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Ruth B. Langton
Colby College

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An Honors Thesis in Biology
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Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. It is of particular interest because of its supposed role in the processes of learning and memory, and also because of its potential toxic effects that have been linked to neurodegenerative diseases, such as Alzheimer's and Parkinson's. Although glutamate is necessary for normal cell functioning, high levels of glutamate receptor activation can result in cell death, a phenomenon known as excitotoxicity. It has been suggested that glutamate also plays an important role in the insect and crustacean nervous systems, allowing for the examination of excitotoxicity in these organisms. The current study aims to determine the effect of high concentrations of glutamate on the neurite outgrowth of cultured fiddler crab (*Uca pugilator*) cells. Cells were obtained from the x-organ, a neurohemal organ located in the crustacean eyestalk, and were cultured for 24 hours in simple culture medium. After 24 hours, cells exhibiting neurite outgrowth were photographed and treated with one of four concentrations of glutamate. The treatment groups included: control with 0 mM glu, 0.1 mM glu, 1 mM glu, and 10 mM glu. After another 24 hours, the cells were photographed a second time and the neurite outgrowth was measured and compared. Higher concentrations of glutamate had a negative effect on neurite outgrowth, causing the neurites to retract or slow their growth. Glutamate receptors were also located in the x-organ cells using immunocytochemistry with fluorescence. This study provides insight into the workings of the crustacean nervous system and shows that fiddler crabs are a model organism in which to examine the effects of excitotoxicity, which may lead to future knowledge about the mechanisms of neurodegenerative diseases.

Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (Sheldon and Robinson, 2007). It is an amino acid that acts predominantly on depolarizing post-synaptic receptors (Nicholls, 1993). Compared to all other neurotransmitters, the levels of glutamate are extremely high in the mammalian central nervous system, about 1000-fold higher than many other important neurotransmitters, such as dopamine and serotonin (Sheldon and Robinson, 2007). Neurotransmitters such as glutamate have been implicated in the development of neuronal morphology and brain neuroarchitecture, and are important to the overall

functioning of the nervous system (Mattson, 1988). Glutamate is of particular interest because of its supposed role in the processes of learning and memory (Bliss and Dolphin, 1982; Collingridge and Singer, 1990) and because of its potential toxic effects that are associated with neurodegenerative diseases, such as Parkinson's and Alzheimer's (Rothman and Olney, 1986; Choi, 1988; Meldrum and Garthwaite, 1990; Nicholls, 1993; Sheldon and Robinson, 2007).

It has been shown that excessive activation of glutamate receptors can result in cell death, a phenomenon known as excitotoxicity (Choi, 1992; Coyle and Puttfarcken, 1993; Doble, 1999; Sheldon and Robinson, 2007). This excitotoxicity has been associated with a number of acute and chronic neurodegenerative diseases. These diseases include: amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease and Parkinson's disease, as well as a few more acute insults such as epilepsy and cerebral ischemia (Rothman and Olney, 1986; Choi, 1988; Mattson et al., 1988; Meldrum and Garthwaite, 1990; Sheldon and Robinson, 2007). Since there is no evidence for extracellular metabolism of glutamate, it is believe that this amino acid needs to be cleared from the extracellular space by transporters (Sheldon and Robinson, 2007). Alterations in the expression or function of these glutamate transporters have been implicated in a variety of neurological disorders, but it is not clear if changes in glutamate transporters precede neurodegeneration or result from it (Sheldon and Robinson, 2007).

There is some evidence that antioxidants can rescue cells from death induced by excitotoxicity. Ueda et al. (2002) show that increased levels of extracellular glutamate at the initiation seizures induced with kanic acid is associated with the generation of free radicals which cause oxidative stress. Antolin et al. (2002) show the protective effects of

melatonin in an experimental model of Parkinson's disease. It was hypothesized that cell death is derived from free radical damage and that anti-oxidants, such as melatonin, may reverse these effects (Antolin et al., 2002).

