SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
CELL BIOLOGY
CELLULAR NEUROSCIENCE

Received
20 December 2013

Accepted
12 March 2014

Published
11 April 2014

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Regeneration of *Aplysia* Bag Cell Neurons is Synergistically Enhanced by Substrate-Bound Hemolymph Proteins and Laminin

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We have investigated *Aplysia* hemolymph as a source of endogenous factors to promote regeneration of bag cell neurons. We describe a novel synergistic effect between substrate-bound hemolymph proteins and laminin. This combination increased outgrowth and branching relative to either laminin or hemolymph alone. Notably, the addition of hemolymph to laminin substrates accelerated growth cone migration rate over ten-fold. Our results indicate that the active factor is either a high molecular weight protein or protein complex and is not the respiratory protein hemocyanin. Substrate-bound factor(s) from central nervous system-conditioned media also had a synergistic effect with laminin, suggesting a possible cooperation between humoral proteins and nervous system extracellular matrix. Further molecular characterization of active factors and their cellular targets is warranted on account of the magnitude of the effects reported here and their potential relevance for nervous system repair.

Development of the nervous system is a complex series of events coordinated on the scale of the entire organism. Neurons extend axons and dendrites, thin processes collectively called neurites, that travel precise pathways to establish an intricate web of synapic connections[1]. Growing neurites are guided to their destinations by combinations of soluble and immobilized chemical guidance cues[1–3]. Understanding how neurites elongate and find their targets is critical for developing treatments to restore lost connectivity following nervous system injury[4–6]. The sea hare *Aplysia californica*, like many invertebrates, has a remarkable ability to regenerate injured portions of the central nervous system[7–9]. This regenerative capacity, along with the readily identifyable individual neurons and populations, has allowed researchers to recreate specific snaptic circuits *in vitro*[10,11]. Isolated *Aplysia* neurons have also become valuable tools for investigating the basic biology of neurite growth and regeneration.

The ability of neurons from the *Aplysia* central nervous system to regenerate both *in vitro* and *in vivo* has prompted a search for endogenous neurotrophic factors. Some efforts have focused on material derived from the nervous system and associated tissues. Enhanced regeneration of dissociated bag cell neurons was observed in the presence of central nervous system (CNS) sheath cells or arterial cells[12], or material remaining after such cells were killed[13]. Coverslips treated with conditioning factors released from mollusk ganglia also enhance regeneration of isolated neurons[14–17]. Although the molecular identities of these factors have not been reported, collagen-like peptides have been identified in the material produced by arterial and sheath cells[13].

Hemolymph, the circulatory fluid of *Aplysia*, has also been investigated as a source of neurotrophic factors. In animals with open circulatory systems, hemolymph directly bathes the internal organs including ganglia and connectives. In fact, sinuses within the abdominal ganglion, where hemolymph could come in direct contact with neurons, have been described[18]. Peptides released by the nervous system are distributed systemically by secretion into hemolymph[19–21], and the neuromodulator serotonin is present in nanomolar concentrations[22], suggesting exchange of material between circulatory and nervous systems. Several studies have found that hemolymph can effect regeneration of isolated neurons. Growth media supplemented with hemolymph promoted neurite outgrowth and synaptogenesis in abdominal ganglion neurons *in vitro*[10,23]. Hemolymph-supplemented media or coverslips treated with poly-l-lysine (PLL) and hemolymph also accelerated outgrowth of *Aplysia* dopaminergic

neurons relative to PLL-treated coverslips alone[24]. This effect was attributed to acetylcholinesterase (AchE), which is present in hemolymph[25,26]. A similar effect of substrate-bound hemolymph components has been reported for bag cell neurons[27].

Several protein components of *Aplysia* hemolymph, in addition to AchE, have been identified. The most abundant is the respiratory protein hemocyanin, which assembles into complex quaternary structures- decamers, didecamers, and multidecamers- from a 400 kDa subunit[25,28]. The second most abundant protein in *Aplysia* hemolymph is haemoporin, a homopentamer made up of 70 kDa subunits. Its function is unknown[29]. Agglutinating activity possibly associated with endogenous lectins has also been reported[30,31].

Aplysia bag cells extend neurites up to 1 mm long within the bag cell cluster and into the sheath surrounding the connective[32]. Basement membranes have been described in the abdominal ganglion where it comes into direct contact with neurites[18,21]. Laminin, a ubiquitous component of basement membranes[33], is known to promote neurite outgrowth in many contexts[2,34–36]. We have used laminin extensively as a growth substrate for bag cell neurons and have found that its presence is required for growth in response to serotonin[37,38]. These observations prompted us to investigate hemolymph as a source of growth factors which are effective in the laminin background.

We show here that application of hemolymph to the growth substrate in the presence of laminin increases growth rate and branching of bag cell neurites relative to laminin or hemolymph alone. Preliminary characterization of the active component of hemolymph suggests that it is a previously unidentified high molecular weight protein or protein complex. CNS-conditioned media has a similar synergistic effect with laminin, suggesting that the interaction between endogenous growth-promoting factors and laminin might be relevant in the *Aplysia* nervous system.

