

Pathological missense mutations of neural cell adhesion molecule L1 affect neurite outgrowth and branching on an L1 substrate

Ling Cheng and Vance Lemmon*

Department of Neurosciences, Case Western Reserve University, Cleveland, OH, USA

The Miami Project to Cure Paralysis, University of Miami, Miami, FL, USA

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A number of pathological missense mutations of L1CAM have been shown to disrupt L1–L1 homophilic binding and/or affect surface expression. To investigate whether these mutations disrupt L1-mediated neurite outgrowth, cerebellar neurons from L1 knockout mice are transfected with WT human L1 or L1 mutant constructs, and grown on an L1 substrate. Various parameters of neurite growth are quantified. Most L1 mutations do not affect neurite length significantly but several mutations cause a significant decrease in branching. Comparison of these data with data on L1 expression levels and homophilic binding strength show that changes in neurite growth cannot be simply explained by reductions in either of these parameters. Our results suggest that a coreceptor is involved in L1-mediated neurite outgrowth. Some pathological mutations have little effect on L1 mediated neurite growth, so it is unlikely that a failure of L1-mediated neurite outgrowth is the principle cause of brain defects in patients with L1 mutations.

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Introduction

L1 is a neural cell adhesion molecule that is critical for the development of the nervous system. L1 has been implicated in a variety of processes in neurohistogenesis, including neurite outgrowth (Lagenaur and Lemmon, 1987), axon fasciculation (Kunz et al., 1998), myelination (Haney et al., 1999) and migration of neuronal precursors (Lindner et al., 1983). Mutations in the human L1 gene cause several X-linked disorders, such as X-linked hydrocephalus, MASA syndrome, agenesis of the corpus callosum and spastic paraplegia type I and L1 syndrome (Fransen et al., 1997; Kamiguchi et al., 1998a; Kenwrick et al., 1996). Remarkably, L1 knockout mice show a phenotype strikingly similar to

human patients with L1 mutations, including hypoplasia of the corticospinal tract, corpus callosum, and cerebellar vermis, as well as hydrocephalus (Cohen et al., 1998; Dahme et al., 1997; Fransen et al., 1998a).

L1 is the founding member of a subfamily within the immunoglobulin superfamily that includes neurofascin, NrCAM and CHL1. All L1 subfamily members share a similar structural organization – an extracellular domain consisting of six Ig domains and five fibronectin type III domains, a single-pass transmembrane domain and a highly conserved cytoplasmic domain. While the 3-D structure of L1 remains to be determined, a model has been proposed with the first four Ig domains forming a horseshoe shape similar to axonin-1 and hemolin (Freigang et al., 2000; Hall et al., 2000; Su et al., 1998). In this model, the sharp bend between the second Ig domain (Ig2) and the third Ig domain (Ig3) creates a U-shaped module in which the Ig1 associates tightly with the Ig4 and the Ig2 associates tightly with the Ig3. However, an extended conformation of L1CAM has also been observed (Drescher et al., 1996; Schurmann et al., 2001). It is still unclear which conformation of L1 is active for cell adhesion (Schurmann et al., 2001).

L1 binds a variety of extracellular partners including L1 itself, other members of the Ig superfamily (Tag-1/axonin-1 and contactin/F3/F11) (Buchstaller et al., 1996; Kuhn et al., 1991), integrins (Felding-Habermann et al., 1997; Montgomery et al., 1996; Ruppert et al., 1995), neuropilin (Castellani et al., 2000, 2002), neurocan (Friedlander et al., 1994; Oleszewski et al., 1999, 2000), and perhaps other extracellular matrix components. Several extracellular interactions have been demonstrated to have physiological significance. For example, L1-neuropilin interaction seems to guide corticospinal tract axons at the pyramidal decussation (Castellani et al., 2000). L1-integrin interactions may also play a role in cell migration and myelination (Haney et al., 1999). Further, it is well known that L1–L1 homophilic interactions can stimulate neurite outgrowth, a critical process during neural development (Lemmon et al., 1989).

The L1 cytoplasmic domain binds to AP-2, ezrin, and ankyrin directly (Dickson et al., 2002; Garver et al., 1997; Kamiguchi and Lemmon, 1998; Kamiguchi et al., 1998c) and can be phosphorylated by several kinases (Schaefer et al., 1999; Wong et al.,

Abbreviations: CAM, cell adhesion molecule; KO, knockout; WT, wild-type; Ig, immunoglobulin; HL1, human L1.

* Corresponding author. The Miami Project to Cure Paralysis, University of Miami School of Medicine, Lois Pope LIFE Center, Room 4-16, 1095 NW 14th Terrace, Miami, Florida 33136. Fax: +1 305 243 3160.

E-mail address: vlemmon@miami.edu (V. Lemmon).

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1996a,b). It is now accepted that by binding to other proteins on either its extracellular domain or intracellular domain L1 can transduce signals in both directions (Long et al., 2001).

