-modules in the culture

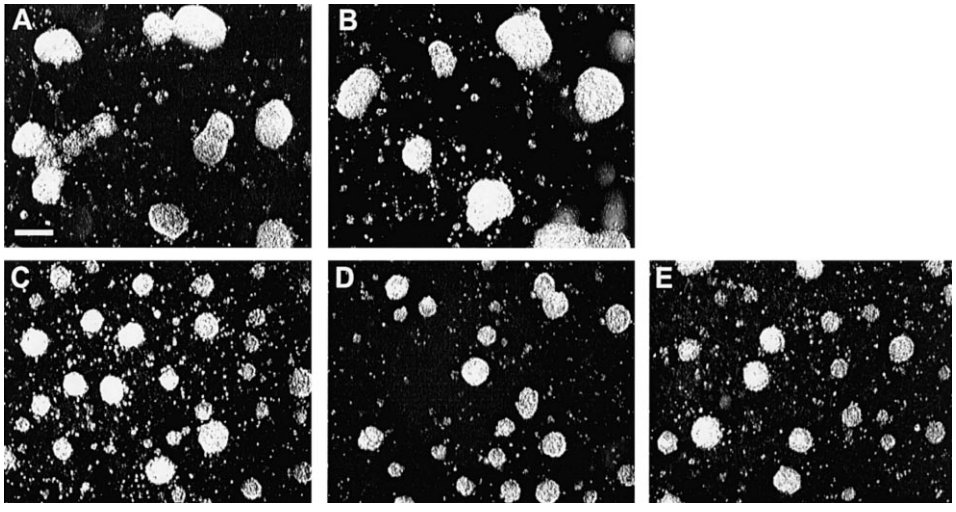

Fig. 1. Effect of recombinant NCAM immunoglobulin (Ig) modules 1, 2 and 3 on aggregation of primary hippocampal cells. Primary hippocampal cells were grown for 24 h in vitro in the absence (A) or presence of 20 μM recombinant titin M5 (B), NCAM Ig1 (C), NCAM Ig2 (D) or NCAM Ig3 (E). Bar: 200 μm.

