Growth cone retraction rates differ in time and on substrata when subjected to sublethal doses of mercury

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Introduction

Background

Growth cones (GCs) are dynamic, microtubule and actin supported protuberances at the end of elongating axons which guide axonal growth during development (Kendal, 2000, p.1070). Developing neurons must extend axons to specific targets which can be meters away, and depend on the motile and sensory abilities of the growth cone to successfully reach the appropriate target. Soluble molecules in the extracellular environment (Kendal, 2000, p. 1070) as well as substrate bound adhesion molecules (Burden-Gulley *et al.*,1995) act as positive and negative cues sensed by actin-rich extensions of the growth cone (filopodia and lamellipodia) which direct the growth cone to advance, retract, or turn.

The degree of advance or retraction depends on the rate of cytoskeleton assembly and disassembly at the tip of the fiolopodium. These cytoskeleton assembly and disassembly units are actin (Mallavarapu and Michison, 1999) and microtubules (Sabry *et al.* 1991).

A study by Leong *et al.* concluded that mercury compounds retard axonal outgrowth of snail (*Lymnaea stagnalis*) neurons by compromising microtubule polymerization (and thus cytoskeletal assemblage). They found the average linear growth rate of GCs to be
-102 μm/h (a retraction rate of 1.7 μm/min) during mercury treatment. Other studies (Kasuya, 1975; Wilke *et al.*, 2003; Nakada *et al.* 1981) have found that various mercury compounds, notably methyl mercury chloride, also inhibit axon outgrowth of vertebrate neurons. The detrimental effects of mercury on axoplasmal cytoskeleton integrity may lead to the high neurotoxicity of mercury, as outlined in the Agency For Toxic Substances and Disease Registry (ATSDR), as well as mercuryÕs especially harmful effect on a fetusÕs developing nervous system (ATSDR).

The working mercury concentration used by Leong *et al.* (100nM), was $2x10^5$ times greater than the mercury concentration found in the most mercury-rich aquatic ecosystems ($5x10^{-4}$nM) (Rickert, 2006), and 20 times greater than the mercury concentration found in the blood of most adults (5nM), approaching the concentration of mercury found in the blood of occupationally mercury-exposed workers (120nM) (Borjesson, 1995). Numerous studies (Brackeman *et al.*, 1997; Kasuya, 1975;Szucs *et al.*,1997; Soderstrom and Ebendal, 1995) have studied the effects of mercury on neurons using toxic does of mercuryÑconcentrations greatly exceeding that found in the average human adult, or the TD_{50} of mercury (500nM) found by Wilke *et al.*(2003).

This study sought to study the neuronal effects of mercury as a property of growth cone movement, using working concentrations closer to those found in unexposed workers (we used a working [Hg^{2+}] of 10nM). Thus, we investigated the possible influence of mercury not only as a neurotoxin, but as a chemorepellant influencing GC growth rates, and thus neuronal development. Concerns for the development of human fetuses in pregnant women with normal blood mercury concentrations (5nM as found by Borjesson, 1995) can then be addressed. The effects of lower mercury concentrations in the surrounding extracellular environment can also help to explain why toxic levels of mercury build up in humans and have detrimental effects when aquatic mercury concentrations are so low.
It was hypothesized that at the lower mercury concentrations used, GC retraction rate would be proportionally slower than the retraction rate as found by Leong *et al.* (2001) and may even show positive, though depressed outgrowth compared to control GCs, which will show positive outgrowth.

in addition to soluble compounds, the substrate used in tissue culture plating has been found to have an effect on GC motilityÑboth direction and rate of growth. A comparison of chick peripheral neurons plated on laminin, conditioned medium, plain glass, and collagen substrata used as substrata for cell plating produce differences in GC advance and the frequency of GC branching (Bray *et al.* 1987).

Thus, the path of a developing axon from cell body to the appropriate target depends on the growth promoting or inhibiting effects of various molecular species in the embryonic environment. These molecules can be categorized into three groups based on their location the subcellular environment: GCs encounter signaling molecules in the membranes of surrounding cells, in the extracellular matrix, and finally, in soluble form (Kandel, 2000, 1074). This study will focus on the combined growth promoting and inhibiting properties of the soluble compound Hg^{2+} in conjunction with two molecules of the extracellular matrix (polyK and laminin), leaving the investigation of mercuryÕs effect on the growth directing properties of cellular surface molecules to Tower (2006) and Fess (2006).