To gain further insight into how L1–L1 homophilic interactions stimulate neurite outgrowth, the set of L1-missense pathological mutations found in human patients provides a valuable tool (<http://dnalab-www.uia.ac.be/dnalab/11/index.html>). At least fifty-five missense mutations have been described, the majority of which affect the extracellular domain. These mutations account for more than one-third of the pathological mutations described and are distributed across the extracellular domain (De Angelis et al., 1999, 2002). Mutations in the extracellular domain are generally correlated with a more severe phenotype than mutations in the cytoplasmic domain (Fransen et al., 1998b; Kamiguchi et al., 1998b; Yamasaki et al., 1997). De Angelis et al. have shown that most extracellular mutations can disrupt L1 homophilic interactions. Many mutations also disrupt the heterophilic interaction with contactin/F3/F11 and TAG-1, which are GPI-anchored, structurally related members of IgCAM superfamily. Some mutant forms of L1 are retained inside the endoplasmic reticulum, instead of trafficking to the cell surface, when expressed in COS cells. Missense mutations of the extracellular domain may influence L1 function in several ways. They might induce protein misfolding, which will cause defects in intracellular processing and surface presentation of the mutant protein. Alternatively they might directly affect the binding sites, altering homophilic or heterophilic binding. Finally, they might influence tertiary structure to disrupt protein–protein interactions indirectly.

Previous work has provided information about how L1 mutations affect L1-mediated adhesion and trafficking. However, these studies did not address how L1 functions in the physiological context of axon growth. To address this issue, we established an assay to investigate how L1–L1 homophilic interactions mediate

neurite outgrowth, one of the most important functions attributed to L1 and a critical process during neural development. The assay involves introduction of pathological missense L1 mutations into neurons from L1-null (L1KO) mice, and growth of the transfected neurons on an L1 substrate. Some mutations were found to disrupt L1-mediated neurite outgrowth (neurite length or branching number) to various degrees. However, reduced homophilic binding or reduced L1 expression did not seem to be solely responsible for these defects. Our data are therefore consistent with the involvement of an unidentified coreceptor in L1-mediated neurite outgrowth.

Results

L1KO neurons transfected with wild type L1 send out neurites on L1 substrates comparable to those of wild type neurons

To establish the neurite outgrowth assay, cerebellar neurons from L1KO mice were transfected with L1 cDNA expression vectors and the transfected neurons were grown on an L1 substrate. Because L1KO neurons lack endogenous L1, all L1 expressed by these neurons is from the exogenous construct, simplifying the interpretation of experiments. Transfected neurons expressing wild-type human L1 (WT HL1) were able to attach and send out neurites on L1 substrates (Fig. 1C), consistent with previous studies showing that L1 can stimulate neurite elongation through a homophilic interaction (Lagenaur and Lemmon, 1987; Lemmon et al., 1989). In contrast, L1KO neurons transfected with a control EGFP vector have almost no neurite outgrowth on the L1 substrate (Fig. 1A). We have previously shown that L1 KO neurons are able to send out neurites on laminin in a similar manner to WT neurons, so they are not inherently unable to extend neurites (Fransen et al., 1998a, Itoh et al., 2004). To compare the transfected L1KO neurons

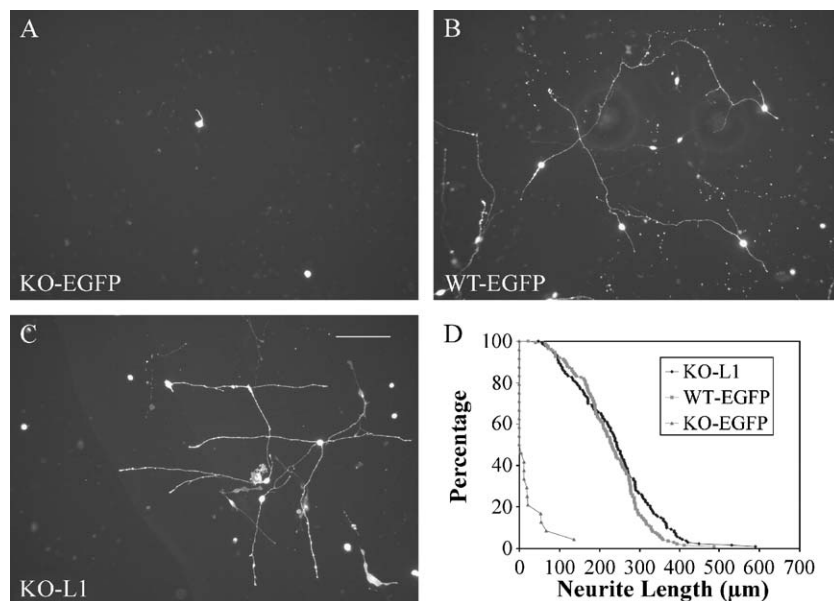


Fig. 1. L1-transfected KO neurons send out neurites on L1 substrate comparable with WT neurons. (A–C) L1KO neurons transfected with EGFP (A) or WT human L1 (C) were grown on L1 substrate. Neurons transfected with EGFP failed to send out neurites but L1KO neurons transfected with L1 sent out neurites comparable with those of WT neurons transfected with EGFP (B) (Bar = 100 μ m). (D) Distribution of neurite lengths of EGFP-transfected L1KO neurons, EGFP-transfected WT neurons and WT L1-transfected L1KO neurons growing on L1 substrate. The X-axis is the neurite length in micrometer. The Y-axis is the percentage of neurons with neurite length longer than a given length.