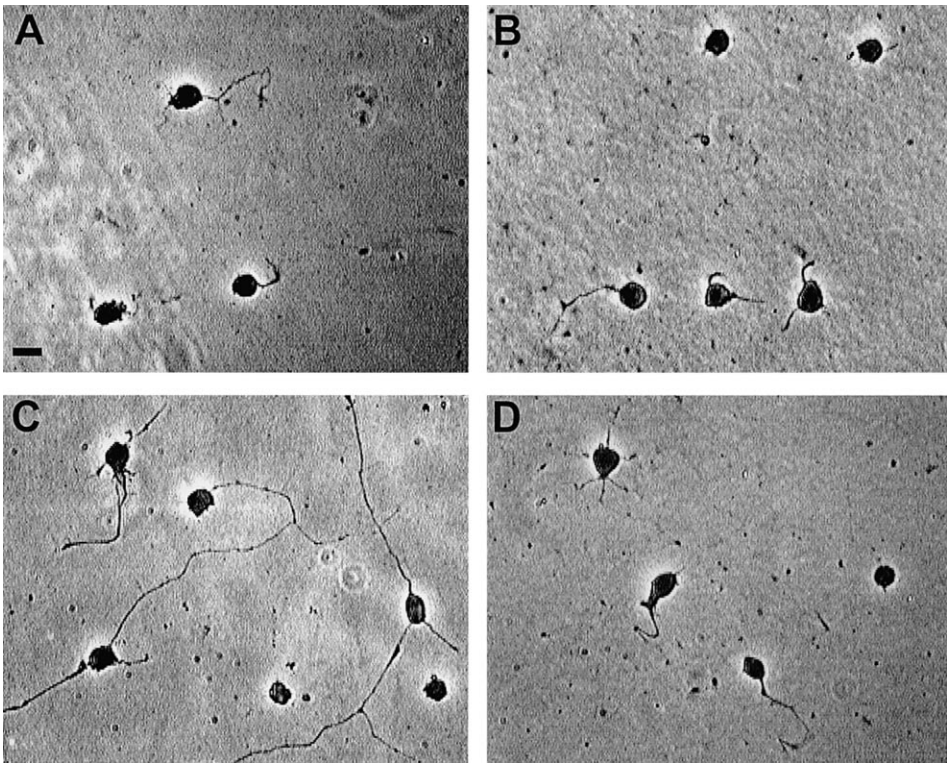

Fig. 2. Neurite outgrowth from primary hippocampal neurons grown in dissociated cell culture for 24 h in the absence (A) or presence of 20 μM NCAM Ig1 (B), NCAM Ig2 (C) or NCAM Ig3 (D). Bar: 20 μm.

medium, see Fig. 1. The addition of similar amounts of the titin M5 Ig-module, which has a similar three-dimensional structure as the Ig-modules 1, 2 and 3 of NCAM [39,40] did not alter aggregation of the cells.

Subsequently, the effect of the three N-terminal Ig-modules of NCAM on neurite outgrowth from primary hippocampal neurons was investigated. Primary rat hippocampal neurons were incubated with 20 μM of each of the recombinant modules. From Fig. 2 it appears that neither the first nor the third Ig-module of NCAM had any neuritogenic effect, whereas the second Ig-module clearly induced neurite outgrowth.

We then investigated the effect of engaging NCAM in homophilic ligation, on the presumed 'assisted homophilic L1-L1 binding'. For this purpose a coculture model based on L1-L1 homophilic binding was established. This type of experimental model allows the analysis of individual molecular binding paradigms due to the selective expression of specific molecules in the substrate monolayer cells. In the present model BT4Cn glioma cells, which are NCAM and L1 negative [41], were transfected with cDNA encoding murine L1, thus establishing the exclusive expression of L1 on the surface of the monolayer cells. Homophilic L1-L1 *trans*-binding subsequently is formed between molecules of the PC12-E2 neuronal cells and the monolayer cells.

Individual Ig-modules in a concentration of 10 μM were added to vector transfected and L1 transfected glioma cells at the time of plating of the neuronal PC12-E2 cells. In Fig. 3A and B, the effect of L1-L1 stimulation on the morphology of the PC12-E2 cells is shown, and in Fig. 3C–F the inhibitory effect of NCAM Ig1 on L1 induced neurite outgrowth is demonstrated using titin as control. From Fig. 4 it appears that in the vector transfected control cells the NCAM Ig-modules 1 and 3 did not alter the neurite outgrowth response from the PC12-E2 cells, whereas we observed a small, but nevertheless significant neurite outgrowth induction by the Ig-module 2. The neurite outgrowth response of PC12-E2 cells grown on an L1 expressing substrate monolayer exhibited a mean stimulation of $>50\%$ above basal level. Addition of titin to the L1 transfected coculture model, did not alter this effect statistically significantly. However, addition of the Ig-modules 1, 2 and 3 of NCAM clearly reduced the neuritogenic effect of L1-L1 stimulation significantly.

4. Discussion

Aggregation of primary hippocampal neurons is known to depend on homophilic NCAM binding [38]. The ability of the recombinant Ig-modules 1, 2 and 3 of NCAM to disaggregate primary rat hippocampal neurons, is therefore an indication of involvement of these Ig-modules in normal homophilic NCAM interaction. By competing for natural ligands, the recombinant Ig-modules of NCAM occupy homophilic binding sites present in the hippocampal cells, thus preventing *trans*-interactions between the cellular NCAM molecules, hereby inhibiting aggregation. The use of a titin M5 Ig-module as a negative control indicates that our observations are due to specific protein interactions between NCAM molecules, rather than merely unspecific effects.

