The receptor protein tyrosine phosphatase HmLAR1 is up-regulated in the CNS of the adult medicinal leech following injury and is required for neuronal sprouting and regeneration

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Abstract

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LAR-like receptor protein tyrosine phosphatases (RPTPs), which are abundantly expressed in the nervous systems of most if not all bilaterian animals thus far examined, have been implicated in regulating a variety of critical neuronal processes. These include neuronal pathfinding, adhesion and synaptogenesis during development and, in adult mammals, neuronal regeneration. Here we explored a possible role of a LAR-like RPTP (HmLAR1) in response to mechanical trauma in the adult nervous system of the medicinal leech. In situ hybridization and QPCR analyses of HmLAR1 expression in individual segmental ganglia revealed a significant up-regulation in receptor expression following CNS injury, both in situ and following a period in vitro. Furthermore, we observed up-regulation in the expression of the leech homologue of the Abelson tyrosine kinase, a putative signaling partner to LAR receptors, but not among other tyrosine kinases. The effects on neuronal regeneration were assayed by comparing growth across a nerve crush by projections of individual dorsal P neurons (P0) following single-cell injection of interfering RNAs against the receptor or control RNAs. Receptor RNAi led to a significant reduction in HmLAR1 expression by the injected cells and resulted in a significant decrease in sprouting and regenerative growth at the crush site relative to controls. These studies extend the role of the HmLARs from leech neuronal development to adult neuronal regeneration and provide a platform to investigate neuronal regeneration and gene regulation at the single cell level.

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Introduction

LAR family RPTPs have been identified as important regulators of neuronal development in studies using different animal models from across the animal kingdom (reviewed in Beltran and Bixby, 2003; Johnson and Van Vactor, 2003; Ensslen-Craig and Brady-Kalnay, 2004; Tonks, 2006). Evolutionarily highly conserved, these receptors are characterized by their cell-adhesion molecule-like extracellular domains (ECD), comprised of distal immunoglobulin (Ig) and membrane-proximal fibronectin-type III (FNIII) repeats, along with a pair of tandem intracellular tyrosine phosphatase domains, D1 and D2.

Mechanistically, LARs are believed to help control F-actin polymerization through dephosphorylation of Ena/Vasp proteins (Wills et al., 1999; Biswas et al., 2002) and helping to regulate Rho GTP-binding proteins (Debant et al., 1996; Bateman et al., 2000). LAR has also been shown to associate with focal adhesions (FAs) in oocytes (Bateman et al., 2001), cultured cells (Hofmeyer et al., 2006) and leech growth cones (Baker et al., 2008), and to work in concert with N-cadherin to direct photoreceptor axons targeting in the optic ganglia of the developing fly (Choe et al., 2006). Likewise, mammalian LAR can associate with FA and cadherin–catenin contact sites in cultured epithelial cells and hippocampal neurons (Serra-Pages et al., 1995; Hoogenraad et al., 2007). Finally, mammalian LAR has been shown to help modulate neurotrophic signaling by associating with the TrkB receptor in neurons (Yang et al., 2006).

Less is known about the role of this class of receptors during neuronal regeneration in the mature nervous system. In the mouse, sciatic nerve crush has been reported to lead to an increase in LAR protein levels among DRG neurons (Xie et al., 2001; Van der Zee et al., 2003), and in LAR-deficient transgenic mice, to a decrease in neuronal regeneration and physiological recovery rates (Xie et al., 2001). Others have documented a complex change in expression among heterogeneous populations of DRG cells following sciatic nerve injury.
with some LAR-like RPTPs increasing while others decreasing their mRNA expression levels (Haworth et al., 1998). Curiously, during regeneration of retinal ganglion cell (RGC) axons across an optic nerve (ON) crush, LAR, which is normally expressed by both RGCs and by ON glia, was found to be down-regulated (Lorber et al., 2004; 2005), whereas regeneration among RGCs from LAR knockout mice was reduced as compared with lesioned wild-type. However, these experiments on RGC regeneration using the mouse knockout are complicated by the fact that an additional effect of the knockout is an increase in the survival rate and/or neurogenesis among RGCs and other neuronal populations (Lorber et al., 2005; Bernabeu et al., 2006). In contrast, RPTPα, which closely resembles LAR, has been shown to slow axon regeneration after peripheral nerve injury and to inhibit axon regeneration following facial and optic nerve crush (Thompson et al., 2003; Sapieha et al., 2005). Significantly, RPTPα appears to be one of the receptors binding with inhibitory components of the glial scar, including chondroitin sulfate proteoglycan (CSPG; Shen et al., 2009).