with normal WT neurons expressing endogenous L1, WT cerebellar neurons were transfected with EGFP and grown on L1 substrates (Fig. 1B). Neurite growth from WT neurons was comparable to that from L1KO neurons transfected with WT L1. To quantify the results, the single longest neurite length from an individual neuron was measured. The distribution of neurite lengths of the L1KO transfected with WT L1, the L1KO transfected with EGFP and the WT neurons transfected with EGFP from one representative experiment are shown in Fig. 1D. The L1KO–L1 curve tracks closely with the WT–EGFP curve, suggesting that the WT L1 expressed in KO neurons is able to support neurite outgrowth to a similar degree as the endogenous L1.

To determine if our transfection method gave an unusually high L1 expression level we transfected L1KO neurons with WT HL1 and stained them with an antibody to the L1CD, which is 100% conserved between human and mouse, and compared this to WT mouse neurons stained the same way. Ratio of the pixel values of L1KO neurons transfected with L1 to normal WT neurons is 1.09:1. A Student T test revealed no significant differences between expression levels in the WT mouse L1 and WT HL1 expressed in L1KO neurons ($p = 0.6945$). Therefore, the transfection approach does not give a higher than normal expression level.

While this system does provide a suitable way to study L1–L1 mediated neurite growth, it is not ideal for studying L1–heterophilic interactions, since those ligands, such as F3/contactin, still are expressed in the neurons. If F3/contactin is used as a substrate, for example, it still binds to F3/contactin in the L1KO neurons and stimulates neurite growth (So and Lemmon, unpublished results) so we cannot easily study those types of interactions. However, if antibodies are used to block the L1 in the neuron, substrate bound L1 does not stimulate neurite growth, arguing that other potential binding partners, such as F3/contactin, play a minor role, at best, in L1 stimulated neurite growth (Lemmon et al., 1989). It is possible that immobilized L1 does not mimic L1 in a membrane as a substrate in all physiological contexts. However, it is well established that even soluble L1 can stimulate neurite growth (Doherty et al., 1995).

We also examined the relationship between the expression level of L1 in neurites and different parameters of neurite growth and found a very poor relationship (correlation coefficients below 0.2) Fig. 2 shows the scatter grams from one representative experiment. In the following experiments, we introduced pathological missense mutations into L1KO neurons and investigated the impact of these mutations on L1-mediated neurite outgrowth.

Effects of mutations on surface expression and homophilic binding

Based on earlier work (De Angelis et al., 1999, 2001), we picked six representative pathological missense mutations for our studies. The location and the properties of the six mutations are summarized in Table 1. Because we are studying neurite outgrowth mediated by L1 homophilic interactions, the selections were based on two features: the strength of homophilic binding and the L1 surface expression level. A reasonable hypothesis is that the more similar a particular mutant is to WT L1 in terms of homophilic binding and cell surface expression, the better it will mediate neurite outgrowth. Quantitative analysis of neurite outgrowth mediated by these mutants may, therefore, provide information about how these two features impact neurite outgrowth. We quantified three parameters of neurite outgrowth—single longest neurite length, branching number, and total neurite length.

Surface expression levels in neurons correlate with those found in CHO cells

Earlier tests of surface expression of L1 mutants were done in CHO cells (De Angelis et al., 1999, 2001). In those studies, they determined the percentage of cells that expressed L1 and it was not clear whether differences observed reflected intrinsic properties of the mutants or peculiarities of heterologous expression. To solve this issue, we measured mutant L1 and WT L1 expression level in transfected cerebellar granule cells. However, measuring expression of mutant L1 has some potential problems. The most obvious is that staining cells expressing L1 mutants with antibodies could give artifactually low results if the mutation disrupts antibody binding to a particular epitope. To attempt to overcome this we stained live cells with both a monoclonal antibody against the extracellular domain of human L1 and a rabbit polyclonal antibody to human L1. Both antibodies give similar results for surface expression levels. We found that surface expression levels of L1 mutants E309K, W1036L, and S542P in cerebellar neurons were significantly reduced compared to WT L1, while levels of D598N, A462D, and I219T on the neuronal surface were similar to those of WT L1 (Table 1). These results show similarities with the results found earlier in CHO cells (De Angelis et al., 1999), and suggest that relative expression levels are properties of the mutant proteins.

We also stained fixed and permeabilized cells to measure the total amount of L1 expressed in the cells. We found there was good correlation between surface expression on neurites and total