The cellular coculture system applied in the investigation of the homophilic L1-L1 interaction stimulated neurite outgrowth from PC12-E2 neuronal cells significantly. Expressing many forms of membrane proteins, including NCAM and L1 [42], the neuronal PC12-E2 cell line is able to make various forms of molecular contacts, involving interactions between

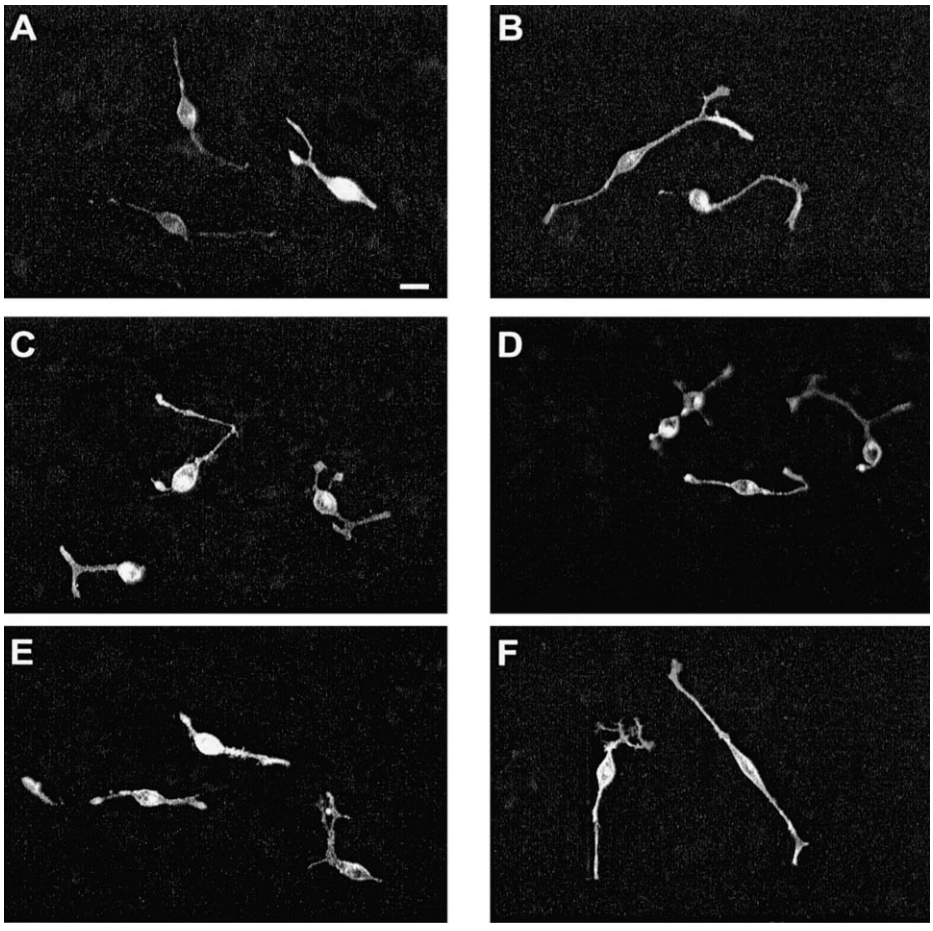

Fig. 3. Neurite outgrowth from PC12-E2 cells grown on monolayers of glioma cells without (A, C, E) or with (B, D, F) expression of mouse L1. Cultures were grown for 24 h in the absence (A, B) or presence of 10 μM NCAM Ig1 (C, D) or 10 μM titin M5 (E, F). Bar: 20 μm.

neighboring cells (*trans*-interaction) as well as between molecules at the surface of the same membrane (*cis*-interaction). PC12-E2 cells growing on a monolayer consisting of L1 expressing cells provide a system where L1 is present on both sides of the interacting surfaces enabling the establishment of homophilic *trans*-interaction between L1 molecules. In the employed set-up, NCAM, however, is only present on the membrane of the PC12-E2 cells, thus excluding homophilic *trans*-interactions, but allowing formation of *cis*-interaction with L1.