The present study explores the possibility that LARs may be involved in neuronal regeneration in the adult leech nervous system. The adult leech CNS, with its easily accessible identified neurons, has been a favored model for examining the capacity of central neurons to regenerate faithfully their central arbors and synapses as well as their peripheral projections (Muller and Carbonetto, 1979; Blackshaw et al., 1982; Macagno et al., 1985; von Bernhardi and Muller, 1995; Duan et al., 2005). On-going studies from our laboratory have documented the developmental expression of two LAR-like RPTPs in the CNS and peripheral organs of the medicinal leech embryo (Gershon et al., 1998a): HmLAR1 is expressed in many central neurons as well as heart and other muscle tissues (Gershon et al., 1998a), and HmLAR2 has a more restricted range of expression, being detected in a few neurons in each segmental ganglion in the embryo and in the developing germinal plate in a set of unique glia-like cell populations, the Comb Cells (CCs; Jellies and Kristan, 1988; 1991; Gershon et al., 1998a). Knocking down HmLAR2 levels in the CC significantly reduces the rate of growth of its processes (Baker and Macagno, 2000), and interfering with receptor ectodomain signaling in the embryo with the injection of interfering antibodies or Fc-chimeric proteins, leads to pronounced navigational errors by the cell (Gershon et al., 1998b; Baker and Baker, 2000). These findings prompted us to ask if the HmLAR receptors play a role in neuronal regeneration in the adult animal. We first describe the changes in CNS gene expression of HmLAR1 that we observed as a result of crushing or cutting central nerves, and then provide evidence that, in an identified central neuron, regeneration of extra-ganglionic projections is significantly decremented if LAR expression is knocked down via single-cell RNAi at the time that these projections are severed by a nerve cut or crush.

Results

In situ hybridization labeling of neural HmLAR1 mRNA increases following CNS injury

The leech central nervous system (CNS) is comprised of head and tail ganglia plus 21 segmental ganglia joined by paired connective nerves. The segmental ganglia are very similar to one another, each containing about 400 neurons (Macagno, 1980), 10 macroglia and a few muscle cells, as well as many microglia and supportive cells (Muller et al., 1981). Neuronal regeneration in the adult leech is known to be accompanied by sprouting of supernumerary branches and the robust regeneration of severed projections (Blackshaw et al., 1982; Macagno et al., 1985; Duan et al., 2005). As a first step in assaying whether the HmLARs might have a role in this response, we examined the distribution of HmLAR1 mRNA in adult leech ganglia using fluorescent in situ hybridization (FISH). Animals were fixed, embedded and sectioned 48 h following either a lesion to the central connective or a control sham operation to the over-lying ventral body wall. Tissue samples containing sectioned ganglia from segments adjacent to or distal from the site of the lesion were treated for FISH using either antisense or sense HmLAR1 riboprobe. In the experimental animals with a partially cut connective nerve, strong labeling by the antisense HmLAR1 riboprobe could be seen near the lesion in the cut nerve (Fig. 1C), indicating that HmLAR1 mRNA may be accumulating in cut axons at the site of regeneration. Granular labeling was also observed in the perinuclear region of many neuronal cell bodies in the ganglia proximal to the lesion (Fig. 1E) but not in a ganglion several segments away (Fig. 1G). In contrast, labeling in the connectives of sham-operated animals was low or absent (Fig. 1D) and when it was present in the ganglion it was in noticeably fewer neurons and at lower intensity than the ganglion next to the cut (cf. Figs. 1E–F and 1E to 1F, H). These results suggest that HmLAR1 mRNA levels are significantly up-regulated by the lesion to the CNS in neurons in ganglia near the lesion, and that this mRNA is transported to the regenerating ends of the lesioned axons in the affected nerve.

QPCR analysis of normal ganglionic expression of HmLAR1

Given the observation of a probable over-expression of HmLAR1 mRNA from the FISH experiments, we then chose to confirm and quantify these effects using a quantitative technique, QPCR. To optimize conditions for measuring gene expression using this approach, we first tested different primer sets on serial cDNA dilutions made from whole leech ganglia to generate different amplification plots. For most primer pairs tested, linear regression analysis of the plots showed a high linear correlation between moderate Ct values and the serial dilutions. Examples of the results obtained for HmLAR1 and leech ribosomal 60S (R60S) are shown in supplemental Fig. 1. Dissociation curves of the PCR products also showed that the amplicons had melting temperatures corresponding to the theoretically calculated temperatures and the melting temperatures obtained from plasmid cDNA templates (supplemental Fig. 1). Controls in which reverse transcriptase was omitted were also performed in order to exclude the possibility of DNA contamination. Using these procedures, we also attempted to measure HmLAR2 gene expression, but despite trying many different primer sets designed to different regions of the gene, we were unable to show a linear correlation between Ct values and dilution of cDNA. (data not shown), and these experiments were discontinued.