It was hypothesized that GCs would advance in control conditions, and retract in experimental mercury conditions. Due to the growth promoting effects of laminin as a neuronal growth factor, it was predicted that in identical control conditions, GCs would advance faster on laminin versus polyK substrata, and that in identical mercury conditions GCs would retract slower on laminin versus polyK. Thus, we hypothesized that GC growth rate would be less inhibited on laminin than on polyK substrata during mercury treatment, predicting that signals from laminin to advance would counteract signals from mercury compounds in solution to retract.

Materials and Methods

Cell Culture

Primary cultures of chick embryonic peripheral neurons were made using a procedure modified from Peter J. Hollenbeck of Purdue University. Dissected orsal root ganglia (DRGs) and sympathetic nerve chains from 9 day chick embryos were washed with Hanks Balanced Salt Solution (HBSS) solution and trypsinized during a 15-20 minute incubation at 37¼C in HBSS solution containing 25% trypsin. A suspension of dissociated cells in F+ medium (100ml Liebovitz L-15, 2mM glutamine, 0.6% glucose, 100 µg/ml penicillin/streptomycin, 10% Fetal Calf Serum, 50 µg/ml nerve growth factor) was made using the trypsinized cells and injected on to 25 mm petri dishes containing 22 mm coverslips coated with either polylysine only, or polylysine coated with laminin.
Laminin-coated coverslips were prepared by placing one side of a sterile glass coverslip for 20 minutes in a 1 mg/ml polylysine solution followed by 20 minutes in a laminin in HBSS solution. The coverslip was kept wet in regular HBSS solution until cells were ready to be plated. Polylysine only coverslips did not undergo the second 20 minute laminin coating.

Cells were plated at 0.5X density (1/2 DRG per plate) on both the laminin and polylysine coverslips, and allowed to grow in a 37¼C humidified incubator. Neurites on both laminin and polylysine covered coverslips were adherent after 12 hours of incubation.

Application of Mercury and Control Solutions

Leong et al. used a working solution of 0.1 µM $HgCl_2$ in *Lymnaea* saline, a dose which is just under the TD_{50} of 0.5µM $HgCl_2$ found by Wilke et al. for rat DRGs equivalent to the U.S. Environmental Protection Agency (EPA) reference dose (RfD) for methyl mercury (MeHg) at 0.1 µg/kg/day (Stern 2005). After consulting *Principles and Applications of Aquatic Chemistry*, which used a standard mercury concentration of 1nM to chart the inorganic speciation of mercury in an estuary, it was decided to modify the working concentration of Leong et al. to 10 nM. A 10 µM stock solution of mercury chloride was diluted in a 1:500 dilution of 0.5% HCl in HBSS to obtain a working solution of 20 nM mercury chloride in HBSS. A control solution was also prepared of a 1:500 dilution of 0.5% HCl in HBSS without mercury chloride.

After a 1-3 day incubation period, the cell culture dish was stripped of F+ medium and replaced immediately with 1ml

of regular HBSS to prevent drying. The culture was placed on the inverted scope where a representative neurite with many growth cones was found and kept in focus throughout the 1 hour mercury treatment. After the neurite was in focus, 1ml of 20 nM mercury chloride solution was added dropwise to the cells already bathed in HBSS. This step, while not ideal, eliminates the possibility of decreased Hg action through chelation by the albumin in the F+ medium containing 10% fetal calf serum. Thus, the neuritis were bathed in a 10 nM concentration of $HgCl_2$ in a 1:1000 dilution of 0.5% HCl in HBSS even though the working solution was 20 nM. The neurons were allowed to incubate in the mercury chloride solution for 1 hour next to a heater giving off approximately 27¼C heat to the surroundings, as monitored by a temperature-sensitive camera. The experimental $HgCl_2$ solution was replaced by the control solution of 1:500 dilution of 0.5% HCL in HBSS with no mercury chloride in the control step, and the same procedure followed as outlined above.

Microscopy and Imaging

Growth cones were viewed at 20X magnification using a Nikon Eclipse TS100 inverted microscope. A time-lapse video recording was taken of the neuron during the initial 3 minutes of incubation in the mercury or control solutions using BTVpro software. BTVpro was also used to take still images at the initial time (t0) prior to the addition of experimental and control solutions and every ten minutes during the 1 hour incubation.

3 growth cones were imaged for the laminin experimental neuron, 7 for the laminin control neuron, and 4 for both the polylysine experimental and control neurons.

Determination of Net Growth Cone Retraction Rate

Since growth cones are an extension of the axon, net growth cone retraction rate was determined by charting the changes in axon length over time. The end region of every axon was identified as a growth cone, as well as the end region of any segments which branched from the main axon. Any segment was considered a branch of an axon if it was not covered up when the mouse cursor was placed over it; thus it had to be longer than the cursor from growth cone end to the branching point.