Results

Substrate-bound *Aplysia* hemolymph and laminin have a synergistic effect on bag cell neuron regeneration. Previous work by Burmeister *et. al.* suggested that *Aplysia* hemolymph could contain growth-promoting molecules that act in a substrate-bound manner[27]. To test whether hemolymph components could enhance regeneration in commonly used tissue culture backgrounds, acid-washed glass was first coated with PLL then with either laminin, hemolymph, or laminin followed by hemolymph. Neurons on PLL or PLL + laminin typically grew a small number of process that did not advance far from the cell body (Fig. 1a,b). The addition of hemolymph caused more extensive neurite elongation (Fig. 1c). Neurons grown on the combination of laminin and surface-adsorbed hemolymph components grew extremely long neurites and complex branching patterns that exceeded any of the other conditions (Fig. 1d). Consistenly, neurons from the same individual animal showed significantly more outgrowth in the hemolymph and laminin condition than in all other conditions.

Neurite regeneration was quantified after 24 hours in culture. This time point was chosen because it was long enough to clearly differentiate between conditions, while at longer time points neurites from neighboring cells would often begin to fasciculate with each other. Two measures were used to quantify the extent of neurite regeneration. Since neurites frequently crossed and fasiculated, tracing individual neurites to measure length was not possible. Instead we measured the neuronal territory, which is the area of the smallest convex polygon that contains the entire neuron. We used 'effective radius', which is the square root of the neuronal territory divided by π, to quantitatively compare neuronal outgrowth between cells. We also used Sholl analysis to compare branching patterns[39]. To quantify the influence of different conditions on branching, the maximum number of intersections measured by sholl analysis was compared (See Methods).

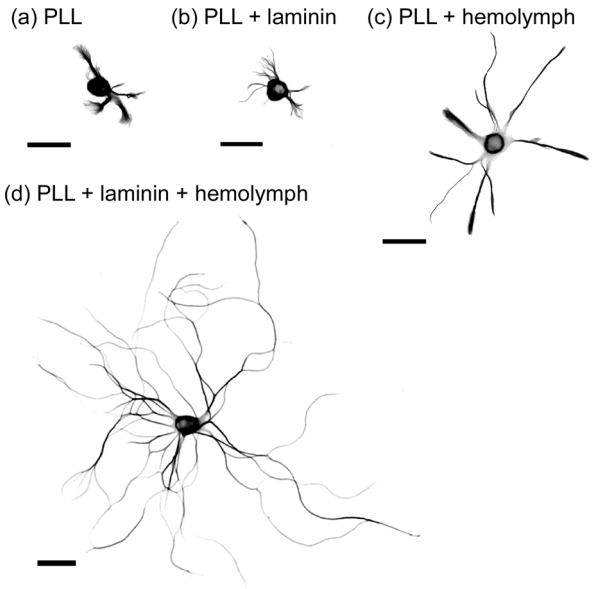

Figure 1 | Substrate-bound laminin and *Aplysia* hemolymph have a synergistic effect on bag cell regeneration. Representative morphologies for bag cells grown on (a) PLL-coated glass, or PLL-coated glass and substrate-bound (b) laminin, (c) hemolymph, (d) laminin and hemolymph. All scale bars are 100 μm.

Laminin did not significantly increase outgrowth relative to PLL alone, but hemolymph components increased effective radius relative to both PLL and laminin (Fig. 2a). Neuronal morphologies in Figure 1 are examples of the groups analyzed in Figure 2. The combination of laminin and hemolymph components increased effective radius relative to all other conditions. Sholl plots show that neurites in this condition extended much farther from the cell body and developed many more branches (Fig. 2b). The maximum number of intersections was significantly higher with the combination of hemolymph components and laminin than in all other conditions (Fig. 2c). Since laminin is commonly used as a growth substrate for neurons and the increase in outgrowth on laminin with the addition of hemolymph components was both substantial and consistent, this is the effect we chose to focus on for the rest of this study. In the remainder of this study glass coverslips coated with PLL and laminin are the common background for all experiments.

To compare growth rates between cells on laminin with cells on laminin and adsorbed hemolymph components, we observed growth cones at high magnification and tracked growth over a two hour period 10 hours after plating (Fig. 3a,b). The mean growth rate on laminin-treated substrates was 0.8 +/− 1.0 μm/hr (N = 13), while the average growth rate on laminin and hemolymph-treated substrates was 16.3 +/− 8.6 μm/hr (N = 14) (Fig. 3c). The latter rates are to our knowledge faster than any previously published rates for bag cell growth cones.

The presence of hemolymph components also qualitatively changed the organization of growth cone actin and microtubule cytoskeletons. On laminin-treated substrates most growth cones were fan shaped with compact central domains and relatively few long filopodia (Fig. 3d). On substrates treated with laminin and hemolymph, growth cones had irregular shapes and long filopodia that extended far beyond veil regions of the peripheral domain (Fig. 3e; arrowheads). Microtubules were often splayed out in the central domain (Fig. 3e), although this difference was less marked.