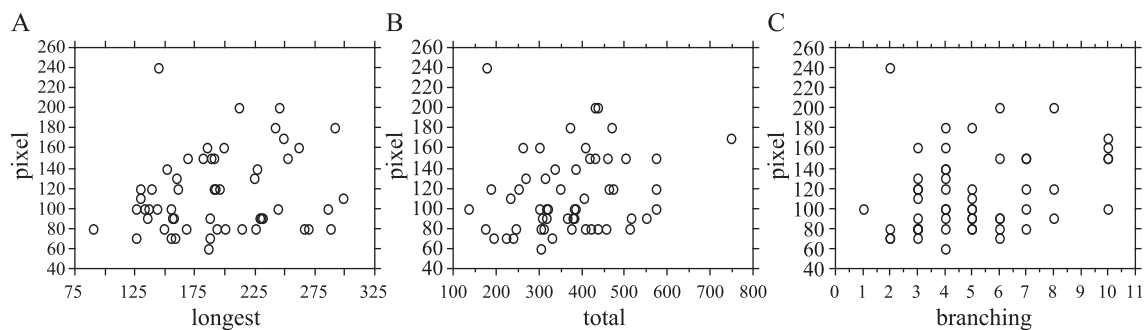


Fig. 2. Scattergrams of L1 expression versus neurite growth. (A) Average pixel intensity is plotted versus the length of the longest neurite. (B) Average pixel intensity is plotted versus total neurite length. (C) Average pixel intensity is plotted versus the number of branches. Pixel values were obtained from 8-bit images with a maximum value of 255 and length is in microns.

Table 1
A summary of the L1 binding and neuronal expression of L1 pathological missense mutations

Mutation	Domain	% L1 binding ± SEM	% CHO expression ± SEM	% Neurite total expression ± SEM	%Neurite surface expression MaHL1 ± SEM	%Neurite surface expression RaHL1 ± SEM	Severity of phenotype in humans
D598N	Ig6	82 ± 1.4	79 ± 10.2	83.50 ± 4.8%	95.78 ± 7.4%	114.34 ± 7.7%	mild ¹
A426D	Ig5	18 ± 4.0	98 ± 13.9	82.01 ± 4.0%	84.41 ± 5.5%	147.09 ± 12.0%	mild ²
I219T	Ig2	20 ± 1.5	119 ± 13.9	90.20 ± 1.9%	165.21 ± 18.4%	119.32 ± 14.1%	severe ³
E309K	Ig3	96 ± 5.8	50 ± 15.5	35.20 ± 1.1%	49.00 ± 7.2%	32.19 ± 4.4%	mild ¹
W1036L	FN5	99 ± 4.2	12 ± 3.0	38.67 ± 3.1%	28.10 ± 3.1%	23.51 ± 4.8%	severe ²
S542P	Ig6	44 ± 5.1	26 ± 7.6	36.91 ± 4.3%	20.68 ± 13.7%	37.57 ± 4.1%	severe ²

The L1 binding values and expression are expressed as mean ± SEM. The % L1 binding and % CHO expression are taken from previous publications from the Kenrick laboratory (De Angelis et al., 1999, 2002). CHO expression is the percent of transfected cells expressing cell surface L1. Neurite expression (total or surface) is expressed as a percentage compared to L1KO neurons transfected with WT HL1. Neurite total expression is from neurites fixed and permeabilized and then stained with a Mab that recognizes human L1 (7B5). Surface expression is from neurites of live cells stained with either a rabbit anti-HL1 antibody or a Mab (7B5) that binds to an epitope on the L1 extracellular domain. Severity of the human phenotype is based on the following: ¹Yamasaki et al., 1997; ²De Angelis et al., 2002; ³Saugier-Verber et al., 1998. However, the number of human individuals with these mutations is very few so strong conclusions about correlations are not possible.

expression of neurites, as assessed using permeabilized neurites. For WT HL1 the correlation coefficient was 0.96 and 0.85 in two different experiments. Even for the three mutants with reduced expression, there was good correlation between surface expression and total expression in neurites (correlation coefficient E309K:0.86, W1036L:0.83, S542P:0.75). Moulding et al also studied D598N and they found it gave reduced cell surface expression in astrocytes (Moulding et al., 2000). However they expressed L1 using virus and measured expression with Mab 5G3, which binds to an epitope that might be altered by the D598N mutation, but western blots using a rabbit anti-L1 antibody indicated equal expression to wild type L1, so these technical differences could explain the different reported expression levels.

Reduction in L1 homophilic binding does not correlate with decreased neurite outgrowth promotion

The D598N mutant has normal homophilic binding and normal surface expression levels in CHO cells and neurons (De Angelis et al., 1999, 2001) (Table 1). Neurite outgrowth from neurons expressing D598N is comparable to that seen with WT L1, for all parameters measured (Fig. 2A; Fig. 3, Fig. 4, and Supplemental data). The A426D mutant, in contrast, has significantly reduced homophilic binding (18% relative to WT) and normal surface

expression (De Angelis et al., 1999) (Table 1). Neurite outgrowth mediated by A426D is nonetheless comparable to WT L1 for all three parameters measured (Fig. 3). In fact, the single longest neurite length is even statistically greater (114%) than that seen with WT L1. The longest neurite length of another mutant, I219T, with significantly reduced homophilic binding and normal surface expression (De Angelis et al., 1999, 2001) (Table 1), is also comparable to WT L1, although the branching number of I219T is significantly reduced (see below). Therefore, large reductions in homophilic binding do not necessarily adversely affect L1-mediated neurite outgrowth.