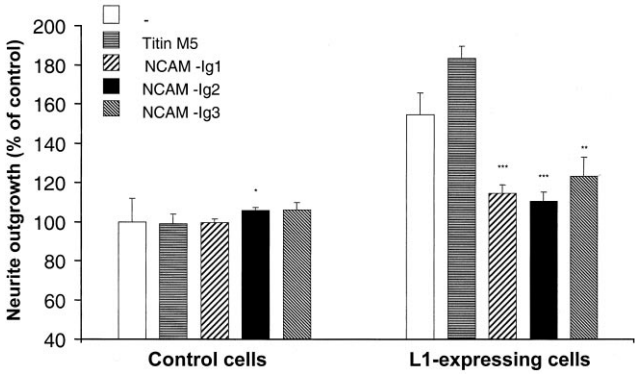

Fig. 4. Neurite outgrowth from PC12-E2 cells grown on monolayers of glioma cells without (control cells) or with expression of mouse L1 (L1 expressing cells). Neurite outgrowth is given as total neurite length per cell as percent of control. Asterisks indicate $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***), paired *t*-test, $n = 4$–7 for each construct.

To stimulate outgrowth of neurites from neuronal cells, L1 and NCAM presumably interact with the fibroblast growth factor receptor FGF-R [43,44]. However, the sequence of events leading to intracellular activation and neuronal outgrowth is unclear and the structural interaction of the CAMs with the FGF-R is at present unknown. Possibly, the interaction with the FGF-R is only one of two separate ways for NCAM and L1 to stimulate neurite outgrowth, as these CAMs might contain an intrinsic potential of signaling via their intracellular domains and separate signaling cascades.

In this study, we have shown that L1 stimulated neurite outgrowth is highly dependent on the presence of juxtaposed NCAM molecules not involved in homophilic NCAM-NCAM *trans*-ligation. The presence of Ig-modules from NCAM in the culture medium may be assumed to interfere with the L1-NCAM *cis*-interaction in various ways. When recombinant NCAM Ig-modules are added to the culture medium, ligation between these modules and their specific binding partners, available in intact NCAM protein on the neuronal cells, takes place and the interaction between L1 and NCAM is affected. This may either be due to occupation of the specific sites in NCAM interacting with L1, or due to physical hindrance of the interaction and/or conformational changes of NCAM after homophilic binding. Alternatively the

recombinant Ig-modules might interact with L1 directly, and thereby, by an unknown mechanism, inhibit its signaling. Whereas the Ig-modules 1 and 2 are not likely targets for glycosylation, the third Ig-module contains a sequence that might be glycosylated in *P. pastoris* (Asn-222, positioned between the A and A′ β-strands). Hence the Ig-module 3 might express oligomannosidic glycans which would interfere with normal NCAM-L1 interaction.

Primary hippocampal neurons, grown on fibronectin, were not affected by addition of the first and the third Ig-modules whereas the second Ig-module stimulated neurite outgrowth. Induction of neurite outgrowth was likewise observed for the second Ig-module in the coculture system, confirming the presence of a neuritogenic sequence in this module. This is in accordance with an observation made by Rønn et al. [45]. Presumably, the effect of the second Ig-domain on neurite outgrowth is separate from the mechanism that underlies NCAM's interaction with L1.

As studies of L1 stimulated neurite outgrowth previously have been performed in cellular systems using either primary neuronal cells or neuronal cell lines containing both NCAM and L1 in their membrane, it is difficult to distinguish between pure L1 stimulated neuritogenesis, and NCAM-dependent L1 stimulation of neurite outgrowth. Based on our results we propose that L1 signaling through the FGF-R, and subsequent stimulation of neurite outgrowth from PC12-E2 cells is dependent on *cis*-binding with 'free' NCAM.

In conclusion we suggest that L1 stimulation of neuronal cells is mediated by binding to NCAM in a *cis*-conformation in the membrane. This *cis*-conformation between L1 and NCAM is highly sensitive to the binding state of NCAM itself, and is neutralized when NCAM is involved in homophilic *trans*-binding. This might be part of a regulatory mechanism for the very sensitive and fine-tuned regulation of neuronal differentiation in development and regeneration.

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