The toxic effects of high concentrations of glutamate, particularly with respect to neurite outgrowth, have been examined in a number of studies. Diffusible factors such as neurotransmitters are known to regulate both the rate and direction of neurite outgrowth in culture (Ryan et al., 2006). Mattson et al. (1988) found that dendrites of isolated hippocampal pyramidal neurons were suppressed by glutamate application. Owen and Bird (1997) investigated the effects of glutamate on the development of spinal cord neurons grown in culture and found that the application of glutamate resulted in a reduction in both the rate of outgrowth and the motility of neurites. Ryan et al. (2006) demonstrated that focal application of glutamate to neurite growth cones of descending brain neurons from larval lamprey inhibited neurite outgrowth or caused neurite retraction. This study suggested that these effects were due to calcium influx via both voltage-gated calcium channels and ligand-gated channels (Ryan et al., 2006). Graf and Cooke (1994) also found that calcium metabolism influenced neurite outgrowth in cultured crustacean x-organ neurons, the subject of this study.

Rival et al. (2004) examined the toxicity of glutamate in the insect brain by silencing a high-affinity glutamate transporter in *Drosophila*. The experimenters observed behavioral changes, a poor ability to fly, and contracted neurites in sampled brain sections (Rival et al., 2004). This evidence suggests that decreasing glutamate buffering capacity in the *Drosophila* brain has neurotoxic effects similar to those seen in mammals. Although the insect and crustacean nervous systems are similar, the potential

neurotoxic effects of glutamate in the crustacean brain have not been previously studied. It is known, however, that glutamate is an integral part of the crustacean nervous system and that the motorneurones in the crayfish locomotory system are glutamatergic (Watson et al., 2000). Watson et al. (2000) show that glutamate may have more than one role in the arthropod central nervous system, with glutamate-immunoreactive synapses representing a mixture of excitatory and inhibitory connections.

Glutamate receptors mediate most excitatory transmission in the brain (Nicholls, 1993; Sheldon and Robinson, 2007). There are both ionotropic and metabotropic glutamate receptors and there is evidence that both types of glutamate receptors exist in the invertebrate brain (Wafford and Sattelle, 1989; Brugess and Derby, 1997; Cleland and Selverston, 1998; Duan and Cooke, 2000; Krenz et al., 2000; Levi and Selverston, 2006). Ionotropic receptors consist of groups of transmembrane ion channels that open and close in response to a chemical messenger such as a neurotransmitter. Metabotropic receptors are indirectly linked to ion channels through signal transduction mechanisms, often G-proteins. Ionotropic glutamate receptors are broken into two categories: NMDA (*N*-methyl *D*-aspartate) and AMPA/kainite (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) that both have glutamate-gated ion channels (Nicholls, 1993). These receptors are made up of structurally related subunits, GluR1-7 (Nicholls, 1993; Santa Cruz Biotechnology, 2008). AMPA/kainite receptors are primarily responsible for fast, excitatory neurotransmission by glutamate and consist of subunits GluR1-4, where as NMDA receptors, subunits GluR5-7, are characterized by slow kinetic and a high permeability for Ca^{2+} ions (Nicholls, 1993; Santa Cruz Biotechnology, 2008).

Duan and Cooke (2000) suggest that ionotropic glutamate receptors exist in the x-organ/sinus gland of crab peptide-secretory neurons. The x-organ/sinus gland is a neurohemal organ located in the crustacean eyestalk just beneath the exoskeleton (Bunt and Ashby, 1967; Nagano, 1986). The x-organ is comprised of a cluster of about 150 large neurons that sends axons to the sinus gland via the sinus gland nerve (Cooke 1985; Nagano and Cooke, 1987; Cooke et al., 1989). The x-organ/sinus gland in the crustacean nervous system is analogous to the hypothalamus in the mammalian brain (Weatherby, 1981; Nagano, 1985; Cooke, 1985; Nagano and Cooke, 1987; Duan and Cooke, 2000) and releases several different types of neurohormones that control processes such as molting and growth, gonadal development, limb regeneration, and the metabolism of proteins and lipids (Cooke, 1985; Weatherby, 1981). This structure provides an excellent model for the study of hormones and neurotransmitters because x-organ cells can be cultured and grown in simple medium. Cooke et al. (1989) found that these cells show immediate outgrowth after adhering to the substrate and can continue to grow for about seven days. It was suggested that these cells may exhibit this immediate outgrowth as a result of already active synthetic, transport, and secretory mechanisms for growth.