Next, we sought to measure the normal HmLAR1 levels in individual ganglia from midbody segments 9 to 13 in order to determine whether segmental variation might be a significant factor in further studies. HmLAR1 gene expression levels were measured using oligo dT primed cDNA transcribed from total RNA extracted from individual whole ganglia freshly dissected from segments 9, 10, 12 and 13. No statistically significant differences in amounts of HmLAR1 mRNA were detected among these four segments when normalized against R60S mRNA levels (values ranging from 0.6 to 0.75, N = 3; ANOVA = 0.898).

QPCR analysis of HmLAR1 expression following CNS lesion

To assay HmLAR1 expression in individual ganglia following mechanical trauma, the anterior and posterior connectives adjacent to ganglion 9 were crushed with forceps in intact animals. In most cases, the root nerves of ganglion 9 were cut as well. After 48 h, ganglia 9, 10, 12 and 13 were dissected from the animal, cDNA prepared and HmLAR1 expression measured using QPCR (Fig. 2). When normalized with respect to R60S expression, levels of HmLAR1 were found to rise very significantly (over 9-fold) in the injured ganglion 9 after the lesion, compared to values from sham-operated animals (p = 0.003; n = 5). No significant differences in receptor expression between sham and...
lesioned animals were detected for ganglia 10 and 12, but, unexpectedly, there was a significant increase in receptor mRNA levels in ganglion 13 after the lesion (p=0.03; n=5).

We next sought to determine whether other key elements of tyrosine phosphorylation pathways also showed changes in gene expression following mechanical tissue damage. Based on the results of genetic and biochemical interaction studies in *Drosophila* (Wills et al., 1999), it is thought that at least one aspect of *Drosophila* LAR function, modulation of F-actin assembly, is likely to be brought about via the coordinated catalytic action of the RPTP together with the cytoplasmic Src-like kinase, Abelson, which together help to control the state of tyrosine phosphorylation of the F-actin modulating protein Ena/Vasp. Accordingly, we chose to measure gene expression levels of the leech Abelson kinase, and of the leech Ena/Vasp homologue, Lena, following the crush (Biswas et al., 2002). Two receptor tyrosine kinases were also chosen for our survey; an insulin-like RPTK (LPTK2) which is highly expressed by the giant packet glia cells in each ganglion of the embryonic leech, and a fibroblast-like RPTK (LPTK18) which is expressed pan-neuronally in the animal (both in the CNS and the PNS; Nitabach and Macagno, 1995). As shown in Fig. 3, leech Abelson kinase expression did increase significantly in ganglion 9 following trauma compared to controls (p = 0.002; n = 4), but no significant changes were observed for either LPTK2, LPTK18 or Lena (Fig. 3).

We also tested whether *Hm* LAR1 gene expression was altered following *in vitro* ganglionic culture. Leech ganglia survive and display morphological, as well as, electrophysiological recovery of damaged pathways and neuronal circuits when placed into culture (e.g., Wallace et al., 1977). As shown in Fig. 4, *Hm* LAR1 expression levels also rose significantly when ganglia were cultured for up to 48 h as compared with freshly dissected ganglia from matching segments. Thus, this increase in gene expression can also occur following ganglionic culture which permits greater access to and the easier experimental manipulation of *Hm* LAR1 at the level of single cells.

**P neurons express *Hm* LAR1, but Retzius neurons do not**

In order to explore potential roles of the evoked changes in *Hm* LAR1 expression in the response to mechanical trauma, we then chose to bring our studies to the cellular level. Based on previous observations of *in situ* hybridization labeling for *Hm* LAR1 using whole leech embryos (Gershon et al., 1998a; unpublished observations), we identified the bilateral dorsal and ventral pressure neurons (*P* D, *P* V) as likely expressers of *Hm* LAR1, at least during development. These

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**Fig. 1.** Fluorescent *in situ* hybridization for *Hm* LAR1 in thin sections of a paraffin-embedded adult leech CNS. (A) Schematic drawing of a leech nerve cord. The arrowhead indicates the relative position of the cut made to a midbody ganglionic connective nerve distal to segment 7. Only one half of the nerve was severed to avoid retraction of the nerve and distortion of the CNS. Labels on the schematic refer to ganglia adjacent to (g1) or distal from (g5) the lesion or sham-operated segment, and are the sources of the images displayed in panels B–H. (B) Sense RNA control labeling in a section alternate to that shown in panel E, revealing background staining levels. The locations of the ganglionic capsule (arrowhead) and the neuropil (np) are indicated in this panel as well as panels E–H. (C) *Hm* LAR1 antisense labeling in the cut connective. Note that the signal is particularly intense at the cut ends (arrow). (D) *Hm* LAR1 antisense labeling along a connective from a sham-operated animal. Note the absence of label in comparison to panel C. (E) *Hm* LAR1 antisense labeling in the ganglion (g1 in panel A) adjacent to the cut, 48 h after the lesion. Strong granular label is present in the perinuclear region of many neurons, probably associated with ER/Golgi complex, as would be expected for translation of trans-membrane receptor proteins. (F) *Hm* LAR1 antisense labeling in a ganglion proximal to the skin opening from a sham-operated animal, showing much lower labeling in comparison to panel E. (G) *Hm* LAR1 antisense labeling in a ganglion distal to the lesion site (corresponding to g5 in panel A). Labeling is much less than that seen adjacent to the cut (cf. panel E). (H) *Hm* LAR1 antisense labeling from the a sham-operated animal in a ganglion distal to the skin opening (corresponding to g5 in panel A). Labeling is comparable to that seen in a ganglion distal from the cut (cf. to panel G). Scale bar = 40 μm.
Mechanosensory neurons, which form elaborate tiled sensory arbors in the leech body wall, have been the subjects of many studies exploring cellular interactions governing dendritic arbor formation (Gan and Macagno, 1995; Wang and Macagno, 1997; 1998). As controls, we chose the largest neurons found in segmental ganglia, the Retzius (Rz) cells, which did not appear to express the HmLAR1 receptor.