Characterization of the active component(s) in hemolymph. To test whether the active component(s) in hemolymph are proteins, hemolymph was heated to a range of temperatures between 40°C and

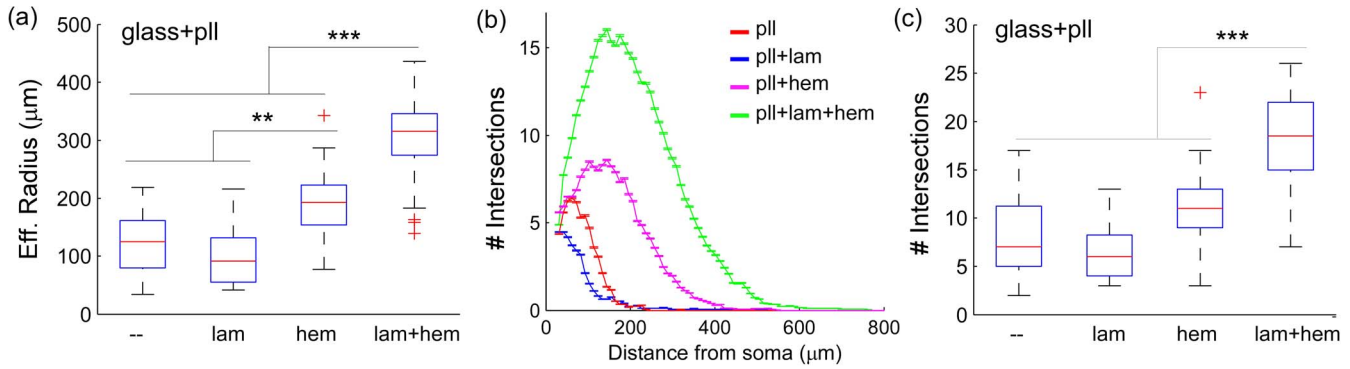

Figure 2 | Substrate-bound laminin and *Aplysia* hemolymph components increase outgrowth and branching. (a) Box plot of effective radius for bag cells grown for 24 hours on PLL-coated glass (N = 31) and with the addition of substrate-bound laminin (N = 30), hemolymph (N = 36), or both laminin and hemolymph (N = 39). (b) Sholl analysis of bag cells grown on pll coated glass with the addition to the substrate of laminin, hemolymph, or both laminin and hemolymph. (c) Box plot of maximum number of intersections from Sholl analysis in (b). *** = $p < 0.001$, ** = $p < 0.01$.

75°C with a gradient thermocycler. Up to 50°C, heat-treated hemolymph components behaved the same as control, significantly increasing effective radius relative to laminin alone, but at 56°C effective radius was not significantly different than with laminin alone (Fig. 4a). The heat inactivation temperature is therefore between 50°C and 56°C, strongly suggesting that the active component is a protein. Within this temperature range no precipitate formed, however large amounts of white material precipitated when hemolymph was heated to 75°C.

Hemolymph components were concentrated 5-fold using a 100 kDa molecular weight cutoff (MWCO) centrifugal filter to test whether an increased concentration could further enhance outgrowth. Laminin-coated substrates were treated with either the filter flow-through, the 5x-concentrated retentate or retentate diluted to the original concentration in ASW. Neurons grown on substrates treated with retentate had significantly higher effective radius than neurons on substrates treated with the flow-through (Fig. 4b). This suggests that the active factor is either a single high molecular weight

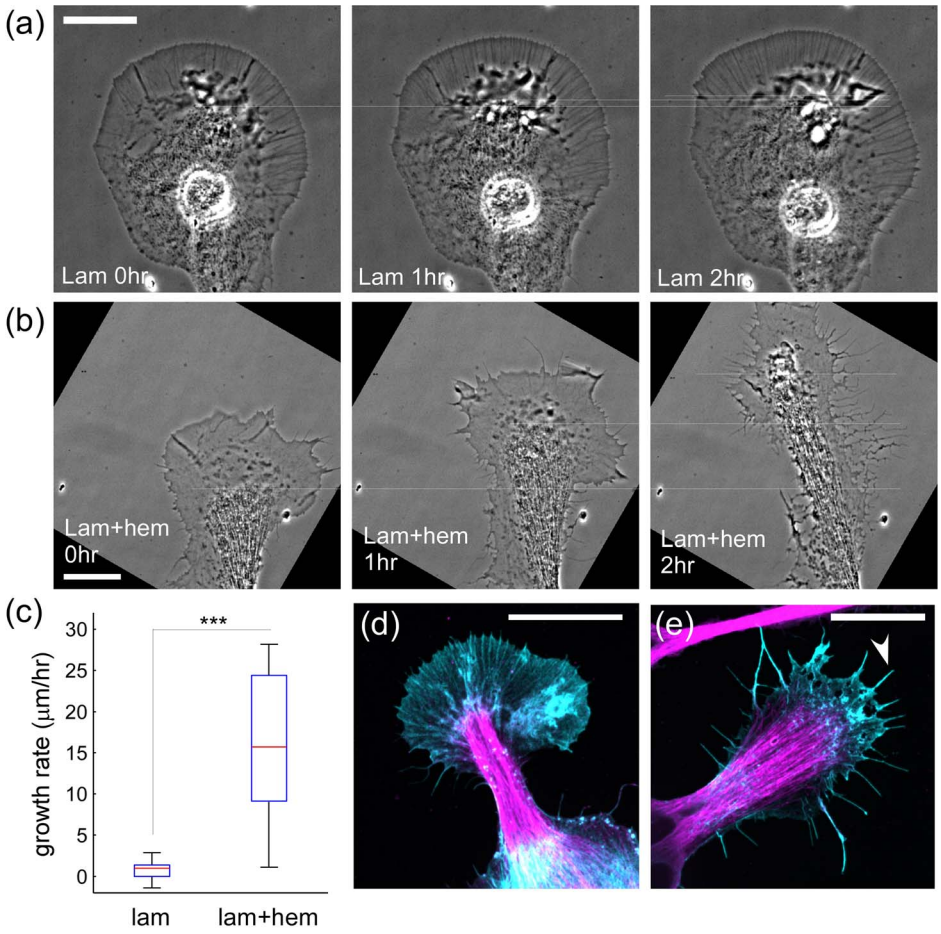

Figure 3 | Hemolymph components increase growth cone migration rate. (a) An example bag cell growth cone growing on laminin substrate followed for 2 hours. (b) An example bag cell growth cone on laminin substrate with hemolymph components followed for 2 hours. Scale bars are 20 μm. (c) Box plot of growth rates for growth cone on laminin (N = 13) or laminin and hemolymph proteins (N = 14). *** = $p < 0.001$. (d) Example of growth cones on laminin-treated substrates. (e) Example of growth cones on laminin and hemolymph-treated substrates. Scale bars are 20 μm.