Ionotropic glutamate receptors may exist in the x-organ/sinus gland of the crab nervous system. It was determined that γ-amnio-butyric acid (GABA), the primary inhibitory neurotransmitter in the mammalian central nervous system, and glutamate activate different receptors that mediate Cl^- conductance within the cell based on a difference in desensitization during Glu and GABA application (Duan and Cooke, 2000). A number of studies suggest the existence of metabotropic glutamate receptors (mGluR) in the spiny lobster's stomatogastric ganglion and that these mGluRs are similar to those

described in mammals (Levi and Selverston, 2006; Krenz et al., 2000). Although there is evidence suggesting that both ionotropic and metabotropic glutamate receptors exist in the crustacean nervous system, they have not been previously located using immunocytochemistry.

The current study aims to examine the potential neurotoxic effects of glutamate in the crustacean brain and to locate the ionotropic glutamate receptors in cultured x-organ/sinus gland cells of fiddler crabs. Specifically, I wanted to determine if the presence of glutamate in high concentrations affects neurite outgrowth of cultured *Uca pugilator* cells.

Methods

Crab housing and care. Fiddler crabs (*Uca pugilator*) were purchased from Gulf Specimen Marine Laboratory (Panacea, FL) and housed in clear plastic tanks with Croalife® artificial seawater with both terrestrial and aquatic terrains available. Crabs were fed Purina Cat Chow® every other day, and water was changed after each feeding.

Tissue Dissection. Dissection procedures were adapted from Cooke et al. (1989). Sterile crab saline solution for crab dissection was prepared by combining 500 ml HPLC chromasolv water, 14.3 g NaCl, 0.41 g KCl, 0.95 g $CaCl_2$, 2.64 g $MgCl_2$, and 1.19 g HEPES. The solution was adjusted to pH 7.4 with 1 M NaOH, vacuum-filtered, treated with 10 mL stabilized penicillin-streptomycin-neomycin solution and stored at 8ºC.

Prior to performing the eyestalk dissection, the fiddler crabs were anesthetized by cooling at 8ºC for at least 10 minutes. All dissection tools and plates were rinsed in 70%

ethanol prior to dissection. Eyestalks were removed and immediately submerged in sterile crab saline. Incisions were made through the outer carapace on the lateral sides of each eyestalk up to the cornea, and the top portion of the carapace was lifted to reveal the underlying eyestalk muscular and neural tissue. The neural tissue was dissected away from the eyestalk and examined to locate the light blue, opalescent sinus gland on the medulla terminalis.

X-organ-containing tissue on the opposite side of the medulla terminalis was removed and placed in Ca^{2+} and Mg^{2+} free saline with 0.1% trypsin. The sterile Ca^{++}-free, Mg^{++}-free saline was prepared by combining 500 ml HPLC chromasolv water, 15.45 g NaCl, 0.41 g KCl and 1.19 g HEPES. The trypsin digest solution was adjusted to pH 7.4 with 1 M NaOH, vacuum filtered and stored at 8ºC. X-organ tissue was gently stirred in the trypsin solution for 1 hour. The trypsin digested protein attachments between cells, allowing the cells to dissociate without being damaged. Prior to dissection, the trypsin solution was prepared by combining 200 µl trypsin 10x solution and 5 ml of Ca^{2+} and Mg^{2+} free saline in an Erlenmeyer flask and swirling to mix.

Cell culture. Culture medium was adapted from Cooke et al. (1989). To prepare the culture medium, a concentrated crab saline solution was made by combining 500 mL HPLC chromasolv water, 16.609 g NaCl, 0.476 g KCl, 1.109 g $CaCl_2$, 3.066 g $MgCl_2$, and 2.303 g HEPES. The super concentrated crab saline solution was then combined with 500 ml L-15 medium leibovitz and 21.624 g D-glucose for a final concentration isosmotic to crab hemolymph, 850 mOsm. The culture medium was adjusted to pH 7.4 with 1 M NaOH and vacuum filtered. Ten ml of 200 mM L-glutamine and 10 mL

penicillin-streptomycin-neomycin solution was added to the final solution, which was stored at 8ºC.

