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Neural cell adhesion molecule L1 modulates type I but not type II inner ear spiral ganglion neurite outgrowth in an *in vitro* alternate choice assay

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Abstract

L1, a neural cell adhesion molecule of the immunoglobulin superfamily, is widely expressed in the nervous system and important in axonal outgrowth, guidance, synapse formation, and signaling. Gene deletion studies emphasize the significance of L1 during development of the central nervous system and L1 is crucial for the topographic targeting of retinal axons.

In contrast to the brain and retina, the role of L1 in the inner ear is largely unknown. While previous studies have localized L1 in the developing inner ear of the chicken and mouse, its function during the innervation of the cochlea still remains largely unclear. We therefore investigated the functional role of L1 in the mammalian inner ear. Our aim was to determine whether or not L1 can modulate type I and/or type II spiral ganglion neuron outgrowth using an *in vitro* alternate choice assay. We found that L1, presented in stripe micropatterns, provide directional cues to neonatal rodent type I but not type II inner ear spiral ganglion neurites.

The results suggest that L1 may play a role in axonal pathfinding of type I spiral ganglion dendrites toward their inner hair cell targets, but not of type II toward the outer hair cells.

Keywords

axonal guidance; axonal outgrowth; inner ear; L1; spiral ganglion

Introduction

Neural cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily are important mediators of nervous system development with multiple functions as guidance cues and receptors for neurons and their axons [reviewed in Brummendorf and Rathjen, 1996]. L1 is such a neural CAM of the Ig superfamily. It is widely expressed in the nervous system and is important in axonal outgrowth, guidance and fasciculation (Burden-Gully et al., 1997; Castellani et al., 2000; Kamiguchi, 2003; Whittard et al., 2006; Chen et al., 2007),

synapse formation (Godenschwege et al., 2006), and signaling (Panicker et al., 2003). Exogenously applied L1 enhances the growth of neuronal axons *in vitro* (e.g. Chen and Charness, 2012), while cells expressing L1 have been found to promote spinal cord regeneration (He et al., 2012).

Gene knockout studies also emphasize the significance of L1 during development of the central nervous system. Mice deficient in L1 showed dilated ventricles, vermis hypoplasia, impaired exploration patterns (Fransen et al., 1998), and abnormalities in neural process extension and hippocampal development (Demyanenko et al., 1999). Furthermore, mutations in the human L1 gene are responsible for an X-linked neurological disorder with major deficits in brain development, including hydrocephalus, agenesis or hypoplasia of the corpus callosum and corticospinal tracts, mental retardation, spastic paraplegia, and adducted thumbs (Kenwrick et al., 2000; Weller and Gärtner, 2001; Schäfer et al., 2010). The abnormalities of neural growth due to loss of function of the L1 protein testify to the importance of L1 in nervous system development.

It was previously shown that L1 is crucial for the topographic mapping of retinal axons to their targets in the mouse superior colliculus. Temporal retinal axons of L1 null mice bypassed correct target locations in the anterior superior colliculus, forming termination zones at incorrect posterior sites – indicating that retinal axons require the function of L1 to achieve proper target identification (Demyanenko and Maness, 2002). The retina shares many common characteristics and developmental determinants with the cochlea, the auditory end organ. Both the retina and the inner ear arise from neuroepithelium and harbor sensitive sensory cells together with supporting cells. Both organs display a complex and highly organized microarchitecture. Therefore, it is not surprising that the molecular events involved in axon growth and pathfinding in the inner ear share similar features to those in the retina.

In contrast to the situation in the brain and retina, the role of L1 in the inner ear is largely unknown. Previous immunocytochemical and immunohistochemical studies have localized L1 in the developing inner ear of the chicken and mouse (Hrynko et al., 1998a, 1998b; Mbiene et al., 1989; Whitlon et al., 1999). At the time of late innervation of the cochlear sensory epithelium in the chicken embryo, L1 was present on auditory ganglion neuron dendrites, which extended to the presumptive synaptic zone beneath the hair cell layer in the basilar papilla. However, L1 was not observed during the early phase of papilla innervation (Hrynko et al., 1998a). Anti-L1 Fab fragments produced a defasciculation of fibers in chick co-cultures of auditory ganglion and basilar papilla *in vitro* at early to intermediate phases of innervation of the embryonic otocyst (Hrynko et al., 1998b). Based on their observations, Hrynko et al. hypothesized that L1 may be involved in fiber penetration into the otic epithelium, target cell recognition, and synapse formation.