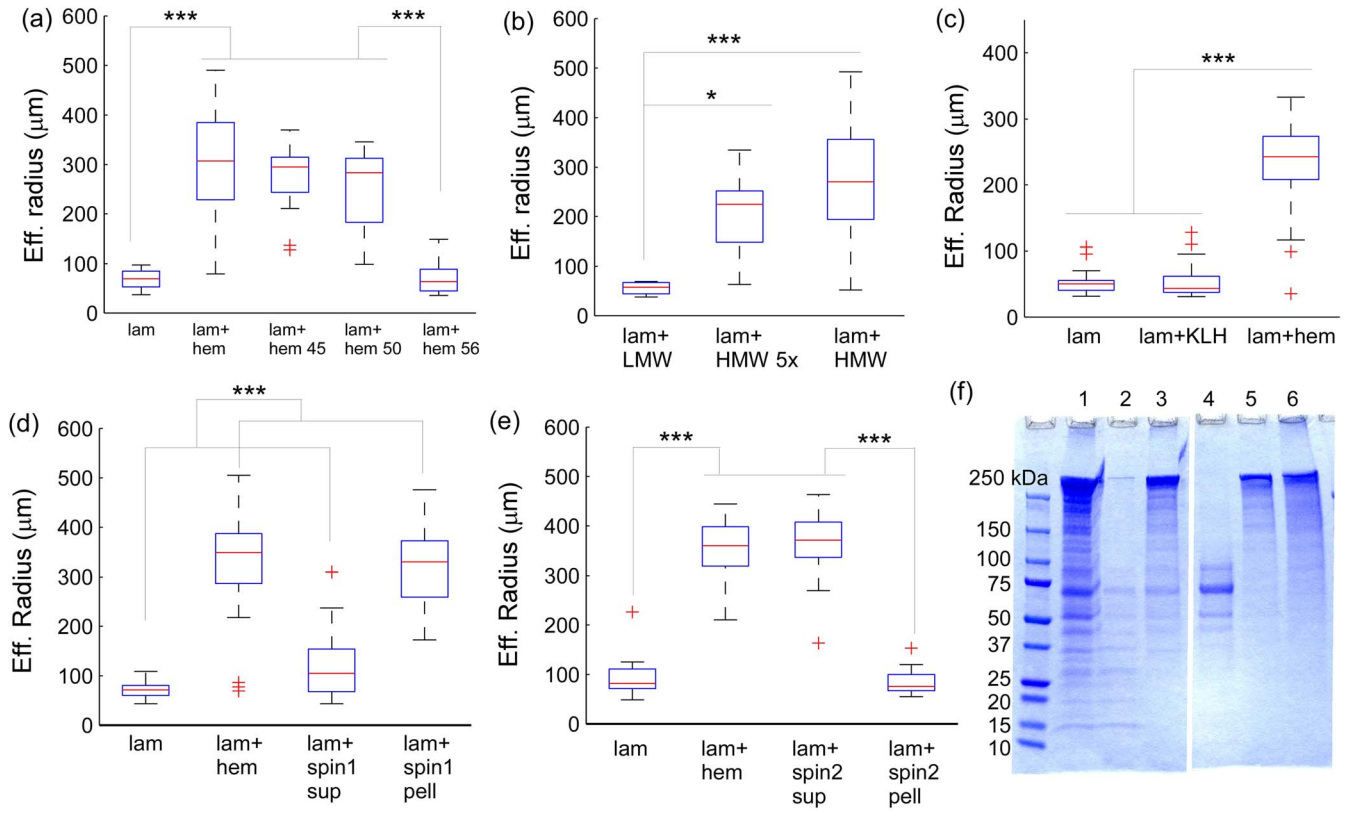

Figure 4 | Characterization of active factor(s) from *Aplysia* hemolymph. (a) Box plot of effective radius of bag cells grown for 24 hours on substrates coated with laminin (N = 28) or laminin and hemolymph proteins either unheated (N = 54) or heated to 45°C (N = 31), 50°C (N = 33), or 56°C (N = 25). (b) Active factor(s) in hemolymph are retained by a 100 kDa MWCO centrifuge filter. Lower molecular weight flow-through (LMW) (N = 5) is compared to high molecular weight retentate (N = 32) and retentate concentrated 5× (N = 23). (c) Box plot of effective radius of bag cells grown for 24 hours on substrates coating with laminin (N = 26), laminin and keyhole limpet hemocyanin (KLH) (N = 25), or laminin and hemolymph proteins (N = 43). (d) Box plot of effective radius for bag cells grown for 24 hours on substrates coated with laminin (N = 19), laminin and unfractionated hemolymph (N = 43), laminin and supernatant from the 193,000 RCF spin (N = 37), and laminin and pellet from 193,000 RCF spin (N = 27). (e) Box plot of effective radius for bag cells grown for 24 hours on substrates coated with laminin, laminin and unfractionated hemolymph, laminin and supernatant from 88,000 RCF spin, and laminin and pellet from 88,000 RCF spin. *** = $p < 0.001$, * = $p < 0.05$. (f) Protein profiles of pellets and supernatants from a two-step ultracentrifugation protocol visualized by SDS-PAGE analysis on 4–15% gradient gel and coomassie stain. Contents of lanes are as follows: 1) unfractionated hemolymph, 2) supernatant from 193,000 RCF spin, 3) pellet from 193,000 RCF spin, 4) supernatant from 88,000 RCF spin, 5) pellet from 88,000 RCF spin, 6) keyhole limpet hemocyanin. Representative examples of neuronal morphologies resulting from these treatments are shown in Supplementary Figure 1.