Significant reductions in neuronal surface expression levels have little effect on L1-mediated neurite outgrowth

The E309K mutant has normal homophilic binding but the surface expression level is reduced by about 50%, in both neurons and CHO cells (De Angelis et al., 1999, 2001) (Table 1). In our outgrowth assays E309K-expressing neurons show slight reductions in single longest neurite length (93%), branching number (93%), and total neurite length (85%) compared with WT L1. The other mutant W1036L with reduced expression and normal homophilic binding also has normal longest neurite length (96%), although the branching number is significantly reduced

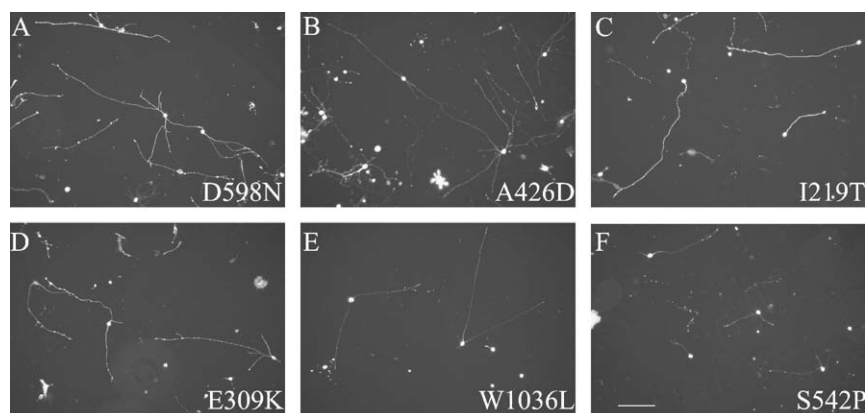


Fig. 3. L1KO neurons transfected with missense mutations send out neurites on an L1 substrate. (Bar = 100 μm). D598N mimics WT HL1 in this assay since it has normal L1 homophilic binding and similar expression levels to WT HL1. (A) D598N. (B) A426D. (C) I219T. (D) E309K. (E) W1036L. (F) S542P.

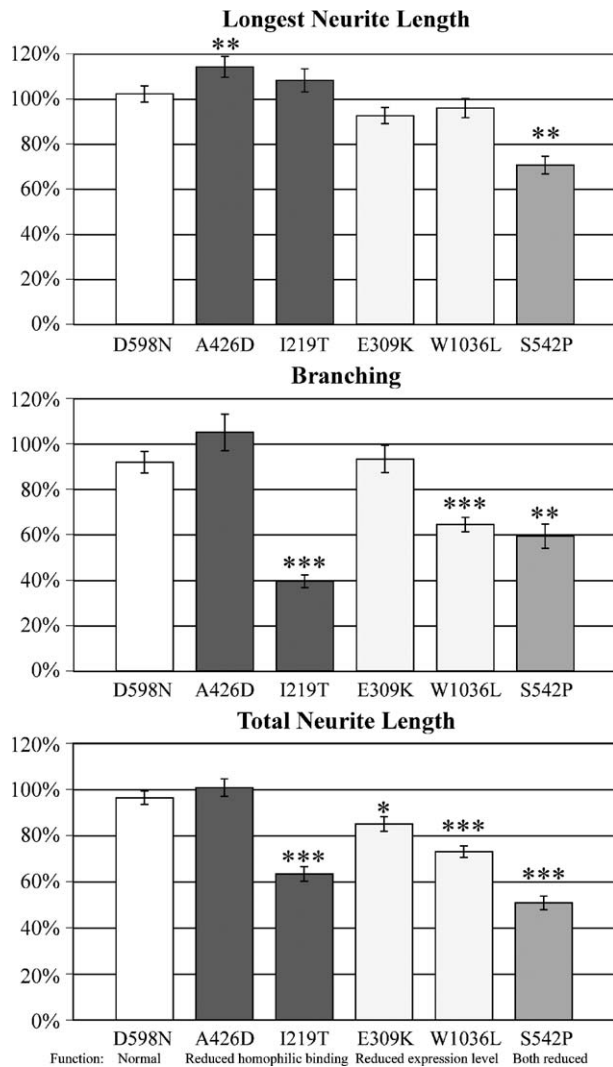


Fig. 4. The effects of pathological missense mutations on L1-mediated neurite outgrowth. The predicted function for each mutation based on L1-binding and L1-expression level is indicated at the bottom. L1KO neurons were transfected with missense mutations and grown on an L1 substrate. Total neurite length, longest neurite length and branching number were quantified. The mean value \pm SEM of the mutant-transfected neurons were always normalized by the mean values of neurons transfected with WT HL1 in the same experiment. Values shown are the average of the mean \pm SEM percentage values from three experiments. Analysis of variance (ANOVA, Fisher's PLSD) was done using Statview 4.5. Statistical significance is shown. ***: $P < 0.001$ in all three experiments. **: $P < 0.05$ in all three experiments. *: $P < 0.05$ in two of the three experiments.

(see below). These results suggest that low surface expression level has a relatively minor impact on neurite length.