In the newborn mouse cochlea, L1 was localized on nerve fibers in the spiral ganglion (SG) and along the spiral lamina. Interestingly, in the organ of Corti (OC), L1 labeling was observed only in the inner hair cell (IHC) region. There was no labeling in the region of the outer hair cells (OHCs). At postnatal day 7 (p7), strong labeling was observed along the dendrites leading up to the IHCs and in the IHC region, the inner spiral plexus. In contrast to the IHC region, there was only weak L1 labeling beneath the OHCs (Whitlon et al., 1999).

Given the importance of L1 in nervous system development and its crucial function to achieve proper topographic mapping in the retina, we decided to investigate the functional role of L1 in mammalian inner ear innervation, during a period when the projections of afferent dendrites from these bipolar neurons approach their mature configurations and segregation between hair cell types in the OC (Defourny et al., 2011; Barclay et al., 2011).

The aim of this study was to investigate whether or not L1 is able to modulate type I and/or type II SG neuron fiber outgrowth in an *in vitro* alternate choice assay. Based on the distribution of immunolabeling observed by Whitton et al. (1999), we hypothesized that only type I SG neurites would respond to L1.

Materials and Methods

Preparation of tissue culture plates

To prepare stripe molds, templates were generated by photolithography to produce parallel stripes of photoresist compound 100 μm wide, 100 μm high, and 100 μm apart. Silicone rubber (Sylgard®184 Silicone Elastomer, Dow Corning Inc., Midland, MI, USA) was then applied to the templates to generate stripe molds. After sterilization with 100% ethanol for 20 min and air drying inside a sterile hood (The Baker Company Inc., Sanford, ME, USA), 4 $\times$ 7 mm pieces of the resultant silicone molds were placed groove side down into the center of individual wells of a 24-well cell culture plate (Costar®, Corning Inc., Acton, MA, USA), as described previously by Evans et al. (2007). As an initial step, 5 μl of Laminin (40 $\mu\text{g}/\text{ml}$ in phosphate buffered saline (PBS), Gibco by Invitrogen, Carlsbad, USA) from Engelberth-Holms sarcoma (Sigma-Aldrich, St. Louis, USA) was applied to the edges of the mold that were parallel to the channels for 2 hours. With the mold in place, the wells were then rinsed 3 $\times$ with PBS. Immediately thereafter, 50 μl of L1 protein solution (R&D Systems, Minneapolis, USA, 1 $\mu\text{g}/\text{ml}$ in PBS) was placed along one edge of the mold perpendicular to the channels, and drawn into the mold's channels by suction applied from the other side of the mold. The plate was then incubated overnight at 37°C. Each well was then washed twice with PBS with the molds in place as described above. The molds were removed, followed by an additional wash with PBS. Wells were then filled with 300 μl of poly-L-lysine solution (PLL, 5 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and incubated at 37°C for 1h. The wells were washed twice with PBS. Since it is unlikely that L1 would have occupied all of the potential binding sites on the culture surface, it is therefore likely that L1 stripes contained PLL as well. For control experiments, molds were treated identically except that PBS alone was substituted for the L1 solution.

To prepare uniformly coated 24-well cell culture plates (Costar®, Corning Inc. USA), wells were filled with different concentrations of L1 solution (0.1 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$, 5 $\mu\text{g}/\text{ml}$, respectively) and incubated overnight at 37°C. The wells were washed twice with PBS. Controls were filled with PLL solution (5 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and incubated at 37°C for 1h and washed twice with PBS.

Prepared wells were then filled with 170 μl of primary attachment medium, containing DMEM, 10% fetal bovine serum (Sigma-Aldrich), 25mM HEPES buffer (both Gibco) and 300 U/ml Penicillin (Sigma-Aldrich).