Axon lengths per growth cone were found at t0, t10, t20, t30, and t40 by quantifying still images taking at that moment in time. Using the segmented line option of the ImageJ software and the mouse curser, the axon length was traced from the tip of every growth cone (endmost point of axon) to where the axon met the cell body. This was done by using the mouse curser to click along the entire segment of the axon to be measured. Clicks were kept as close together as possible to decrease experimental error from the curvature of the axon (each segment between mouse clicks represents a straight line).

Once the desired axon length was traced, the Measure option under the Analyze menu was selected, which summed the length between clicks and immediately displayed the selected length in pixels (abbreviation for picture element). These are the many tiny dots which make up the representation of a picture in the computerÕs memory. Three different trial measurements were performed per growth cone for each measurement and averaged to decrease experimental error.

Measurements could then be converted from pixels to μm using a conversion factor of 15.7 pixels/1μm. This factor was determined by using ImageJ to measure the distance in pixels of a 10 μm segment of a stage micrometer taken with BTVpro at 20x magnification. 8 different trial measurements were averaged to obtain a final measurement of 156.5 pixels per 10 μm, giving a conversion unit of 15.7 pixels/ μm.

Net retraction rate can be calculated for each growth cone between any time interval from t0 to t40 by finding the change in axon length over the change in time, giving a net retraction rate in μm/min. Data were more easily interpreted by using the average of the axon lengths measured per t0, t10, etc, to find an average net GC retraction rate of the laminin ctrl, laminin exp, polyK ctrl, and polyK exp cells.

Results

Influence of mercury treatment on overall cell appearance

Cells observed after less tan 24 hours clearly showed robust outgrowth on both laminin and polyK cover slips. Neurites were strongly adherent to the substrata, and axonal processes were clearly observable. Processes seemed to be longer in general on the laminin coated coverslip, but this conclusion was not supported by the limited number of cells used in this experiment.

To directly observe the effects of control and experimental solutions on developing neurons, still images of each cell at the different conditions were taken before application and for every subsequent ten minutes. A three minute live video was also taken during the dropwise addition of experimental and control solutions to the normal HBSS solution which was used to replace the F+ medium.

Pre-experimental tests applying 10nM mercury solutions directly on the cells after F+ removal showed detergent-like diminishing effects on the axons within 3 minutes, but we did not see the same dramatic effects from the dropwise application of mercury solution to the HBSS. We did observe that control and experimental cells on both polyK and laminin substrata were shaken by the sweeping force of direct application of control and experimental solutions. In spite of the disturbance, all cells remained adherent to the substrata and the axon length was not affected in any way by the movement of the solution over the cover slip.

Although the drastic changes in contrast of the previous tests were not seen, allowing the mercury and control solutions to diffuse through a neutral media (normal HBSS) may have only slowed the effects; still images of both control and mercury treatment show that the cells were negatively affected by both treatments after only 10 minutes, and drastically affected at the end of the 40 minute treatments (Fig 1-4). Contrast of the images decreased, so that in most cases, the cell body as well as the axons became more dim, suggesting that the cellular membrane was weakened by the new solutions, or that membrane integrity was diminished, perhaps due to the mercury-induced microtubule disintegration as observed by Leong *et al.* (2001). The collapse of GC cytoskeletal structure was observable in some of the images at t40, especially for the polyK experimental cell (Figure 4).

Laminin Control Cell

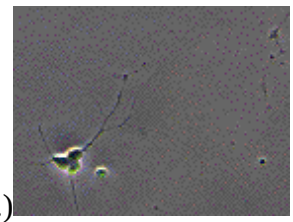
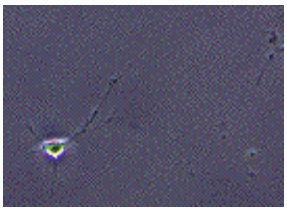
A.)  B.) 

Figure 1. Cell on laminin during control treatment at time t0min. (A) and at time t40min.

Laminin Experimental cell (mercury treatment)

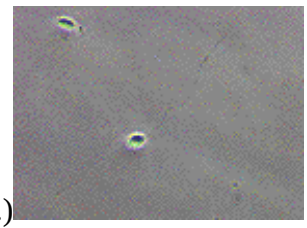
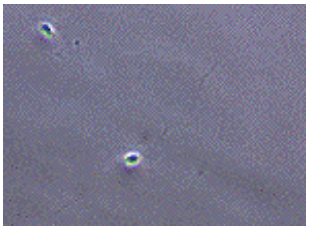
A.)  B.) 