To measure gene expression in identified neurons, we isolated from adult ganglia a minimum of 5 individual P neurons (PV or PD) or 5 Rz neurons for each experiment, synthesized cDNA and used it to screen for HmLAR1, Abelson kinase, Lena, and R60S. As shown in Fig. 5, all mRNAs were detected in the P neurons (N=3), while Abelson kinase, Lena and R60S but not HmLAR1 were detected in the Rz neuron (N=3).

The endogenous expression of HmLAR1 by the P neurons was additionally confirmed by testing the effects of injecting individual P cells with siRNAs directed against HmLAR1 or with a control siRNAs (targeting a Xenopus GAPDH gene). Cells were injected in individual ganglion isolated from the animal and then placed in culture for 48 h before the P neurons were removed and receptor levels measured by QPCR. Compared with the control injected cells those receiving HmLAR1 siRNAs were found to have mRNA levels reduced by up an average of 80% (normalized with respect to R60S expression; p=0.003; n=3).

Thus, single-cell QPCR results confirm that the P neurons express HmLAR1 in the adult animal and reveal that RNAi injections can effectively reduce receptor mRNA levels in single cells.

Receptor knockdown impairs regeneration of P cell projections

Leech ganglia placed in organ culture with defined media show a robust regenerative sprouting response and, if allowed sufficient time for recovery, display precise synaptic reconnectivity (Wallace et al., 1977; Ready and Nicholls, 1979). Accordingly, we placed chains of 3 ganglia with intact connective nerves in culture and severed the P neuron projections extending from the middle ganglion by crushing the adjacent connective nerves with forceps. Individual P neurons in the middle ganglion were then injected with a combination of siRNAs directed against HmLAR1 or Xenopus GAPDH and neurobiotin (to allow tracing of the cell’s projections). After 48 h in culture, the P neuron’s projection across each of the crush sites was measured by staining the neurobiotin with a Cy3-conjugated strepavidin.

Control PD neurons injected with either neurobiotin alone or with dsRNA corresponding to Xenopus GAPDH showed similar rates of collateral sprouting and regeneration (Fig. 6B). De-novo growth was usually characterized by the presence of supernumerary and collateral sprouting from the lesioned processes, in both the severed dorsal peripheral root (which were usually seen to extend retrogradely along the outside of the cut end of the peripheral root; supplemental Fig. 2A), as well as, at the crush site (Fig. 6A). After 48 h there was usually complete traversing of the crush site by the regenerating fibers. Following HmLAR1 RNAi, however, all these aspects of growth were found to be significantly curtailed. There were fewer collateral sprouts at the lesion site and at the proximal stump of the severed peripheral projection out the lateral nerve root (supplemental Fig. 2B), and a significant reduction in both the number of collateral

Fig. 2. HmLAR1 gene expression is altered following a crush to the CNS connective. (A) View of a young adult leech 48 h after the connective was crushed next to ganglion 9 and the roots exiting the ganglion were cut. An incision has been made along the ventral midline to expose the underlying CNS. (B) Gene expression in crushed and sham-operated animals after 48 h. HmLAR1 was normalized against R60S measured from the same cDNA sample (n=5). Receptor levels were found to be significantly elevated in ganglion 9 and 13, as compared to sham. Bars represent standard error of mean. *p<0.03, **p<0.003 vs. sham and segmentally matched ganglia. Scale bar = 3 mm.