Each piece of x-organ tissue was transferred to a 200 μL drop of culture medium (osmolality 840 mM) in 35mm MatTek Corporation a glass-bottom culture dish. The tissue was then broken apart using forceps. Cells were allowed to adhere to the glass-bottom and then approximately 1 hour later 3 mL of culture medium was added to each dish. The cultures were housed in darkness and high humidity.

Treatments. A stock solution of 10 mM glutamate was made and combined with regular culture medium to attain the desired glutamate concentration for each treatment group. Treatment groups included: control (0 mM glu), 0.1 mM glu, 1 mM glu, and 10 mM glu. Cells were cultured and allowed to grow for 24 hours in regular culture medium. After the first 24 hours, the original culture medium was removed using a glass Pasteur pipette and was replaced with the control or glutamate solution of the appropriate concentration. Cells were allowed to grow for another 24 hours while bathed in the glutamate solution. Five trials were conducted with each of the four treatment groups.

Measurement of neurite growth. Cells were viewed with a Zeiss Axiovert 200 microscope with phase contrast and photographed with AxioVision software; an example of one of these images can be seen in Figure 1. Photographs were taken immediately after 24 and 48 hours of growth, before and after glutamate application respectively. Approximately 30 cells from each dish were photographed, totaling about 150 cells per

treatment group. With AxioVision software, the perimeter of the neurite-encompassing area around each cell was traced as well as the cell body (example in Figure 2).

Analysis. In order to standardize measurements to account for differing cell sizes, cell body measurements were subtracted from the neurite area and then divided by the cell body area.

Statistical Analysis. A two-way ANOVA was used to determine the differences among treatments (before and after glutamate application) and among culture dishes within treatments, with the Holm-Sidak method for pairwise comparisons when the data did not pass the Equal Variance Test. Data was indexed and the factors used for this test were: treatment group (24 or 48 hours) and dish number within each group (dishes 1-5). An overall comparison of the four treatment groups was done using a one-way ANOVA and Dunn's method for multiple comparisons. Statistics were performed using SigmaStat software, version 2.0.

Glutamate and Melatonin. One trial run was conducted to determine if melatonin has a protective effect on cells treated with glutamate. Cells were cultured in simple medium following the procedure outlined above and were left to incubate for 24 hours. Cells were photographed, as described above. Culture medium was removed using a glass Pasteur pipette and was replaced with 3.2 mL of 10 mM glutamate stock solution and 3.6 μL of 1 mM melatonin. Culture dishes were left to incubate for another 24 hours and were photographed using the procedure above.

Immunocytochemistry with fluorescence. Cells were stained for glutamate receptors using a rabbit antibody GluR (H-301 that was purchased from Santa Cruz Biotechnology, Inc.) and was prepared at a dilution of 1:50 when combined with PBS buffer. This antibody was used for the detection of ionotropic glutamate receptors 1, 2, 3, and 4. FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) was prepared with a 1:50 dilution.

Fixing cells. Cells were dissected in the same method as stated above and were fixed using formaldehyde. Culture medium was removed from the dish using a glass Pasteur pipette and cells were treated with 3.7% formaldehyde for 15 minutes. Formaldehyde was removed and cells were rinsed with PBS buffer. The buffer was removed and 1M glycine was added for 15 minutes and then removed. Cells were rinsed with PBS buffer, PBS was removed, and 0.5% triton-X 100 was added for 10 minutes. Triton-X 100 was removed and cells were rinsed with PBS buffer. PBS was removed and 1% goat serum (10 μL serum into 1mL of PBS buffer) was added for 30 minutes. Cells were rinsed with PBS buffer.