Figure 2. Cell on laminin during mercury treatment at time t0min. (A) and at time t40min. (B).

PolyK Control cell

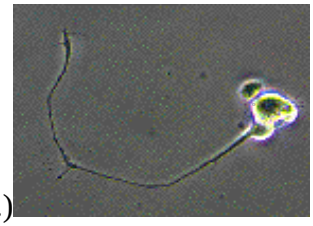
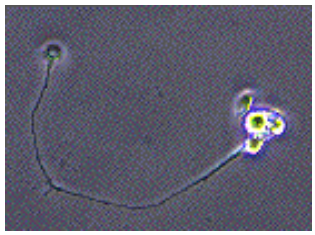
A.)  B.) 

Figure 3. Cell on polyK during control treatment at time t0min (A) and at time t40min. (B).

PolyK Experimental Cell

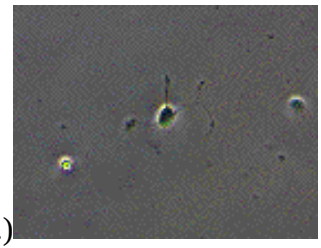
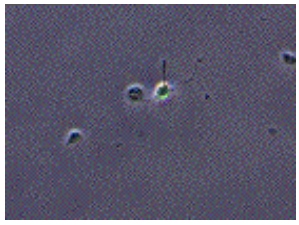
A.)  B.) 

Figure 4. Cell on polyK during mercury treatment at time t0 (A) and at time t40min. (B).

*All of the above images were taken on a Nikon eclipse TS100 inverted microscope using BTVpro software.

This negative effect on all observed cells (laminin ctrl and exp, polyK ctrl and exp) increased from t0min to t40min, the image being dimmest at t40min (Figures 1b, 2b, 3b, and 4b). Cells in all conditions show signs of being in distress after 40 minutes of treatment: along with a noticeable GC retraction (especially for polyK experimental and laminin control cells, Figure 1, 4), cell body shrank considerably, membrane integrity was compromised, and blebs appearedÑa sign of osmotic stress. The degree of distress between control cells and mercury treated cells was almost indistinguishable.

On average, growth cones on polyK retract faster during mercury treatment

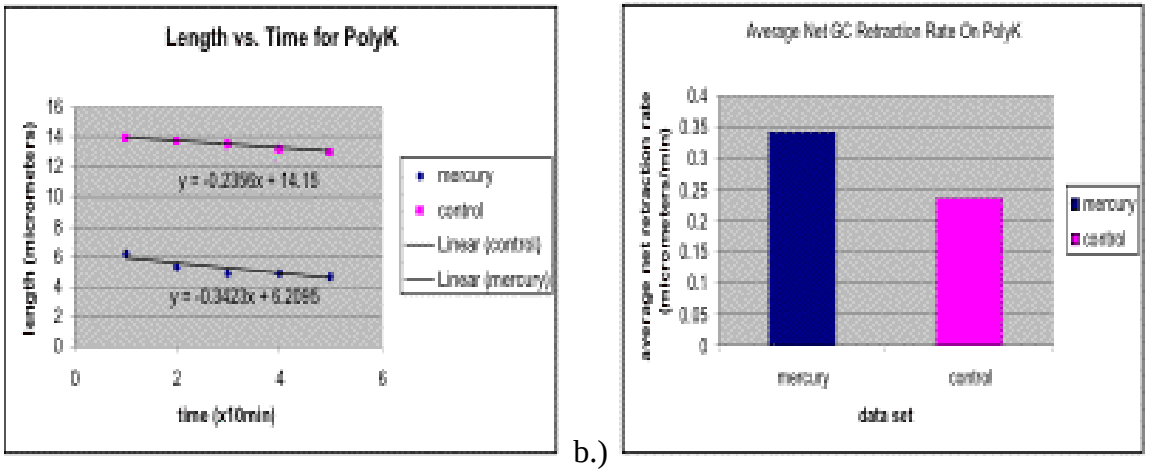
To determine direction and degree of GC movement, images form t0 to t40 (Figures 1-4 and those in between not shown), were quantified for axon length from growth cone to cell body. Results show that axon length consistently decreased for nearly all 4 cells in all 4 conditions (laminin ctrl and exp, polyK ctrl and exp) (Table 1). Thus, growth cones universally displayed negative growth and thus retracted in all conditions. However, some GCs showed greater retraction rates than others.