Fig. 3. Expression of leech Abelson kinase (Abi), leech insulin-like RPTK (LPTK2), leech fibroblast-like RPTK (LPTK18) and leech Ena-Vasp (lena) in individual ganglia 9, 48 h following the crush. Gene expression was normalized against R60S measured from the same cDNA sample (n=5). Compared to sham-operated animals, Abelson kinase expression increased significantly, but not the levels of LPTK2 and 18. Lena expression however was not significantly elevated in the operated ganglion. Bars represent standard error of mean. **p<0.002 vs. ganglion 9 from sham animals.
sprouts at the crush site and the extent of anterograde growth, with no examples of the nerve crush site being traversed (Fig. 6; N = 5). Thus, knockdown of HmLAR1, and presumably also prevention of its increased expression in response to trauma, strongly negates the regenerative response that mechanical damage normally elicits in the P neurons.

Fig. 4. Expression of HmLAR1 mRNA in whole adult ganglion after in vitro culture. (A) Individual ganglia with their connectives and roots cut distally and pinned to Sylgard-coated plates. The connectives were then crushed proximally to the ganglion to maximize the axonal injury and it was cultured in defined media for 48 h. Normalized gene expression (against R60S) comparing freshly dissected ganglia with those cultured for 24 h (n = 3). *p < 0.02. Scale bar = 200 μm.

Fig. 5. RT-QPCR gene expression in pooled groups of individually isolated Pd and Pp (n = 5) and Retzius neurons (Rz; n = 5). (A) Ventral view of a whole adult leech ganglion. The paired bilateral pressure cells (Pd, and Pp) and the Retzius (Rz) neurons are marked. (B) List of genes examined and their absence or presence in the P and Rz neurons. HmLAR1 was expressed in the P neurons (n = 3), along with Abelson and Lena, but not in the Rz neuron, which expressed only Abelson and Lena. (C) Representative amplification plots for the P and Rz neurons. Note that whereas both neurons show CT values for Abelson, Lena and R60S between 30 and 38, the P neuron shows a CT for HmLAR1 at 26.
Discussion

In the CNS of annelids, as in those of insects and vertebrates, many neurons express high levels of RPTPs during development (Gershon et al., 1998a; Chagnon et al., 2004; Tonks, 2006). This common property raises an interesting question: are these receptors also present at high levels during CNS repair in the adult, given that several studies have linked RPTP function to cellular adhesion and F-actin signaling, processes common to both developing and regenerating neurons (e.g. Debant et al., 1996; Wills et al., 1999; Bateman et al., 2000; 2001; Biswas et al., 2002; Choe et al., 2006). In the work presented here, we have shown that HmLAR1 is indeed normally expressed by select neurons in the adult leech CNS and that when neuronal processes are damaged, receptor levels are significantly increased. Furthermore, we showed that an increase in the expression of HmLAR1 is required for the P3 neuron to regenerate its severed projections: RNAi of HmLAR1 significantly reduced sprouting by the severed neuronal process and regeneration across the crush site. Interestingly, observations of the cut end using FISH suggest that HmLAR1 mRNA may be transported to the regenerating ends of lesioned axons, an observation that bears further detailed analysis. By comparison, very little is known about HmLAR1 function in the CNS. Binding studies in vitro have indicated that HmLAR1 probably does not act as its own ligand (Baker et al., 2000), and the present QPCR results reveal that the non-receptor Src tyrosine kinase, Abelson, is also significantly up-regulated following the lesion. This may suggest a common signaling pathway, perhaps through Lena, although gene expression of Lena does not significantly change with the crush (at least at the whole ganglion level). It would be interesting to repeat our P3 regeneration experiments, but following RNAi of Abelson, to see if its absence can phenocopy that of the loss of HmLAR1, or perhaps when done in conjunction with HmLAR1 RNAi, to reverse the effects of receptor knockdown as documented for Drosophila LAR and Abelson among motor axons (Wills et al., 1999).

Fig. 6. Knocking down HmLAR1 receptor levels in the P3 neuron hinders neuronal regeneration and sprouting. (A) The left P3 neuron was injected with siRNAs directed against a Xenopus GAPDH gene while the P3 neuron on the right with siRNAs directed against HmLAR1. The anterior connective was then crushed with forceps and after 48 h in culture the P3 neurons were injected with neurobiotin and visualized with strepavidin-Cy3. Images are from collapsed confocal image stacks displayed as black on white to improve contrast. Regeneration across the crush site is reduced in the receptor knockdown cell compared with the control cell. (B) Quantification of the regeneration lengths (measured as the length of the anterior process at the crush site with lateral sprouts), and number of sprouts (C) at the crush site following dye injection only (n=5), frog GAPDH (n=4) or HmLAR1 siRNAs (n=5). Values represent means ± SD. *p<0.02. Scale bar=200 μm.