Spiral ganglion dissection

The local animal subjects committee of the VA San Diego Healthcare System approved the surgical procedures in accordance with the guidelines laid down by the National Institute of Health regarding the care and use of animals for experimental procedures. Neonatal (postnatal day 5, p5) Sprague–Dawley rats (for type I SG neuron experiments) or neonatal (p2) C57/BL6 mice (for type II SG experiments) were euthanized. The temporal bones were removed and further dissected similar to the method described by Van de Water and Ruben (1971). Briefly, the cochlear capsule was opened and the membranous labyrinth was removed from the modiolus. The spiral lamina containing the SG was carefully separated from the modiolus and transferred immediately into primary cell culture medium, where it

was then cut into equal portions of 300 μm to 500 μm before transfer into the prepared culture plates.

Cell Culture

SG explants was placed in the center of each well, on the L1, or control stripe pattern. They were first incubated for 24h at 37°C in the primary attachment medium, before the culture medium was changed to serum-free maintenance media (DMEM (Gibco), 25mM HEPES-Buffer (Gibco), 6mg/ml glucose (Gibco), 300 U/ml penicillin (Sigma-Aldrich) and 30 $\mu\text{l/ml}$ N2-supplement (Gibco). For trophic support of SG neuron survival and optimization of neurite outgrowth in the stripe assays, maintenance medium was supplemented with 10 ng/ml (for rat SG neuron experiments) or 30 ng/ml (for mouse SG neuron experiments) of recombinant BDNF (R&D Systems, Minneapolis, MN, USA), which we have found to be optimal dosages for each species (Barclay et al., 2012; Kondo et al., 2013). Cultures were kept in a humidified incubator at 5% CO_2 and 37°C for 72h.

Immunohistochemistry

After fixation with 4% paraformaldehyde for 20 min at room temperature (RT) and two washes with PBS (Gibco), the explants were permeabilized with 5% triton X-100 (Sigma-Aldrich) for 10 min, washed twice with PBS and blocked for non-specific antibody binding with 5% donkey serum (Sigma-Aldrich). Neurites were labeled for neurofilament using a rabbit polyclonal 200kDa anti-neurofilament primary antibody (1:400; Sigma-Aldrich), or a polyclonal chicken anti-peripherin antibody (1:1000; Thermo Scientific, Rockford, IL, USA). L1 stripes were visualized using polyclonal rabbit antibodies against L1 (1:400; Sigma-Aldrich). Laminin along the mold edges was visualized using a rabbit polyclonal antibody against laminin (1:400; Sigma-Aldrich) After primary antibody incubation overnight at 4°C, followed by two PBS washes, the neurites and stripes were visualized by 2.5h of incubation with fluorescein isothiocyanate (FITC) or Texas-red (TR) conjugated secondary antibodies (1:100; Jackson ImmunoResearch, West Grove, PA, USA) against the species of the respective primary antibody. Specificity of staining was confirmed by a series of negative control staining without primary antibodies.

Data Analysis

Digital images were obtained on an inverted fluorescence microscope (Olympus FSX100, Center Valley, PA, USA) equipped with appropriate excitation and emission filters for FITC and TR. For the stripe assay, stripes were either directly visualized using the L1 antibody, or their locations were determined from the laminin placed along the mold edges. In the latter case, necessary when L1 was omitted as a control, a stripe image produced using L1 without an explant was merged with the explant image using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) to indicate the position of the stripes. For publication in this manuscript, images were optimized to achieve uniform brightness and contrast using Photoshop. The number of neurites terminating on each individual stripe substrate was counted. Data for each experiment showed normal distribution (in a Kolmogorow-Smirnow test) and homogenous variances so that data were compared by Mann-Whitney-U-test using Statview 5.0 (StatSoft Inc., Tulsa, OK, USA). Neurite outgrowth from the SG was evaluated by measuring the number and lengths of the processes. Images of the immunostained cultures were analyzed by using NIH ImageJ software (NIH, Bethesda, MD, USA). Each neurite was traced and the number of neurites and average lengths of neurites per explant were analyzed using a one-way analysis of variance followed by a Bonferroni-corrected least-significant-difference post-hoc test. Data presented in the text and figures are means and standard deviations. Results were considered to be significant when the likelihood for a type 1 error was less than 5% ($p < 0.05$). 16-20 SG explants were analyzed per experimental condition.