Table 1. Average length of axons per growth cone at 10 minute intervals for laminin and polyK cells

Time (min)	Laminin		Poly-L-lysine	
	Control length (µm)	Experimental length (µm)	Control length (µm)	Experimental length (µm)
t0	10.475	13.988	6.165	13.865
t10	8.547	13.950	5.320	13.732
t20	7.944	14.170	4.887	13.476
t30	7.311	12.769	4.855	13.176
t40	6.465	12.385	4.686	12.965

Excluding the average axon length of growth cones on laminin at t20, all average axon lengths consistently decreased form t0 to t40.

Comparing changes in axon length over time for control and experimental GCs on polyK substratum (Figure 5a) shows that the net retraction rate for the 40 minute treatment was greater for mercury than for control solutions. As shown in Figure 5b, growth cones retracted at an approximately 1/3 greater rate during the 40 minute mercury treatment verses the control treatment.

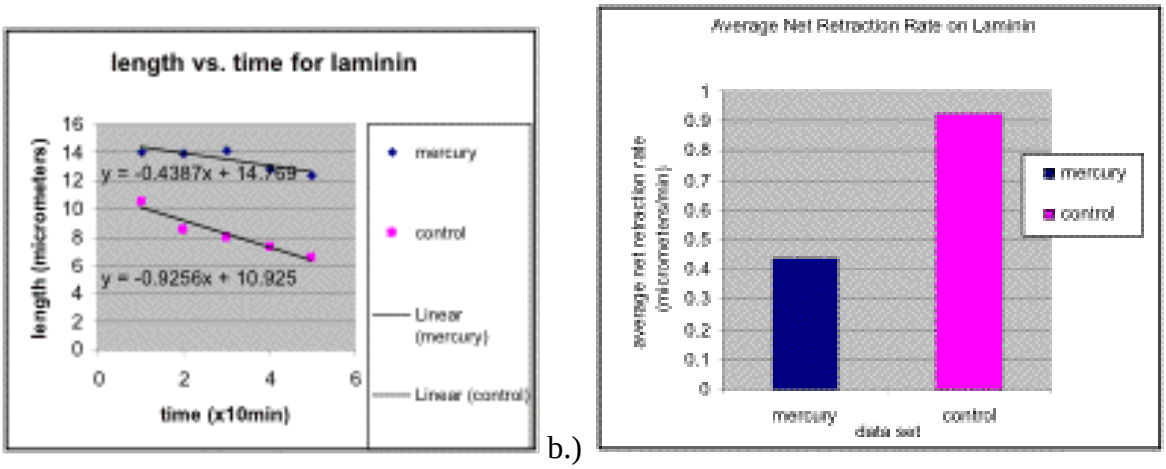


a.) b.)

Figure 5. Length of axons from cell body to tip of growth cone was averaged for all growth cones on polyK substrata at t10, t20, t30, and t40. The average axon length was plotted against the times at which they were measured (a). Trendlines show that the net growth cone retraction rate during mercury treatment was 0.3423 µm/min, compared to the slower net retraction rate of 0.2356 µm /min for growth cones during control treatment. A bar graph representation of growth rates for experimental verses control conditions on polyK is show in Figure 5b.

On average, growth cones on laminin retract slower during mercury treatment.

Surprisingly, growth cones actually exhibited a slower average net retraction rate during the mercury treatment as compared to the control treatment. Linear regression analysis of the change in axon length from t0 to t40 shows that growth cones retracted by an average of 0.4387 µm/min when subjected to the mercury solution, while growth cones retracted by an average of 0.9256 µm/min when subjected to the control solution (Figure 6a). This is a two fold increase in average net growth cone retraction rate for the control condition (Figure 6b).



a.) b.)

Figure 6. . Length of axons from cell body to tip of growth cone was averaged for all growth cones on laminin substrata at t10, t20, t30, and t40. The average axon length was plotted against the times at which they were measured (a). Trendlines show that the net growth cone retraction rate during mercury treatment was 0.0.439 µm/min, compared to the faster net retraction rate of 0.9153 µm /min for growth cones during control treatment. A bar graph representation of growth rates for experimental verses control conditions on laminin is show in Figure 5b.

Growth Cones exhibited greater retraction rates on laminin than on polyK in both experimental and control conditions

A comparison of the average net retraction rate (from t0 to t40) of growth cones from cells grown on laminin versus cells grown on polyK shows that on average, growth cones on laminin retracted at a greater rate than those on polyK for both experimental and control conditions (Figure 7).