Based on the observations presented here for HmLAR1 plus results of our earlier studies of the functions of HmLAR2 in the growth and arborization of the leech Comb Cell (CC; Gershon et al., 1998b; Baker et al., 2008), it would appear that both LAR-like leech receptors are involved in promoting cellular growth, albeit in different cells. A great deal lot is known about the molecular biology of HmLAR2, especially in the CC. HmLAR2 can be localized to integrin puncta within the cell’s growth cones and at the tips of its filopodia (Baker et al., 2008). Single-cell RNAi experiments have revealed that HmLAR2 promotes cellular adhesion and filopodial extension and that the effects of receptor knockdown can be mimicked by interfering with focal adhesion function (through RNAi targeting of CC paxillin), and F-actin signaling (RNAi of CC Lena). On the outside of the cell, it appears that HmLAR2 can function as its own ligand, and binding studies and experiments with soluble ectodomain proteins and antibodies recognizing the receptor ectodomain have indicated that ectodomain signaling promotes isocellular repulsion, helping to prevent CC arbor process overlap, and hence organizing body wall coverage by the CC, a process known as tiling (Gershon et al., 1998b; Baker et al., 2000). By comparison, very little is known about HmLAR1 function in the CNS. Binding studies in vitro have indicated that HmLAR1 probably does not act as its own ligand (Baker et al., 2000), and the present QPCR results reveal that the non-receptor Src tyrosine kinase, Abelson, is also significantly up-regulated following the lesion. This may suggest a common signaling pathway, perhaps through Lena, although gene expression of Lena does not significantly change with the crush (at least at the whole ganglion level). It would be interesting to repeat our P3 regeneration experiments, but following RNAi of Abelson, to see if its absence can phenocopy that of the loss of HmLAR1, or perhaps when done in conjunction with HmLAR1 RNAi, to reverse the effects of receptor knockdown as documented for Drosophila LAR and Abelson among motor axons (Wills et al., 1999).

Current experiments are also underway to use QPCR and single P3 and Rz cells to survey gene expression for candidate adhesion molecules and other RPTPs and kinases in the same experimental conditions.
Our results do suggest a role for HmlAR1 in neuronal regeneration, underscoring the importance of knowing which other neurons express the receptor, where it is normally localized in the cells and how receptor distribution may change following lesion and regeneration. Unfortunately, the supply of an antiserum raised against the N-terminal Ig domains of the receptor (Gershon et al., 1998a) has been exhausted and recent efforts to generate a new one have not been successful. Availability of a high affinity antibody to HmlAR1 would help answer our incongruous result reported here concerning the up-regulation of HmlAR1 after the crush in ganglion 9 and 13 but not ganglia 10, and 12. One plausible explanation for this finding would be that not all the somatic ganglia, 9 through 13, are identical in their quotient of HmlAR1 expressing neurons, particularly following a lesion. For example, there could reside neurons in ganglion 13 which project processes anteriorly through ganglion 9 but which normally in the adult have to negligible levels of HmlAR1 expressed, but that following the lesion become significantly elevated.

Attempts to date at over or ectopically expressing HmlAR1 transgenes in adult neurons have meet with only limited success, but such experiments may allow a discretion of the necessary receptor components involved in this response. For example, ecto-endodomain deletion constructs of HmlAR1 could be tested to see if they promote or hinder regeneration and/or sprouting by the P0 neuron. It should be noted that a role for a LAR-like receptor’s ECD independent of the intracellular catalytic activity is not without precedent: genetic rescue experiments in Drosophila have shown that LAR ECD expression helps rescue lethality and aspects of photoreceptor cell pathfinding after LAR knockout (Maurel-Zaffran et al., 2001). Recently it has also been shown that the ECDs of certain LAR-like RPTPs may function independently of their PTPase domains by conferring rigidity and spacing constraints between the membranes of opposing cells through their cell–cell trans-homophilic binding (Aricescu et al., 2007). Adhesive role for the receptor has been documented for both leech and fly LAR-like RPTPs as well as in mammalian cells (e.g. Baker et al., 2008; Hofmeyer and Treisman, 2009). Additionally, by ectopically expressing the receptor in neurons like the Rz cell, we could test the generality of its actions on growth among cells which do not normally express the receptor.

Sprouting and axonal regeneration by leech neurons across a crush site, including the axon of the P0 neuron, have been shown to be dependent upon an accumulation of microglia cells to the injury site (Ngu et al., 2007). This recruitment, directed by nitric oxide (Duan et al., 2007). An adhesive role for the receptor has been proposed as being membrane and ECM heparin sulfate proteoglycans (Fox and Zinn, 2005; Johnson et al., 2006). Thus, it would be interesting to examine where and to what the HmlAR1 ectodomain binds with on a crushed connective and whether binding changes in the absence of microglia accumulation.