molecule or a large complex. There was no significant difference between the outgrowth stimulated by the two concentrations of retentate.

Hemocyanin is the most abundant protein in *Aplysia* hemolymph and the most extensively characterized. We therefore explored this high molecular weight protein as a candidate for the active factor in hemolymph. Hemocyanin from the keyhole limpet *Megathura crenulata*, another marine gastropod, is commercially available in purified form. Typical total protein concentrations in *Aplysia* hemolymph were 0.2–1 mg/mL. Surface adsorption of of 1 mg/mL keyhole limpet hemocyanin (KLH) in the presence of laminin did not increase effective radius relative to laminin alone (Fig. 4c). Lower concentrations also has no effect on outgrowth (data not shown).

Because the active factor in hemolymph was thought to be a high molecular weight protein but not hemocyanin, we used ultracentrifugation to separate the active factor from hemocyanin. First, hemolymph was centrifuged at 193,000 RCF with a sucrose cushion to pellet high molecular weight proteins and complexes. SDS-PAGE analysis confirmed that proteins over 50 kDa were contained in the pellet (Fig. 4f). Supernatant and pellet fractions were used to treat PLL and laminin coated coverslips for cell culture. The growth promoting factor was contained in the pellet (Fig. 4d). A previous study showed that divalent cations could induce hemocyanin to form large complexes[40]. 100 mM $CaCl_2$ and 100 mM $MgCl_2$ was added to the pellet fraction, which was then centrifuged again at 88,000 RCF. After this step, the protein profile of the pellet closely resembled that of keyhole limpet hemocyanin, showing that this method effectively pelleted the hemocyanin. The active factor was retained in the supernatant (Fig. 4e), which contained a small number of bands corresponding to proteins between 37 and 100 kDa (Fig. 4f). This result further indicates that hemocyanin is not responsible for the activity. Efforts to further separted these components were were unsuccessful or resulted in loss of activity.

Growth media conditioned by incubation with CNS tissues has been shown to promote neurite outgrowth in *Aplysia* and other systems[14–17,41]. Similar to the present study, the factor that enhances regeneration in the snail *Helisoma* is thought to be a protein acting in a substrate-bound manner[15–17]. CNS-conditioned media was prepared by incubating the CNS of one adult *Aplysia* in L15-ASW for 72 hours[14]. Conditioned media was then applied to coverslips in a similar manner as hemolymph. Conditioned media did not enhance outgrowth relative to laminin, but conditioned media and laminin together significantly increased outgrowth relative to either laminin or conditioned media alone (Fig. 5).

Discussion

We have shown a robust and significant synergistic effect of substrate-bound *Aplysia* hemolymph proteins and laminin on bag cell neurite outgrowth and branching (Fig. 1–3). Hemolymph treatment of the substrate accelerated outgrowth of individual growth cones over 10× relative to laminin alone. The activity could be inactivated by heating, suggesting that it is one or more proteins (Fig. 4a). The activity was retained by a 100 kDa MWCO centrifuge filter and not present in the low molecular weight flow-through indicating that it is a high molecular weight protein or protein complex (Fig. 4b). We found that factors secreted by the CNS also have a synergistic effect with laminin on outgrowth of bag cell neurons. The identity of the protein or proteins responsible for accelerating growth remains unknown, but the results presented here narrow the field of candidates and suggest strategies for purification.

Hemocyanin is the most abundant protein in hemolymph and the best characterized. For these reasons it was our first candidate protein, even though it has no known cell adhesion functions. Three lines of evidence suggest that the active factor is not hemocyanin. Commercially prepared keyhole limpet hemocyanin, which shares 58% sequence identity with the *Aplysia* protein, could not replicate the effect of *Aplysia* hemolymph (Fig. 4c). Further, the hemolymph growth-promoting activity inactivated at 56°C (Fig. 4a), much lower than the 80°C denaturation temperature of hemocyanin from another marine gastropod, *Rapana thomasiana*[42]. A protocol for promoting the formation of large hemocyanin multimers by increasing the concentraion of divalent cations[40] allowed protein bands corresponding to hemocyanin to be pelleted by ultracentrifugation, while the active factor remained in the supernatant (Fig. 4d–f).

A previous study observed increased outgrowth of dopaminergic neurons in the presence of hemolymph-supplemented media[24]. This effect was attributed to AchE since it could be blocked by BW284c51, an inhibitor of the peripheral anionic site AchE, and replicated by purified human AchE. This possibility intrigued us, since AchE is known to bind to laminin[43,44] and can enhance cell adhesion and neurite outgrowth[45–47]. A synergistic effect of laminin and AchE on neurite outgrowth has also been demonstrated in the R28 neuronal cell line[48]. These functions are attributed to the peripheral anionic site are unrelated to AchE enzymatic activity[46,49]. AchE from mammals inactivates at a similar temperature to the active factor in *Aplysia* hemolymph[50]. Preliminary experiments with AchE inhibitors were inconclusive (data not shown), but AchE nonetheless remains a strong potential candidate for the active component.