L1-dependent neurite branching is regulated differently from neurite length

The W1036L mutant has normal homophilic binding but the surface expression level is significantly reduced (De Angelis et al., 1999, 2001) (Table 1). Neurite growth mediated by this mutant is affected in a complex way. The length of the single longest neurite is similar to WT (96%) but the branching number is significantly reduced (64%); most neurons have few branches (Fig. 2E). As a result, the total neurite length is significantly reduced (73%). The

I219T mutant has significantly reduced homophilic binding (20% relative to WT) and normal surface expression level (De Angelis et al., 1999, 2001) (Table 1). Expression of this mutant results in significantly reduced branching number (40%) and therefore total neurite length (63%), while the longest neurite length is slightly higher than with WT L1 (108%). Taken together, results with W1036L and I219T indicate that branching and neurite length are differentially regulated by L1, and that neither homophilic binding nor surface expression are key features determining WT branching function.

The S542P mutant mediates poor neurite growth

The S542P mutation has reduced homophilic binding (44%) and reduced surface expression level (De Angelis et al., 1999, 2001) (Table 1). Expression of this mutant results in reductions in longest neurite length (71%) and in branching number (59%). This mutation has the most severe impact on neurite outgrowth of those we tested.

Discussion

We have developed an assay to analyze the impact of L1 mutations on neurite outgrowth mediated by L1–L1 homophilic interactions. Our assay has some major advantages. First, the primary neurons we use are more relevant to the in vivo situation than cell lines, and also grow neurites rapidly. Second, we are able to express WT L1 or mutant L1 proteins in an L1KO background, thus eliminating the contribution of endogenous L1, simplifying interpretation and eliminating potential dominate-negative interactions. One concern with a transfection assay of this type is that inappropriate expression levels or distributions of L1 could make interpretation more difficult. However, we were able to show that neurons transfected with WT L1 behave like WT neurons in our assay and the expression level is very close to the endogenous L1 expression level in normal WT neurons.

Human mutations of L1, especially the missense mutations, highlight critical individual residues. Each of these mutations is associated with various neurological diseases in human patients. While it has been shown that there is a correlation between the type of mutation and the severity of the disease in humans (Kamiguchi et al., 1998b; Yamasaki et al., 1997), to understand how different mutations produce a particular phenotype, in vitro assays and functional assays need to be performed. Previous studies have investigated effects of pathological L1 mutations on homophilic and heterophilic binding as well as cell surface expression (De Angelis et al., 1999, 2001). Mutations were found to affect each of these, and it was suggested that changes in homophilic binding, heterophilic binding, and surface expression were likely to affect the contributions of L1 mutations to neuropathology. However, the binding assay was performed with recombinant proteins coated on the fluorescent microspheres. It is unclear how well this in vitro assay correlates with the in vivo situation. To investigate the effects of these pathological missense mutations in a more physiological context, we used primary neuron cultures to study the impact of mutations on L1-mediated neurite outgrowth. Of particular interest we wanted to learn if reduced homophilic binding and/or reduced surface expression altered neurite growth (Fig. 4).

In terms of single longest neurite length, our results suggest that reduced homophilic binding or reduced expression level alone

have minor effects on neurite length. Both mutations with reduced homophilic binding, A426D and I219T, have good single neurite outgrowth. The neurite lengths of the two mutations with reduced expression, E309K and W1036L, were either unaffected or slightly reduced. It seems that reducing homophilic binding by 80% or reducing surface expression by as much as 50–80% have little affect on neurite length. The results of S542P are consistent with this and suggest that there may be a synergistic effect when both the homophilic binding and surface expression level is reduced. S542P, with both reduced homophilic binding and reduced surface expression, has the worst neurite outgrowth in all six mutations. We analyzed our data from LIKO neurons transfected with WT L1 and found a remarkably poor correlation (correlation coefficients less than 0.2) between neurite growth and cell surface expression. This raises the possibility that L1 mediated neurite growth is stimulated once a certain threshold of L1 signaling inside the cell is achieved. This is consistent with work by Doherty and associates who showed that soluble L1 could stimulate neurite outgrowth at very low concentrations (Doherty et al., 1995).

The significantly reduced branching number in I219T, W1036L and S542P is unexpected. Neither reduced homophilic binding nor reduced expression level alone seems to play a role in branching. Both I219T and A426D have homophilic binding reduced to a similar degree but only I219T has a significantly reduced branching number. Similarly, both E309K and W1036L have reduced expression level but only W1036L has significantly reduced branching number.

Therefore, normal L1–L1 homophilic binding does not seem to be required for the regulation of branching. There are different ways to explain these results. One possibility is that some mutations alter L1 conformation and disrupt interactions with the intracellular machinery that regulate branching. Some intracellular proteins, especially Rho family proteins and other molecules related to cytoskeleton, have been shown to play a role in branching. Another study from our lab (Cheng et al, unpublished results) demonstrates that the L1 cytoplasmic domain (L1CD) is involved in branching and the interaction between L1 and the actin cytoskeleton is critical for the regulation of branching. Some extracellular mutations, such as I219T and W1036L, may alter the interaction between L1 and the actin cytoskeleton by changing L1 conformation. All the extracellular mutations studied in this paper have an intact cytoplasmic domain, suggesting that L1CD is not the only regulator of branching. Another possibility is that branching is regulated by L1 interacting in cis with some coreceptor via the L1 extracellular domain, presumably through some heterophilic interactions. It is possible that I219T and W1036L may disrupt interactions with the coreceptor or other proteins that interact with L1 in cis and can thereby regulate branching. Another mutation S542P, with both reduced homophilic binding and reduced surface expression, also has significantly reduced branching number. Although reduced homophilic binding or reduced surface expression alone does not seem to affect branching, the combination of both may have a synergistic effect. However, I219T, with normal expression, did reduce branching more than S542P. Finally, some mutations could disrupt L1–L1 cis interactions that could somehow regulate branching.