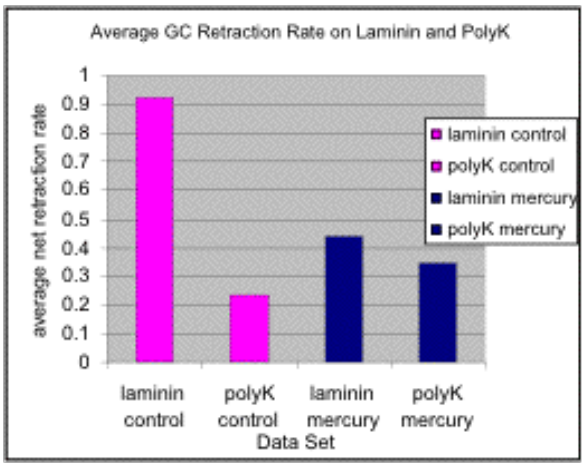
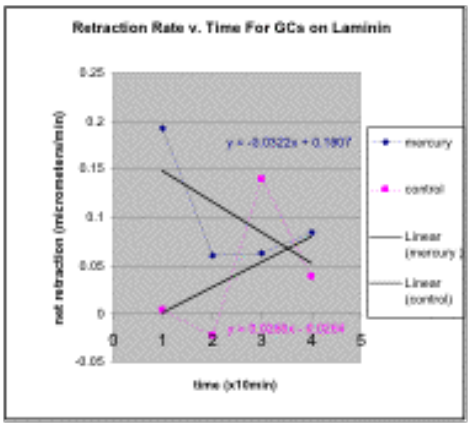


Figure 7. Data from figures 5b and 6b were combined into one chart for comparison of cells on laminin versus cells on polyK.

Retraction rate accelerated during control treatment, and decelerated during mercury treatment

Data set of GC deceleration on laminin substrata (Figure 8a) looks very similar to data set of GC deceleration on polyK (Figure 8b). Regardless of the substrata used, growth cones show the greatest retraction rates during the first 20 minutes of mercury treatment, after which the retraction rate levels off, and approaches the retraction rate of growth cones in the control solution. Acceleration of GC retraction was steeply negative during the mercury treatments, and only slightly positive during the control treatments.

a.) 

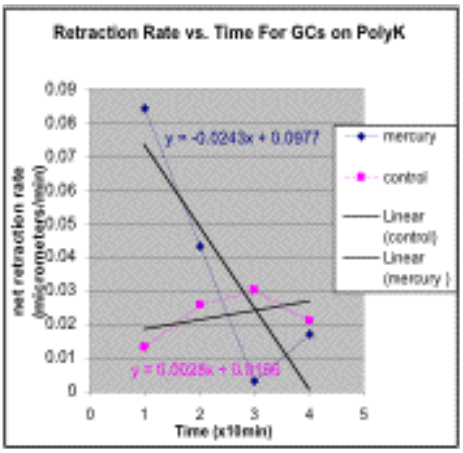
b.) 

Figure 8. Average net retraction rate was plotted against time for GCs on laminin (a) and GCs on polyK (b)

Discussion

Toxicity of low doses of mercury on neruite growth

Results of this investigation suggest that sublethal blood mercury concentrations have minimal negative effects on the developing nervous system, but do not rule out the possibility of these low mercury concentrations in inhibiting membrane integrity and normal GC outgrowth.

Original experiments applying mercury solution directly on to plated cells resulted in images of neurites with axons so diminished that they were virtually invisible on the inverted scope after 5 minutes (data not shown). Previously, Leong *et al.* (2001) discovered that growth cones exhibited robust microtubule collapse within minutes of mercury exposure, causing the axon membrane to collapse around the remaining actin filaments. This membrane collapse may have occurred in our experiments, resulting in a change in contrast which caused the axons to disappear in the viewfinder of

the inverted scope. Revising the procedure to deliver the solutions at a slower rate (i.e. by adding control and experimental solutions dropwise to normal HBSS solution) was successful in slowing the diminish of axons in the still images, but did not entirely eliminate this effect (Figures 1-4). GCs after mercury treatment where still visible in images taken by Leong *et al.*(2001) using a Zeiss (Axiovert Model 135) inverted scope. The diminish in clarity of our images may be caused by the inability of our microscope (Nikon EclipseTs100) to account for the change in contrast due to membrane collapse. Possibly, microtubule disintegration was more dramatic for our procedures than for Leong *et al.*(2001). Although Leong *et al.* (2001) used a 10 fold greater mercury concentration, they delivered it in a steady stream, simultaneously providing a constant flow of sterile *Lymnaea* saline through the culture dish during the mercury exposure. We were unable to provide this steady flow of growth media, so the cells were subject to a static solutions throughout the 40 minute experiment.