Results

Preferential termination of neonatal rat SG neurites on L1 versus PLL stripes, and neurite extension along L1 stripes

More neurites extending from rat SG explants terminated upon L1 stripes (1 $\mu\text{g/ml}$) than on PLL stripes (5 $\mu\text{g/ml}$) substrate (Figure 1, Mann-Whitney-U-test, $p < .0061$). In addition, many (but not all) neurites turned onto, and then elongated along, L1 stripes (Figures 2,3). Omission of L1 in the L1 coating solution (PBS control), served as a negative control. In this condition, there was no significant difference in the number of SG neurites ending on the stripe regions of the control pattern (Figure 1, Mann-Whitney-U-test, $p = .5004$). Explants grown on control patterns showed a radial outgrowth pattern and did not turn on or otherwise react to the pattern (Figure 4). In the L1 stripe condition, some neurites that did not terminate on L1 were observed to be noticeably thinner than the majority of fibers (e.g. Figure 3). While this morphology is consistent with Type II SGN fibers, in the absence of a specific labeling agent we could not identify the type of these neurites. However, Type II SGNs make up only 5-10% of the ganglion at this age (Nayagam et al., 2011; Barclay et al., 2012). Given the high proportion of neurites that responded to L1 stripes, response by Type I fibers can safely be inferred. To address the issue of Type II neurites, we used P2 explants from mouse, in which Type II neurons can be differentiated in culture using peripherin labeling, and in which Type II neurons make up the majority of neurites that are extended from explants (Barclay et al., 2012).

No preferential termination on or extension along L1 versus PLL stripes by neonatal mouse type II spiral ganglion neurites

In contrast to type I SG neurites, type II SG neurites showed no preferential tendency to end on either L1 (1 $\mu\text{g/ml}$) or PLL (5 $\mu\text{g/ml}$) stripes (Figure 5, Mann-Whitney-U-test, $p = .69044$). In addition, there was no evidence of stripe tracking by type II SG neurites (Figure 6). Omission of L1 in the L1 coating solution, serving as a negative control, showed the identical result (data not shown).

L1 does not influence type I SG neurite number or length

Because L1 influences neonatal rat neurite direction and/or termination preference, we evaluated whether it might also affect neuritogenesis or neurite growth rate. When SG explants were cultured on a uniform L1 coated surface (0.1, 1 or 5 $\mu\text{g/ml}$, respectively), the number of neurites per explant did not differ from the number on control explants grown on 5 $\mu\text{g/ml}$ PLL (Figure 7, ANOVA, $p > 0.05$ for all conditions). Figure 8 shows a representative image of an explant cultured on 5 $\mu\text{g/ml}$ L1 (Figure 8a) and of an explant cultured on 5 $\mu\text{g/ml}$ PLL (Figure 8b). Similarly, the length of neurites did not differ between growth on L1, as above, and control explants grown only on 5 $\mu\text{g/ml}$ PLL (Figure 9, ANOVA, $p > 0.05$ for all conditions).

Discussion

At present, relatively little is known about the function of L1 in the inner ear. Hrynyk et al. (1998b) showed in antibody perturbation experiments that L1 inhibition produced defasciculation of chick auditory ganglion fibers but did not affect neurite outgrowth. In the developing mouse cochlea, immunohistochemistry demonstrated differences in the expression of L1 in the IHC and OHC region. Interestingly, L1 labelling was much stronger in the area near the IHC when compared to the OHC region (Whitlon et al., 1999). The authors suggested that molecular differences at or near the cell surface of IHCs and OHCs may be one way in which growing neurites distinguish different environments to determine

their growth routes and synaptic partners in the cochlea. The source of L1 would presumably be the supporting cells through which the neurites extend to reach the HCs.