Since a variety of lectins bind to bag cell membranes and couple to growth cone retrograde flow[51], it would not be surprising if endogenous lectins could function as neuronal adhesion molecules. Lectins from *Aplysia* hemolymph have been characterized[30,31], and a lectin from *Aplysia* gonad promotes neurite outgrowth[52]. However, the endogenous agglutinating activity in hemolymph inactivates between 60°C and 70°C[30], suggesting that the active factor is not a lectin. The abundant 70 kDa protein, haemoporin, is another potential candidate, but very little is known about it[29].

The synergistic effect of laminin and the growth-promoting protein(s) from hemolymph suggest that these two proteins interact through either direct binding or indirectly by modifying the cell's signaling background. Direct binding between hemolymph proteins and laminin could modify the way these factors are presented to cell surface receptors. A variety of growth factors and axon guidance molecules are known to influence cell behavior while immobilized on the substrate[53–57]. The hemolymph-laminin interaction presented here could be related to an emerging paradigm in which the interaction between ECM and secreted growth factors modulates adhesion-dependent growth.

Is the interaction between hemolymph and neuronal tissue relevant in a physiological context? The observation of sinuses within the abdominal ganglion suggests that the hemolymph might come in direct contact with the CNS or at least with the extracellular matrix surrounding the ganglia[18]. The sheath is known to contain basement membranes, of which laminin is one component[18]. These structures have not been extensively investigated and this interpretation of their function is speculative. However, even if a barrier exists between hemoceol and the CNS during normal development, nervous system injury could expose neurons to hemolymph, making the interaction between the two relevant in the context of regeneration[9]. The similarity between the response to factors in hemolymph and conditioned media raises the intriguing possibility that growth promoting factors in hemolymph are originally released by the central nervous system (Fig. 5). Alternatively, the synergistic effect with laminin could be a common feature shared by growth factors present in both hemolymph and the CNS. In either case, the results presented here are likely to be relevant to the normal physiology of the *Aplysia* nervous system.

The effects described here could be relevant to other organisms as well. Many families of axon guidance and neurotrophic factors that are expressed in the CNS and conserved across animal phyla have been characterized *in vivo* and in tissue culture. However, humoral factors that influence neuron development and regeneration have received relatively little attention. The majority of animal species have open circulatory systems and the above reasoning for hemolymph's relevance to the nervous system would apply. *Aplysia's* well established neuronal culture systems[58], the ease of collecting large quantities of hemolymph, and the availability of gene sequences[59,60] means that this organism could be ideal for investigation of humoral neurotrophic factors. Unraveling the mechanism behind the syn-

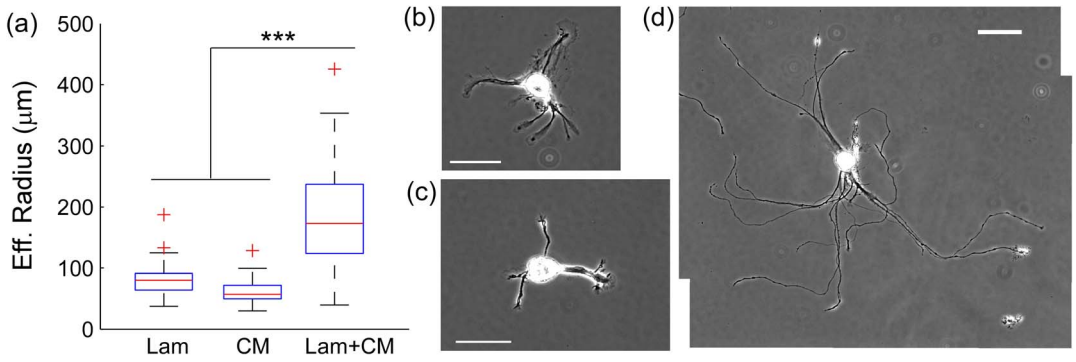

Figure 5 | Laminin and conditioned media have a synergistic effect on neurite outgrowth. (a) Box plot showing square root of the neuronal area for bag cells grown for 24 hours on PLL-coated glass with the addition of substrate-bound laminin (N = 31), conditioned media (N = 31), or both laminin and conditioned media (N = 58). *** = $p < 0.001$. Example images of bag cells grown on (b) laminin, (c) conditioned media, of (d) laminin and conditioned media. All scale bars are 100 μm.

ergistic effect of laminin and hemolymph proteins might contribute directly to our evolving understanding of the common pathways regulating growth cone advance. In addition, identifying the molecules and pathways involved might provide physiological relevance for recent discoveries about cytoskeletal regulation. The magnitude of the effect, its potential novelty, and its physiological relevance make this an important topic for future studies.

In conclusion, we have shown that substrate-bound hemolymph proteins and laminin have a synergistic effect on bag cell neuron outgrowth and branching. Similarly, substrate-bound factors from CNS conditioned media also had a synergistic effect with laminin. These results point to a potential cooperation between humoral proteins and the ganglion ECM in promoting growth and regeneration of neurons. Investigating the mechanism of this cooperation and its impact on cell physiology may suggest new strategies for repairing the injured nervous system.