**Experimental methods**

**RNA preparation, cDNA synthesis**

For RNA isolation from the whole leech ganglia, total RNA was extracted with the Absolute RNA nanoprep kit (Stratagene) according to the manufacturer’s instructions. Individual ganglia were dissected from animals, trimmed of connectives and nerve roots in leech Ringer’s solution (115 mM NaCl, 18 mM CaCl2, 4 mM KCl, 10 mM Tris maleate, pH 7.4; Kuffler and Potter, 1964) and mechanically homogenized in lysis buffer and stored at −80 °C until needed. Total RNA was reverse transcribed using superscript III (Invitrogen) and an oligo dT primer. QPCR was also performed on groups of 4–5 individual neurons. This was accomplished by pinning the ganglion ventral side up in a Sylgard dish using RNase free pins. The ganglion was then de-sheathed using micro-dissection scissors. A mouth suction device and glass capillaries with polished glass tips approximating the diameter of the Pressure (P) and Retzius (Rz) neurons were then used to extract the cells. Neurons were washed repeatedly (3×) in drops of L15 media (~100 μl), before they were placed in 20 μl of lysis buffer and the RNA isolated and cDNA prepared as described above. In some cases, individual neurons were first injected with a mixture of a fluorescent dye (Alexa 568 dextran; Invitrogen) to allow quick confirmation of cellular identity based on cellular morphology and 0.5% Fast Green to aid in visual identification of the same neuron under brightfield illumination after the ganglionic capsule was removed.

**QPCR**

Quantitative PCR reactions were performed in triplicate using an automated Stratagene MX3000P cycler and the following parameters run for 40 cycles: 10 min at 95 °C, 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. At the end of the run, a dissociation profile of the PCR product was obtained by running the temperature from 60 to 95 °C in order to evaluate primer specificity. Each 20 μl of the PCR reaction contained: 10 μl of SYBR Green (BioPioneer), 8 μl of nuclease free water, 1 μl of diluted cDNA and 1 μl of diluted primers for the gene of interest. The optimal cDNA dilutions were determined by performing step dilutions of 1:2, 1:4, 1:8 and 1:16. A dilution of 1:2 was found to produce cycle threshold (Ct) values between 22–32 for individual control ganglia (freshly dissected and untreated) and 33–37 for the individually identified neurons, with no double peaks and a single melting temperature. The genes examined in the study, their primers, amplicon size and melting temperatures are listed in Table 1. As internal controls, we also determined the abundance of several housekeeping genes. These included leech GAPDH, elongation factor 1α (ELF1) and a ribosomal 60S protein (R60S). However, since R60S was observed to be the most invariant, showing no significant change following ganglionic culture or other experimental treatments, it was

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<td>?</td>
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<tr>
<td>Lena</td>
<td>FRWD: 5’GGCGGTTGACCTGTGCTCC</td>
<td>162</td>
<td>89.6</td>
<td>?</td>
</tr>
<tr>
<td>Actin1</td>
<td>FRWD: 5’CTGTTAATCCGAGCAGA GACG: 5’-CAGTGGCTGCGTATCAGA</td>
<td>159</td>
<td>89.1</td>
<td>DG333328</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FRWD: 5’GCTTACCTACCTGCCGAC</td>
<td>164</td>
<td>87.3</td>
<td>?</td>
</tr>
<tr>
<td>ELF1</td>
<td>FRWD: 5’ATTCGAGCTGACTTGGTTGACG: 5’-CGATCGTTGTAGGATTACCA</td>
<td>178</td>
<td>87.3</td>
<td>DG333328</td>
</tr>
<tr>
<td>R60S</td>
<td>FRWD: 5’AGGGAGGTTGCTGCTGCTG</td>
<td>189</td>
<td>85.3</td>
<td>?</td>
</tr>
<tr>
<td>LPTK2</td>
<td>FRWD: 5’ACATCTGACTGACGCTGAA REV: 5’-AAGTGGATGGCACTTGGAC</td>
<td>152</td>
<td>84.1</td>
<td>AAB34824</td>
</tr>
<tr>
<td>LPTK18</td>
<td>FRWD: 5’CCGTCGACTGCTGCTGCA TACAA REV: 5’-AAGTGGATGGCACTTGGAC</td>
<td>156</td>
<td>86.8</td>
<td>AAB34826</td>
</tr>
</tbody>
</table>
used it as an internal control and for normalization in all of our experiments.

Analysis

The Ct values for the expression of the various mRNA were normalized using housekeeping reference genes and the $\Delta\Delta Ct$ method and the following formula: $2^{-\Delta\Delta Ct}$ where the averaged Ct of the gene of interest is subtracted from the housekeeping gene to the power of $2^{-\Delta\Delta Ct}$. All values are presented as mean $\pm$ SD and analyzed by ANOVA Tukey's and or Student's t test.