Antibody Labeling. 200μL of glutamate receptor (GluR-1, 2, 3, 4) primary antibody (GluR (H-301)) was added to each dish. Cells were incubated for approximately 1 hour in the dark at room temperature. Primary antibody was removed and cells were rinsed with PBS buffer. 200μL of rhodamine phallodin (for actin labeling) and 100μL of goat anti-rabbit (secondary antibody) were added to each dish and cells incubated of approximately 30 minutes in the dark at room temperature. Rhodamine and the

secondary antibody were removed and cells were rinsed with PBS. A few drops of Prolong Gold antifade fixative with DAPI (for nuclear staining) was added to each dish. Dishes were stored in an aluminum-wrapped box at room temperature.

Photographing. Cells were located using a Zeiss Axiovert 200 fluorescence microscope. Photographs were taken using Axiovision software. A confocal microscope at the Mount Desert Island Biological Laboratory was used to photograph whole chunks of eyestalk tissue that had been fixed and stained using the procedure above.

Results

Control cells showed more growth after 48 hours than after 24 hours, however, this difference was not significant (Figure 3, $p>0.05$). No significant difference among dishes within each control time period (24 and 28 hours) was found ($p>0.05$).

Cells treated with 0.1mM glutamate showed a slight but insignificant ($p>0.05$) increase after 48 hours of growth when cells were bathed in a 0.1mM glutamate solution at 24 hours (Figure 4). No significant difference was seen between dishes within each treatment ($p>0.05$).

Cells in the 1mM glutamate treatment group also showed a slight but insignificant ($p>0.05$) increase from 24 to 48 hours, after being bathed in 1mM glutamate (Figure 5). No significant difference was seen between culture dishes within each treatment ($p>0.05$).

A significant difference was seen between cells in the 10 mM glutamate group before and after glutamate application (after 24 and 48 hours respectively) ($p<0.01$). A

decrease in standardized neurite outgrowth was seen after the application of 10 mM glutamate (Figure 6). No significant difference was found between culture dishes within each treatment ($p>0.05$).

When comparing the four treatment groups, there was no significant difference seen among any of the groups after 24 hours of growth prior to glutamate application (Figure 7, $p>0.05$). After 48 hours (24 hours after the application of glutamate), neurite outgrowth decreased with increasing concentrations of glutamate, compared to the control, with 10 mM glutamate cells showing the least outgrowth (Figure 8). The 0.1 mM and 1 mM glutamate treatment groups did not differ significantly from the control ($p>0.05$) and are not significantly different from each other ($p>0.05$). The 10 mM glutamate treatment group is significantly different from all other treatment groups and the control ($p<0.05$).

Immunocytochemistry indicated the potential presence of glutamate receptors in the fiddler crab neurons using green (FITC) fluorescence. Glutamate receptors appear to be located on the cell membrane of the cell body and also in the membrane of the neurites (Figure 9). As well as staining the cultured cells, I also labeled larger pieces of tissue from the x-organ to determine the general localization pattern of potential glutamate receptors. Based on the fluorescence staining, the glutamate receptors appear to be located in the x-organ/sinus gland of the fiddler crab eyestalk (Figure 10), as well as in other neural tissue.

Cells in the glutamate plus melatonin trial group showed normal neurite outgrowth after the first untreated 24 hours of growth in simple culture medium. After being treated with melatonin and glutamate, at 48 hours, the cells had almost all lysed

and all that was left was debris from the cell bodies. This pattern recurred in all three different culture dishes.

Discussion

The results of this study suggest glutamate has an effect on neurite outgrowth and also that glutamate receptors exist in fiddler crab x-organ cells. The lower concentrations of glutamate (0.1 mM and 1 mM) had no significant impact on neurite outgrowth, but the highest concentration, 10 mM, significantly decreased outgrowth which is assumed to be a result of excitotoxicity. I expected that lower levels of glutamate would not affect normal cell growth, because they are more normal physiological concentrations at which glutamate is necessary for mammalian cell functioning (Nicholls, 1993). These results show that high levels of glutamate are necessary to cause excitotoxicity in the x-organ cells.