There are two subtypes of SG neurons, the type I and type II SG neurons (e.g. Reid et al., 2004; Nayagan et al., 2011) - which have different cellular targets and functions. The dendrites of type I cells form afferent synapses exclusively with the IHCs, while the dendrites of type II cells exclusively receive synapses from the OHCs (Reid et al., 2004). Given the limited data available regarding the function of L1 in the inner ear and the differences in the expression of L1 in the IHC and OHC regions of the OC, the aim of our study was to analyze whether or not L1 is able to modulate type I and/or type II SG neurite outgrowth in an alternative choice assay, *in vitro*. Due to its expression pattern, we hypothesized that L1 can serve as a guidance cue to type I SG neurons, but not to type II SG neurons, in culture.

It should be noted that in our experiments, cochlear explants were harvested from different species and at different time points. For the type I SG neurons experiments we used p5 rats in which type I SGNs make up >90% of the ganglion. The rationale for this was that the larger cochlea of the rat allowed more tissue to be harvested, thus fewer animals needed to be sacrificed for the experiments. We initially attempted to distinguish type II neurons extending from rat explants using anti-peripheral antibody labelling. However, unfortunately peripherin labelling did not distinguish between type I and type II neurons in the rat *in vitro*, due to up-regulation of peripherin in type I neurons (Lallemend et al., 2007). However, *in vitro* studies have confirmed the utility of peripherin as a marker for type II SG neurites in the mouse (Mou et al., 1998; Schimmang et al., 2002; Huang et al., 2007; Barclay et al., 2012). Therefore, we used the mouse for type II SGN studies. In addition, since the proportion of type II SG neurons rapidly decreases during the first postnatal week, and type II neurons survive preferentially in culture when exposed to BDNF, p2 mice were used to maximize collection of type II data. However, the species and modest age mismatch of the neurons is a weakness of the current study.

In our *in vitro* alternative choice assay, P5 rat SG neurites were attracted by L1 stripes, whereas P2 mouse type II neurites showed no response to L1 stripes. This evidence is consistent with modulation of afferent dendrite outgrowth from type I, but not from type II SG neurons. It can be noted that L1 is positioned to serve as a factor for separating the projections of type I and type II SG dendrites *in vivo*, after they have exited the habenula perforate into the OC (Whitlon et al., 1999). Thus, L1 may serve to attract type I dendrites toward the IHCs while allowing the type II dendrites to pass unaffected toward the OHCs.

The control condition used in the present experiment was the elimination of L1 from the solution used to generate stripe patterns. This raises the possibility that the mere presence of any protein might provide guidance signalling in comparison to PLL. However, we have previously found that stripes of the neutral protein bovine serum albumin do no influence either SG neurite termination or direction (Evans et al., 2007).

The mechanism by which L1 influences type I SG neurite growth is less clear from our study. It might be argued that the apparent preference of L1 versus PLL is due to a slower growth rate of type I SG neurites on the L1 surface compared to PLL. Therefore, the type I SG neurites would spend more time on the PLL surface and be found more often on the PLL substrate. However, the fact that increasing coating concentrations of L1 on a uniform surface compared to PLL controls did not influence type I neurite growth rate argues against this (Figure 9). Another potential explanation for a different distribution of the type I SG neurites on the stripes is branching. If the substrate on one stripe increases branching of the neurites in response to the substrate, more type I SG neurites might be found on that

particular stripe. However, again, increasing coating concentrations of L1 on a uniform surface did not influence type I neurite number compared to PLL controls (Figure 7). Finally, the observation of preferential stripe tracking along L1, as observed in type I neurites, is inconsistent with either mechanism (Figure 3).

In our stripe assay experiments, we used organotypic explants from the cochlear SG. Besides neurons, the explants contained supporting cells, including fibroblasts and Schwann cells, which reside between the neurons *in vivo*. Both cells are known to provide guidance cues to advancing neurites and growth cones, and therefore might have influenced the observed neurite patterns in our experiments. However, these supporting cells grew out in a homogenous, radial way from the explant (Figure 2). This suggests that the response of SG neurites to L1 is more likely to have been mediated directly, rather than via Schwann cells or fibroblasts, although this possibility must still be considered.