Intracellular injection and RNAi

To study P0 neuron regeneration, intact ganglionic chains of 3 or more ganglia were dissected out of the animal and pinned out flat onto a Sylgard-coated dish with supplemented L15 (2% fetal calf serum, 0.6% glucose, and gentamicin (0.1 μg/ml)). This media has been shown previously to support growth and synaptic recovery of leech central neurons resembling that seen in the animal after injury (Wallace et al., 1977; Ready and Nicholls, 1979). The connectives adjoining the middle ganglion are then crushed proximal to the middle ganglion with fine forceps. The P0 neurons of the middle ganglion were then injected as described below, and the chain cultured for up to 4 days. Typically L-15 medium was replaced on a daily basis. Double-stranded siRNAs were made using the Silencer ssiRNA Cocktail kit (Ambion) as previously described (Baker and Macagno, 2010). For HmLAR1, dsRNAs encompassing nucleotides 1–950 was used as DNA template. This same siRNA mixture has been previously shown by us to be effective at knocking down HmLAR1 in individual embryonic neurons, as assayed by in situ hybridization staining (Baker and Macagno, 2010). For a control template, a Xenopus GAPDH template was used which encompasses a 440 bp fragment and which is supplied by the kit. Knockdown of HmLAR1 in the P cell was accomplished using established injection procedures (e.g. Baker and Macagno, 2006). Briefly, a beveled electrode is filled with both the dsRNA and a 2% solution of Alexa 568 Dextran (Invitrogen) to visualize the injectate and the solution pressure injected into a neuron until its primary processes in the ganglion were clearly visible under fluorescent illumination. dsRNA was injected at a final concentration of not less than 1 μg/μl. The ganglia were then cultured for 48 h and the fine morphology of the P0 neuron visualized via a second dye injection, this time using a mixture of Alexa 488 Dextran (Invitrogen) and 5% Neurobiotin (Vector Laboratories). After a further 1 h to allow diffusion of the dye, the ganglia were fixed with 4% paraformaldehyde and the Neurobiotin visualized with a Cy3-conjugated strepavidin (Jackson ImmunoResearch) as previously described (Fan et al., 2005).

Experimental animals and CNS lesion

Leeches used in these experiments were obtained from a mixed Hirudo medicinalis/Hirudo verbana breeding colony maintained in our laboratories. In order to study changes in gene expression that might accompany a lesion to the CNS, we performed surgery on adult animals by anesthetizing them in 10% ethanol in leech Ringer’s for 15 min before pinning the animal ventral side up in a chamber containing ice-cold leech Ringer’s solution. Using the genital pore opened between segments 6 and 7 as reference, segment 9 was identified and a 3–5 mm longitudinal cut was made in the body wall over ganglion 9 using a scalpel. The CNS connectives immediately adjacent to ganglion 9 (anterior and posterior) were then crushed with fine forceps. To further damage the neuronal projections, the lateral nerve roots extending from ganglion 9 to the body wall were also cut (Wang et al., 2005). Following the surgery, animals were kept in leech ringer’s until movement was observed and then thereafter they were maintained at 15 °C in artificial spring water (0.5 g/L of solid sea salts (Instant Ocean) in H2O). After 48 h, the leech was again pinned ventral side up and ganglia 8, 9, 10, 12 and 13 were removed and individually stored in lysis buffer for RNA isolation and cDNA synthesis.

Fluorescent in situ hybridization

To assay the histological distribution of HmLAR1 mRNA following CNS injury, we performed fluorescent in situ hybridization on paraffin embedded sections of the whole lesioned leech. Lesion to the animal was performed as described above except that instead of a crush to the connective, half of the connective was cut completely through with iridectomy scissors. After allowing 48 h for recovery, lesioned and sham-operated animals were fixed in 4% paraformaldehyde (PFA) in PBS and embedded in paraffin. Coronal 7 μm serial sections were then made of the paraffin blocks containing the CNS and processed for mRNA labeling.

Synthesis of the riboprobes

A 451 bp long fragment of the LAR1 sequence was cloned in pGEM-T (forward primer: ATTTGAACGCTCAACACAGCC–reverse primer: TTTCAATCTGGTAATGGA). Linearized plasmid was then used for the synthesis of sense and antisense riboprobes using the DIG RNA labeling mix (Roche) and the RNA polymerases T7 and SP6 (Roche), respectively. Purified probes were diluted in hybridization buffer (HB; 50% formamide, 5× SSC, 50 μg/ml heparin, 0.1% Tween20 and 0.5 mg/ml tRNA). Slides were de-paraffined and progressively rehydrated in PBS. A first post-fixation was performed in 5% PFA for 25 min. Tissue permeabilization was achieved by incubation for 15 min with Proteinase K, 10 μg/ml, followed by a second post-fixation in 5% PFA. Slides were then hybridized with either the antisense or sense probes in HB overnight at 55 °C and labeling was visualized using a sheep anti-DIG primary antibody (Jackson ImmunoResearch) and a donkey anti-sheep secondary antibody coupled to Alexa 555 (Invitrogen). Sections were mounted in glycergel (Dako) and examined using a confocal microscope (Zeiss LSM 510).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2010.08.002.

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References