Despite this additional negative stimulus, all of the GCs observed in this experiment exhibited a slower retraction rate than the rates found by Leong *et al.* (2001). The average GC retraction rates during mercury exposure were 0.0370 μm/min for polyK, and 0.0401 μm/min for laminiñabout 50x less than the 1.7 μm/min net retraction rate found by Leong *et al.* (2001). Thus our hypothesis was supported that lower concentrations of mercury would induce lower GC retraction rates. This relationship seems to be exponential rater than a direct relationship as was hypothesized. This suggests that increasing levels of blood mercury concentrations will cause increasing neurotoxicity to developing fetuses.

Even at extracellular concentrations of 10 nM, close to the normal blood mercury concentration found in adults (5nM as found by Borjesson, 1995), neuronal growth was inhibited due to GC retraction and collapse. However, GCs also retracted during the control treatments, so it is difficult to conclude the degree in which retraction was due to mercury compounds or the HBSS solvent. PolyK data suggests that mercury exacerbated the GC retraction rate, causing further retraction than that found during control conditions. Laminin data, however, offsets these data, suggesting that mercury does not increase GC retration; retraction rates were greater during the control treatment. Since laminins are growth factors found in the basal lamina which surround most vertebrate neurons (Kandel, 2000, p.1074), we can tentatively infer that normal blood mercury concentrations do not have a negative effect on developing fetuses.

PolyK versus Laminin Substra

Compounds in solution, such as mercury, influence growth cone movement; however, cellular adhesion molecules (CAM) and extracellular matrix molecules (ECM) on the surfaces of connective tissue or along basal laminae, also guide growth cone movement (Kandel, 2000, p. 1074; Burden-Gulley, 1995). Collagens, fibronectin, and laminin influence neurite outgrowth *in vitro* and are believed to do the same *in vivo* (Burden-Gulley *et al.* 1995). Growth promoting effects of laminin and polyK are well documented and reportedly attribute their properties to the activity of GCs. Laminins, major extracellular molecules of both vertebrates and invertebrates, are recognized by growth stimulating receptor molecules on the GC membrane called integrins (Kandel, 2000, p. 1074), and polyK, a polypeptide of the negatively charged amino acid lysine, provides an attractive surface used by GCs for traction to propel themselves forward on the underlying surface (Letourneau, 1975; Yavin and Yavin, 1974). The attraction of the GC membrane for various substrates have been found to influence GC movement (Kandel, 2000, p. 1074; Heidemann, 1990; Kleinman, 1981). However, other studies (Lemmon *et al.* 1992) have concluded that the adhesiveness of a substrate has minimal influences on axonal growth rate or degree of fasciculation.

Comparing the effect of different substrata on 15 day chick peripheral neuron growth, Bray *et al.* (1987) found that the mean radial length of outgrowth after 24 hours was greatest for laminin substrata (529$\pm$46.7 μm) verses on conditioned medium (417$\pm$21.6 μm) or collagen (321$\pm$14.3μm). This complies with guidelines for primary culture of chick embryonic peripheral neurons as created by Hollenbeck, which states that neurons grow faster on laminin, but adhere better to collagen. Since polyK serves an adhesive role similar to collagens (Yavin and Yavin, 1974; Kleinman, 1981), the data by Bray *et al.* suggests that in optimal growth conditions, GCs would advance at a faster rate on laminin coated substra as opposed to polyK, leading to our earlier hypothesis that in control conditions, GCs on laminin would show greater growth rates than on polyK. However, our data shows the opposite results: GCs on laminin show less advancement (greater retraction) than GCs on polyK, in both control and experimental conditions. Important differences between the experiment conducted by Bray *et al.* (1987) and this experiment are the clear signs that our cells were in osmotic distress in both control and experimental conditions. Analysis of only control data leads to the

conclusion that the greater growth promoting effects of laminin over polyK (as observed by Bray *et al.*(1987)) are reversed when the cell is in osmotic distress. Perhaps the greater adhesive property of polyK as found by Yavin and Yavin (1974) prevented the GCs of the distressed cell from retracting as quickly as the GCs on laminin.

Effect of substrata on growth-inhibiting properties of mercury

The application of mercury to the two different substrata complicates the comparisons between the growth promoting properties of polyK versus laminin. If various chemical signals in cell membranes, in solution, and in the extracellular matrix work together to guide growth cone movement (Kandel, 2000, 1074), the degree in which polyK and laminin promote growth would not be the same when an additional chemical signal (in this case, mercury) is taken into account, or vice versa. Bozyczko and Horwitz (1986) found that the effect of the monoclonal antibody (Mab) on neuronal adhesion and neurite outgrowth depended on the particular substrate, and similar conclusions can be made from this experiment.