Methods

***Aplysia* bag cell neuron culture.** Adult *Aplysia californica* were obtained either from the National *Aplysia* Resourse at Univerisity of Miami or from Marinus Scientific (Long Beach, CA). All animals used for both cell culture and hemolymph extraction were 100–200 grams. Dissociated cultures of *Aplysia* bag cell neurons were prepared as previously described[61] with the following modifications. Abdominal gangila were partially digested with 125 U/mL dispase (StemCell Technologies, Vancouver, CA) in L15-supplemented artificial sea water (L15-ASW) (400 mM NaCl, 10 mM KCl, 15 mM HEPES, pH 7.8, 10 mM $CaCl_2$, 55 mM $MgCl_2$, and Phenol Red, all from Sigma-Aldrich, St. Louis, MO) for 18–22 hours at 22°C. The bag cell cluster was manually dissected from the ganglia and gently titriated with a 20 μL pipette tip coated with bovine serum albumin (BSA, Sigma) in 35 mm tissue culture dishes also coated with BSA. Bag cells separating from the cluster during tiriation were transferred first to a separate tissue culture dish containing L15-ASW to remove cell debris and connective tissue, then to coverslips coated with substrate proteins. During dissociation, neurons were completely separated from support cells. Typically 40–50 neurons were plated per coverslips.

Acid-washed glass coverslips were pretreated with 20 μg/mL poly-L-lysine (PLL) (Sigma). PLL solution was aspirated from the surface of the coverslip, then 50 μg/mL laminin (Sigma) was added. Hemolymph, keyhole limpet hemocyanin (KLH; Sigma), or conditioned media was added in the same manner either directly on top of PLL or following the laminin-coating step. PLL was allowed to adsorb for 20 minutes, laminin for 2 hours and hemolymph for 30 minutes. All coverslips were extensively washed with either water or culture medium prior to plating. Cultures were maintained at room temperature. Imaging experiments were performed at room temperature in L15-ASW supplmented with 0.5 mM vitamin E and 2 mg/ml carnosine (both from Sigma).

Hemolymph collection. Hemolymph was collected similarly to a previously described protocol[23]. In detail, adult *Aplysia* were kept at 4°C for at least 1 hour to anesthetize. The body cavity was then pierced just behind the head with a syringe and large bore needle and hemolymph extracted by gentle suction. The animal was sacrificed immediately after this procedure. 20–50 mL of hemolymph was typically obtained. Hemolymph was cetrifuged at room temperature for at least 10 minutes at 440 RCF in a table-top clinical centrifuge to pellet hemocytes. The supernatant was decanted and kept at 4°C for several weeks. Hemolymph was discarded if any precipate was observed. Hemolymph never lost activity when stored in this manner, but the precipitate inhibited cell growth and could not be completely removed by centrifugation. High molecular weight hemolymph components were concentrated using 100 kDa Amicon centrifugal filter (Millipore, Billerica, MA) in a Sorval Super T21 table-top centrifuge with swinging bucket rotor (DuPont, Willmington, DE).

Heat inactivation of hemolymph proteins. An Eppendorf MasterCycler gradient thermocyler with the heated lid disabled was used for heat inactivation studies (Eppendorf, Hamburg, Germany). Hemolymph was heated in 50 μL aliquots to temperatures from 40°C to 65°C for 30 min and then immediately cooled to 4°C. Aliquots were then centrifuged at 16,000 RCF in a tabletop microcentrifuge at room temperature for 5 min to remove protein aggregates. Supernatants were removed and stored at 4°C.

Enrichment of active fraction. Ultracentrifugation was used to enrich the active fraction from hemolymph and selectively remove hemocyanin, a respiratory protein that is the major constituent of *Aplysia* hemolymph[25]. Cell-free hemolymph was first centrifuged with a 2 M sucrose cushion at 193,000 RCF for 4 hours in a Beckman Optima L-90K Ultracentrifuge at 4°C with a Ti 50.2 fixed angle rotor (Beckman-Coulter). The pellet fraction, which contained the high molecular weight proteins including hemocyanin, was dialyzed against ASW using 8 kDa MWCO dialysis tubing (BioDesign, Carmel, NY). This was justified on account of the similarity between the ionic composition of hemolymph and sea water[62]. Hemocyanin was induced to oligomerize by the addition of 100 mM $CaCl_2$ and 100 mM $MgCl_2$ to the pellet fraction[40]. Hemolymph with added $CaCl_2$ and $MgCl_2$ was kept at 4°C for six days then centrifuged for 2 hours at 88,000 RCF as above. SDS-PAGE analysis showed that a protein profile resembling hemocyanin was present in the pellet after this step.

SDS-PAGE analysis. Proteins samples were separated by SDS-PAGE on 4–15% gradient gels (Bio-Rad, Hercules, CA) by standard methods[63]. Gels were stained with 0.1% Coomassie brilliant blue R-250 (Sigma) in 10% acetic acid and 40% methanol (both from J.T. Baker), and destained in 10% acetic acid and 20% methanol.

Conditioned media preparation. Conditioned media was prepared in a manner similar to a previously described method[14]. One central nervous system (CNS) was removed from an adult *Aplysia*, rinsed twice in 4 mL L15-ASW and placed in 2 mL of L15-ASW with added Mini-Complete EDTA-free protease inhibitor cocktail (Roche, Palo Alto, CA). Media containing the CNS was incubated at 14°C for 72 hours. The CNS was then removed and the conditioned media stored at 4°C. Conditioned media was used as a substrate coating in the same manner as hemolymph.