Previous studies have also used high concentrations of glutamate and seen a toxic effect on cells. Ryan et al. (2006) used focal application of glutamate at concentrations of 5 mM and 25 mM to larval lamprey descending brain neurons. This study found that the application of glutamate inhibited outgrowth of treated neurites, but did not affect other neurites from the same neuron (Ryan et al, 2006). However, when glutamate was applied directly to the cell body, all the neurites from the cell were inhibited (Ryan et al., 2006), which corresponds with the results of the my study. Owen and Bird (1997) used concentrations of glutamate ranging from 1 to 100 μM when studying its effects on mouse spinal cord neurons and found a significant inhibition of neurite growth. This

suggests that higher concentrations of glutamate are necessary for outgrowth inhibition in crustaceans than in mammals.

Immunocytochemisty with fluorescence was used in this study to attempt to locate glutamate receptors, which appear to have been successfully labeled in both the cell body and the neurites of x-organ cells. This provides support for the inhibitory effect of glutamate on neurite outgrowth because it indicates that glutamate is able to enter and interact with the cell. Further studies are needed to more fully establish the presence of these receptors.

After I determined that 10 mM glutamate significantly decreased neurite outgrowth in cultured cells, I added melatonin as another variable in the experiment to determine if melatonin, an antioxidant, has the ability to rescue cells treated with glutamate. Melatonin is known to have widespread antioxidant actions including the direct scavenging of free radicals and activation of enzymes in antioxidant pathways (Tan et al, 1993; Reiter et al., 2007). Antolin, et al. (2002) suggests that melatonin prevents cell death as well as damage caused by oxidative stress in a mouse model of Parkinson's disease and indicate that melatonin may prevent neurotoxin damage in general.

If cells were rescued from the excitotoxicity caused by glutamate, they would not show reduced outgrowth, or may show more outgrowth after 48 hours. For example, previous studies in the Tilden lab (Cuttler, 2007) showed both a neurite growth enhancing effect and a separate antioxidant, neuroprotective effect of melatonin alone on x-organ cells. However, in a trial run of this experiment, these cells lysed after being treated with both glutamate and melatonin, and all cells that were present in the dish after 48 hours

did not have visible neurites. The reason for this detrimental effect of the combination of melatonin and glutamate is unclear and is an area that requires further research.

In sum, I was able to locate glutamate receptors in the x-organ cells of fiddler crabs, which implicates these crabs as a powerful model system for studying glutamate-mediated neurodegeneration. These fiddler crabs can be used in future studies to add to the body of research about neurodegenerative disease and excitotoxicity.

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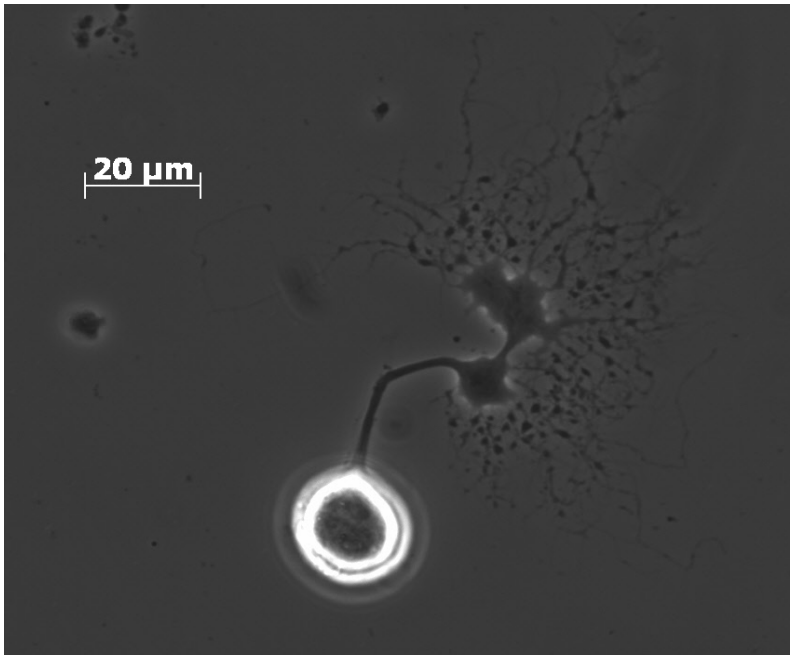

Figure 1. Control cell after 48 hours of growth in simple culture medium. Photographed using a Zeiss Axiovert 200 microscope at 400X magnification.