Therefore, we suggest that L1 serves to guide the direction of type I SG afferent dendrites towards their target zones on the IHCs. However, the morphology and function of the inner ear in the L1-null mouse has not yet been investigated and further studies will be needed to evaluate the role of L1 in the intact animal. Similarly, the cellular mechanism by which L1 influences type I SG neurite growth could be probed using cell signalling inhibitors, as we have used for other guidance factors (e.g. Aletsee et al., 2001; Mullen et al., 2012; Euteneuer et al., 2013). However, L1 modulation of axon guidance is likely to involve cytoskeletal mediated by interactions between L1 and internal ankyrin repeats (Hortsch et al., 2009).

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Animals: p5 rat

Coating L1 Dose: 1 μ g/ml in PBS **Figure 1.**

A. Quantitative analysis of termination by neonatal p5 rat SG neurites on alternating stripes of L1 (1 μ g/ml coating solution) vs. PLL (5 μ g/ml). Neurites showed a clear preference for L1. B. Control stripes (omission of L1; PBS only coating solution) elicited no preferential termination versus PLL (5 μ g/ml); bars represent mean and SD of neurites/explant. * $p < 0.05$, Mann-Whitney-U-test.

Figure 2. Representative example of a rat SG explant cultured on an L1 (1 $\mu\text{g/ml}$) vs. PLL (5 $\mu\text{g/ml}$) alternating stripe pattern for 72 h and stained with an anti-neurofilament antibody (green) to visualize all SG neurites, and with an anti-L1 antibody (red) to visualize L1. There was a strong tendency for neurites to terminate upon and/or grow along the L1 substrate. Stripe width 100 μm .

Figure 3. Higher magnification of a part of the SG explant seen in Figure 2. Most neurites turn along the L1 substrate and extend upon the L1 surface. A very thin neurite (arrowhead) terminates upon PLL. Stripe width 100 μm .

Figure 4.

Representative example of a rat SG explant grown on a control stripe pattern (PBS with omission of L1) vs. PLL (5 $\mu\text{g/ml}$) after 72 h in culture stained with an anti-neurofilament primary antibody to visualize all SG neurons. In this figure, a virtual stripe pattern has been matched to the location of the channels in the stripe mold, using the laminin edge labeling to align the pattern. There was no tendency for neurites to be found preferentially on the stripe locations. Neurites grew in a radial pattern and did not exhibit any turning parallel to the pattern. Stripe width 100 μm .

Animals: p2 mouse

Coating L1 Dose: 1 μ g/ml in PBS **Figure 5.**

Quantitative analysis of the termination of neonatal p2 mouse Type II SG neurites on alternating stripes of L1 (1 μ g/ml) vs. PLL (5 μ g/ml). There was no significant difference between termination on L1 versus PLL. Bars represent mean $\pm$ SD of neurites/explant. Control experiments conducted with omission of L1 from the stripe production solution (PBS only) showed the same result (data not shown).

Figure 6.

Representative examples for SG explants grown on L1 (1 $\mu\text{g/ml}$) vs. PLL (5 $\mu\text{g/ml}$) stripe patterns after 72 h in culture stained with an anti-peripherin primary antibody to visualize type II SG neurons. There was no tendency for type II neurites to be found preferentially on the L1 substrate. Neurites grew in a radial pattern and did not turn towards the L1 stripes. Stripe width 100 μm . Experiments with control stripes with omission of L1 (PBS only) showed the same result (data not shown).

Animals: p5 rat

Figure 7.

Effects of L1 on the number of neurites extending from SG explants cultivated on increasing concentration of L1. Number of neurites did not differ from control explants grown on PLL 5 µg/ml. Mean of neurites/explant $\pm$ SD.

Figure 8. Representative example for SG explants grown on (A) uniform 5 µg/ml L1 or (B) uniform 5 µg/ml PLL, after 72 h in culture and stained with an anti-neurofilament primary antibody to visualize all SG neurons

Animals: p5 rat

Figure 9. Effects of L1 on the average length of neurites per SG explant cultivated on increasing concentration of L1. The average length of neurites did not differ from control explants grown on PLL 5 µg/ml. Mean of neurites/explant $\pm$ SD.