Immunocytochemistry. Bag cells were fixed with 4% formaldehyde (J.T. Baker, Phillipsburg, NJ) in ASW and 400 mM sucrose (J.T. Baker) and permebilized with either 1% Triton X-100 (Sigma) or 0.1% saponin (Sigma) in the same solution[64]. Actin filaments in fixed cells were labeled with 0.66 μM Alexa-594 or Alexa-488 phalloidin (Life Technologies) in PBS-T (PBS + 0.1% Triton X-100) for 1 hour. Cells were blocked for 30 minutes to 1 hour in a solution of 5% BSA in PBS-T. Blocked cells were then incubated for 30 minutes to 1 hour with mouse-anti-α tubulin primary antibody (Sigma) diluted 1 : 100 in blocking solution, washed with PBS-T, then incubated for 30 minutes in either Cy5 goat-anti-mouse (Zymed Laboratories, San Francisco, CA) or Alexa-488 goat-anti-mouse secondary antibody (Life Technologies) diluted 1 : 100 in blocking solution. Cells were washed with PBS and mounted in Mowiol (Calbiochem) and N-propyl-gallate (Sigma).

Image acquisition. High magnification fluorescence images were acquired on a Nikon TE2000E inverted microscope (Nikon, Melville, NY) and an Andor Revolution spinning disk confocal system (Andor, Belfast, UK) equiped with a CSU-X1 confocal head (Yokogawa, Tokyo, Japan) and an Andor iXon^{EM+}888 EM CCD camera. Confocal illumination was with 488 and 640 nm laser lines controlled with an Andor Laser Combiner. Emission wavelength was selected with bandpass filters from Chroma Technology (Bellows Falls, VT) mounted in a Sutter LB10W-2800 filter wheel. Transillumination was with a halogen lamp and SmartShutter (Sutter Instruments, Novato, CA). All hardware and image acquisition were controlled with μ-Manager software[65]. Objectives used were Plan Apo VC 100×/1.4 numerical aperture (NA) and phase contrast Plan Apo 60×/1.4 NA (Nikon). X-Y stage position was controlled with a Newport MM3000 motion controller with 850G actuators (Newport, Irvine, CA).

Low magnification phase contrast images were acquired on a Zeiss AxioObserver.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany) with a Zeiss AxioCam MRc camera and a phase contrast A-Plan 10×/0.25 NA objective. Transillumination was with a halogen lamp. Low magnification fluorescence images were acquired on a Zeiss AxioImager.M1 microscope with a Zeiss AxioCam MRm camera and a EC-plan NeoFluor 10×/0.3 NA Ph1 objective. Epifluorescence illumination was with a X-Cite Series 120Q light source (Lumen Dynamics, Mississauga, Ontario, Canada). Low magnification hardware and image acquisition were controlled with Zeiss AxioVision 4.7 software.

Analysis of neuronal growth and morphology. Morphometric analysis was performed on either 10× phase images of live neurons or 10× fluorescence images of fixed neurons stained with an antibody against tubulin (see above). Neurons were analyzed if they had at least one process at least as long as the diameter of the cell body and were not contacting any other neurons. Because of the complexity of neuronal arbors, 'Effective radius' of neurons was used as a proxy for average length of neurites. Effective radius was calculated as the square root of the area of the convex hull of the neuron divided by pi. The convex hull was constructed either manually in ImageJ[66] or MATLAB (MathWorks, Natick, MA) from phase images or automatically with a custom Matlab routine from thresholded fluorescence images of neurons.

Sholl analysis quantifies neuronal complexity by counting the number of neurites that intersect circles drawn at interval distances from the center of the soma[39]. This analysis was performed with a custom MATLAB routine from thresholded fluorescence images of neurons. The user specifies the radial position of the first circle, the distance between circles, and the radial position of the final circle. The program then displays each image and prompts the user to click on the center of the soma. The program constructs a series of concentric circles from this point. At each circle, the number intersections is counted. All statistical analysis of morphometric data was performed in MATLAB.

Growth was measured from DIC images as displacement of the growth cone's central domain along the inferred growth axis. Retractions were counted as negative growth.

Statistical analyses. All graphing and statistical analyses were performed in MATLAB. For hemolymph outgrowth experiments, which typically involved multiple experimental groups with unequal variances, p values were determined with a Kruskal-Wallis test followed by a Tukey multiple comparison test. Statistical significance was defined as $p < 0.05$. Bar graphs show sample mean with error bars repesenting one standard deviation. In box and whister plots, the center mark is the

median, the edges of the box show 25th and 75th percentiles, whiskers show the limits of the data points not considered outliers, and outliers are marked by '+' symbols. Outliers are those points that are outside 2.7 standard deviations of the mean.

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Acknowledgments

This work was supported by National Science Foundation Graduate Research Fellowship (to C.H.), National Institutes of Health Grants RO1-NS28695 and RO1-NS051786 (to P.F.), and National Science Foundation Grant DBI-0619674 (to E.R.D. and P.F.).

Author contributions

C.H., P.F. and E.R.D. designed research and wrote the paper, C.H. performed experiments, analyzed data and prepared figures.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hyland, C., Dufrense, E.R. & Forscher, P. Regeneration of *Aplysia* Bag Cell Neurons is Synergistically Enhanced by Substrate-Bound Hemolymph Proteins and Laminin. *Sci. Rep.* **4**, 4617; DOI:10.1038/srep04617 (2014).