Our results are not the first to argue for a coreceptor being involved in L1 mediated neurite growth. A number of candidates have been proposed. TAG-1 has been implicated in L1 mediated neurite growth and guidance (Buchstaller et al., 1996). The FGF receptor has also been implicated in L1 mediated neurite growth

(Williams et al., 1994). Neuropilin has been shown to interact with L1 and is important in axon guidance (Castellani et al., 2000). Recently the EGF receptor has been implicated in L1 mediated neurite growth (Islam et al., 2004). Other candidates include EphA4 (Prevost et al., 2002) and integrins (Yip et al., 1998). So there is a substantial set of potential coreceptors and more than one may be involved in the processes we are examining. Given the difficulty of interpreting dominant-negative experiments, it may be necessary to undertake experiments using double-knockouts or RNA knockdown to narrow the list.

This work extends our understanding how these pathological mutations might affect L1 function. All of these mutations cause abnormal brain development in humans although the severity of the problems can vary. Some mutations disrupt L1-mediated neurite outgrowth in our assay and others do not. But all are associated with MASA syndrome or X-linked hydrocephalus. This suggests that the defects in brain development are not exclusively due to a failure of L1 mediated neurite extension. Interestingly, we have recently studied a novel mouse line, the L1-6D line in which the 6th Ig domain of L1 is removed (Itoh et al., 2004). This abolishes L1–L1 binding as well as interactions with some integrins. Amazingly the brains of these mice are substantially normal on the 129sv genetic background while the brains of LIKO mice on the same background are highly abnormal. This independent evidence strongly suggests that loss of L1–L1 based neurite extension is not the underlying basis of brain defects in X-linked hydrocephalus. However, if L1-mediated branching is important in axon guidance functions, the reduced branching we see could have important implications for the correct formation of neural pathways. Interestingly, the three mutations that give reduced branching (I219, S542 and W1036) appear to have more severe consequences than the other three mutations (Table 1). The numbers of affected humans with these mutations are very small so strong conclusions are not warranted. Nonetheless it raises the possibility that L1-mediated branching is a critical aspect of brain development.

In conclusion, we have demonstrated that the six pathological missense mutations on the L1 extracellular domain have distinct effects on L1–L1 homophilic binding-mediated neurite outgrowth. The single longest neurite length does not seem to be affected by either reduced homophilic binding or reduced expression level alone, although the combination of the two seems to have a synergistic effect. Neurite branching is mediated differently from neurite length as loss of L1–L1 homophilic binding or decrease in L1 expression level does not correlate with significantly reduced branching. We propose that a coreceptor for L1-mediated neurite outgrowth is involved and some mutations affect neurite outgrowth or branching by disrupting the interaction with the coreceptor. The fact that mutations with minor effects on L1-mediated neurite outgrowth can nonetheless cause severe phenotypes in human suggests that L1-mediated neurite extension is not the principle cause of brain defects in patients with L1 mutations.

Experimental methods

Materials and animals

The monoclonal anti-human L1 antibody (7B5) against human L1 extracellular domain was produced in the laboratory. The rabbit polyclonal anti-chicken L1 antibodies were described previously

(Lemmon and McLoon, 1986). Fluorescent secondary antibodies were purchased from Molecular Probes, Inc. Tissue culture reagents were purchased from Gibco. Mouse neuron nucleofector kit was from Amaxa. Chick L1 was purified as described previously (Lagenaur and Lemmon, 1987). Coverslips were purchased from Corning Inc. Chemicals are purchased from Sigma and Pierce. The CWRU and Univ. of Miami Animal Care and Use Committees approved all experiments using mice. L1 knockout mice used have been described previously (Fransen et al., 1998a).

DNA constructs

The wild-type human L1 vector in pcDNA3 was described previously (Wong et al., 1995). All the L1 mutant constructs were described previously (De Angelis et al., 1999, 2001).

Preparation of substrate

For these experiments we used Chick L1 (NgCAM, 8D9 antigen) (Hortsch, 2000) for technical reasons. This allows us to study L1 mediated neurite growth and look at L1 expression in the neurons without having the antibodies bind to the substrate and obscure the neurons and neurites. The L1 mutations we study in this paper have conserved residues in Chick NgCAM. Chick L1 was coated to silanized coverslips by a covalent cross-linking method. Clean coverslips (Corning, No.1, 22 × 22 mm) were silanized by incubation in 5% 3-aminopropyltriethoxysilane for 30 min at room temperature. Silicon gaskets (Grace Biolabs) were used to create L1 spots on the coverslips. Purified chick L1 (100 micrograms/ml) was incubated with 0.1 mM EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and sulfo-N-hydroxysulfosuccinimide for 15 min before coating the coverslips. EDAC-attached chick L1 was then added to the coverslips and incubated for 2 h at room temperature. After 2 h, gaskets were removed. Coverslips were washed extensively and blocked with 5% hemoglobin for 1 h at room temperature before plating cells. This method gives improved optics over the nitrocellulose method and also reduces the large variations in concentration that can occur with proteins spotted on nitrocellulose.