Although growth cones subject to the control polyK treatment showed a retraction rate averaged at 0.236 micrometers/minute, growth cones subject to the mercury treatment showed a greater retraction rate averaged at 0.342 micrometers/minute (Figure 5). Thus, growth cones retracted faster in the mercury treatment by 0.107 micrometers/minute, showing that mercury contributed to the negative influences of the control condition, exacerbating the growth cone rate of retraction. This suggests that mercury acted as a chemorepellant for growth cones on polyK substrata.

On the other hand, growth cones on laminin showed a slower mercury retraction rate (0.439 μm/min) during mercury treatment than during the control treatment (0.915 μm/min) (Figure 6). This suggests that mercury did not act as a chemorepellant for growth cones on laminin substrata.

These results support our earlier hypothesis that mercury inhibits GC growth rate to a lesser extent on laminin than on polyK. The growth promoting signals from laminin molecules may have counteracted the retraction signals from mercury compounds in solution. Thus, laminin may serve to protect neurite axons from excessive degeneration.

The chemorepellent effect of mercury decreases over time

An important result of this experiment was the finding that retraction rates for mercury decreased over time, while retraction rates for control solutions remained relatively constant, regardless of the substrate used (Figure 8). From t0 to t20, mercury induced GCs to retract greater than comparable control retraction rates. This effect diminished at t30, when the growth cone retraction rate was lower than that of the controls, and then rose at t40 to meet the retraction rates seen in the controls.

Thus, even though net retraction rate of GCs was greater for control versus mercury treatments on laminin, Figure 8 shows that mercury treatment had growth inhibiting effects on both laminin and polyK during the first 20 minutes. The gradual leveling off of retraction rates to meet the control rates suggests that growth cones resist the negative-growth influences of mercury over time.

Experimental Error

Due to time constraints, we were unable to find the lethal dose of mercury in F+ medium (concentrations which would kill ½ of cells in culture), and thus could not apply the experimental or control solutions directly into the F+ medium. F+ medium is ideal for cell growth, but also contains albumin proteins from the fetal bovine serum which makes up 10% of the solution; this protein has the ability to chelate mercury ions, inhibiting the mercury ions form having an effect on the cells. Thus, it was necessary to replace the F+ medium with HBSS before the application of either mercury or control solutions, so that the concentration of mercury having an effect on the cells was certain. HBSS, however, is not a medium conducive to neurite outgrowth, not having the nutritional supplements found in F+ medium (i.e. glutamine, glucose, and nerve growth factor), a fact which may have resulted in the negative GC movement during the control treatment.

Furthermore, to ensure that images of the same cell were taken every ten minutes, we were forced to keep the cell

culture on the inverted scope throughout the 40 minute experimental period. Even with a heater, the temperature of the culture dish was seen to be around 30¼C (using a temperature sensitive camera)Ñfar below the optimal 37¼C for incubation. Leong *et al.,* working with invertebrate neurons, did not have to worry about the effect of cold, room temperatures on depressed GC outgrowth. However, GC growth rates of vertebrate neurons, such was the chick embryonic peripheral neurons using in this experiment, are inhibited when the temperature drops below 37¼cÑthe typical temperature in vivo (Morris, personal communication).

The non-ideal media and low temperatures may have increased the retraction rate which was recorded in this experiment. Ideally, in the control conditions, GCs should show positive outgrowth comparable to normal neurite development. The fact that GCs in our controls retracted suggested that retraction rates found for experimental conditions were only partly influenced by the addition of mercury. Other factors, including pH, the solvent used, and temperature, also contributed to the retraction rate recorded for cells subjected to $HgCl_2$.

Experimenter error would also a factor in quantifying data; using the mouse curser to measure axon length was subject to change with each trial. This error can be minimized with increasing trial measurements calculated into the average axon length. Further improves on this experiment should use a stronger heater to provide optimal temperatures and a calculation of the lethal dose, so that the working mercury concentration can be revised so that cells can be kept in F+ medium at all times.

Future experiments

An intriguing result on this experiment was the gradual decrease in GC sensitivity to mercury from t0 to t40, shown by the leveling of GC retraction rates to approach those of controls (Figure 8). This mercury desensitization can be further investigated by investigating increasingly larger doses of mercury to mercury-treated Òpre-sensitizedÓ cells. Allowing mercury-treated cells to recover for a certain period of time, and treated again with mercury may show a decrease in GC retraction, and a greater mercury resistance.

Future experiments should also investigate the permeability of neurons to mercury; the quantity of mercury compounds which enter the cell and are able to leave is an important factor in the loss of cytoskeletal integrity which is suggested to be an effect of mercury by Leong *et al* (2001). It may also explain how mercury builds up in larger organisms when environmental concentrations of mercury are modestly low.

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