Cell culture and electroporation

P8 mouse cerebella from wild type mice or L1 KO mice were dissected and dissociated as described previously (Beattie and Siegel, 1993). Cells were counted and resuspended in nucleofector solution (Amaxa) to a final concentration of $2\text{--}4 \times 10^6$ cells per 100 μl nucleofector solution. 10 μg DNA was added to each sample and electroporation was done with the Nucleofector machine (program G13). Cells were immediately added to dishes with L1-coated coverslips. Cells were grown for 48 h in DMEM, supplemented with 10% fetal bovine serum, Glutamax, sodium pyruvate, HEPES, 25 mM KCl and antibiotics.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 1 h at 4°C followed by permeabilization with 0.03% Triton-X-100 in PBS for 1 h at room temperature. Cells were then incubated with primary antibodies (the monoclonal anti-human L1 antibody 7B5 supernatant + 1:500 rabbit anti-chick L1 (to stain the substrate L1)) for 1 h at room temperature, followed by incubation with secondary

antibodies (1:200 Oregon green 514 anti-mouse IgG + 1:200 Texas red-X anti-rabbit IgG) for 1 h at room temperature. The coverslips were mounted onto slides with the SlowFade light kit (Molecular Probes).

Image acquisition and analysis

Images were acquired with a Spot RT slider camera (Diagnostic Instrument Inc.) mounted on a Leica DLMB microscope with a 20× objective (N.A. = 0.7). Images were analyzed with Image J (NIH), or Neurolucida (MicroBrightField Inc.) and figures prepared with Adobe Photoshop 7. To facilitate comparison, mutant samples and the wild-type L1 samples were always analyzed for neurite growth in each experiment. A neurite was defined as a process extending from a neuronal cell body by more than two cell diameters. Only neurites that emerged from a single isolated neuron and did not contact other neurites or cells were chosen for quantification. The percentage of neurons with neurites longer than a given length was plotted versus neurite length (Chang et al., 1987). Neuron tracing was done manually with the Neurolucida program (MicroBrightField Inc.). Analysis of total neurite length, single longest neurite length and branching number was done in the NeuroExplorer program (MicroBrightField Inc.). Total neurite length is the sum of all neuritic branches elaborated by a single neuron. A branching point is the point where a neurite extends from the cell body or from another neurite. To be considered, a branch had to be at least 2 times the soma diameter, so we were unlikely to be measuring filopodia. Branching number is the sum of every branching point from a single neuron. About 80–100 neurons were analyzed for each construct in one experiment and each construct was tested at least three times (see supplemental data). Analysis of variance (ANOVA, Fisher's PLSD) was done using Statview 4.5.

Measurement of expression level

Transfected L1KO neurons were stained 2 different ways. 1) To label L1 expressed on the cell surface, live cells were stained with either a monoclonal antibody, 7B5, to the extracellular domain of Human L1 or with a rabbit polyclonal antibody to Human L1. Live cells were incubated with primary antibody for 1 h at 4°C. The cells were washed and then fixed with 4% paraformaldehyde and incubated with secondary antibodies. 2) To label both the L1 expressed on the cell surface and the total amount of L1 expressed in the cell, live cells were incubated with the primary antibody (7B5 or rabbit anti-human L1) for 1 h at 4°C. Cells were then fixed and permeabilized, followed by incubation with the other primary antibody (rabbit anti-human L1 or 7B5). Cells were incubated with secondary antibodies (1:200 Oregon green 514 anti-mouse IgG + 1:200 Texas red-X anti-rabbit IgG) for 1 h at room temperature. The coverslips were mounted onto slides with the SlowFade light kit (Molecular Probes). To compare WT HL1 expression in transfected L1KO neurons and the normal L1 expression level in WT neurons, transfected L1KO neurons and control WT neurons were fixed and permeabilized, followed by staining with a rabbit polyclonal antibody against the L1 cytoplasmic domain, which is 100% conserved between human and mouse.

All images were acquired on a Leica DLMB microscope with a 20× objective (N.A. = 0.7) coupled to a Spot RT slider camera (Diagnostic Instrument Inc.), with the same exposure time corresponding to unsaturated acquisitions. Pixel values on the

neurite were quantified at six different locations on the shaft of the longest neurite with NIH ImageJ. The average pixel value was determined for each neuron and the background was subtracted. Approximately 20 neurons for each mutation in the same experiment were quantified. Because absolute fluorescence intensity varied among different experiments, the values for the mutant-transfected neurons were always normalized by the values of neurons transfected with WT HL1 stained with the same condition in the same experiment. To compare WT HL1 expression in transfected L1KO neurons and the normal L1 expression level in WT neurons, pixel values of transfected L1KO neurons were normalized by pixel values of control WT neurons stained with the same condition in the same experiment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.mcn.2004.08.005](https://doi.org/10.1016/j.mcn.2004.08.005).

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