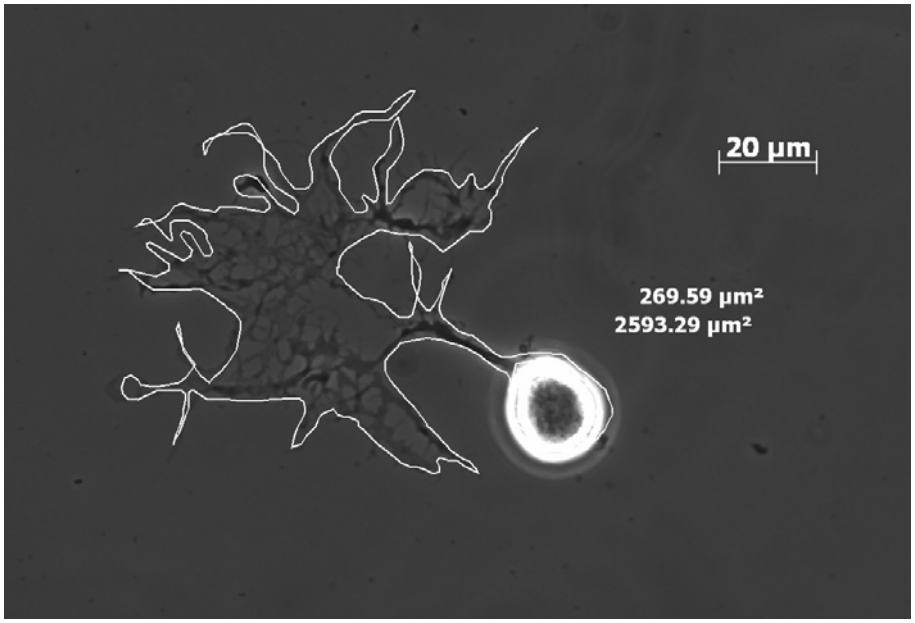

Figure 2. Control cell after 48 hours of growth in simple culture medium. Photographed using a Zeiss Axiovert 200 microscope at 400X magnification and analyzed using AxioVision software. Measurements represent cell body size (269.59µm^2) and total neurite encompassing area (2593.29 µm^2).

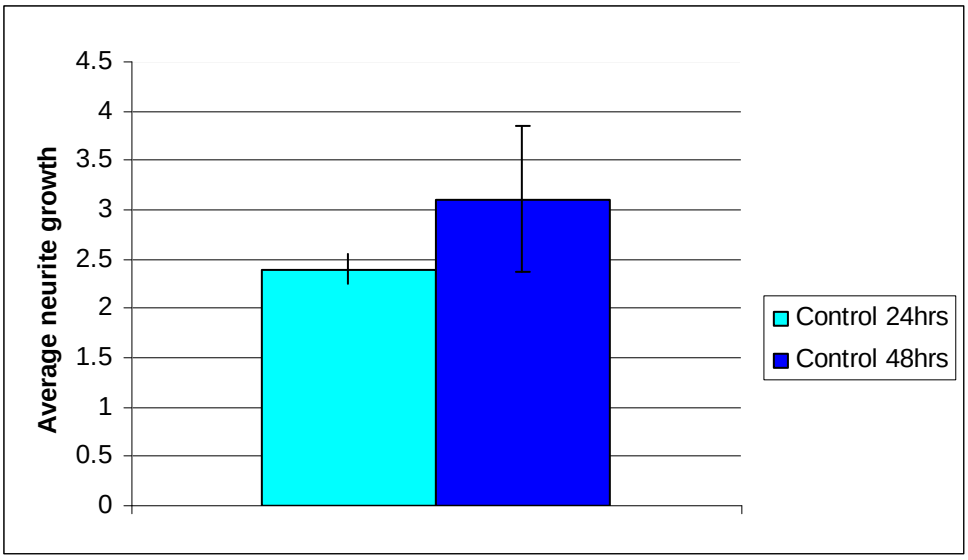

Figure 3. Mean neurite outgrowth of the control cells, standardized for differing cell body sizes, after 24 and 48 hours of growth in simple culture medium (+/-*SE*).

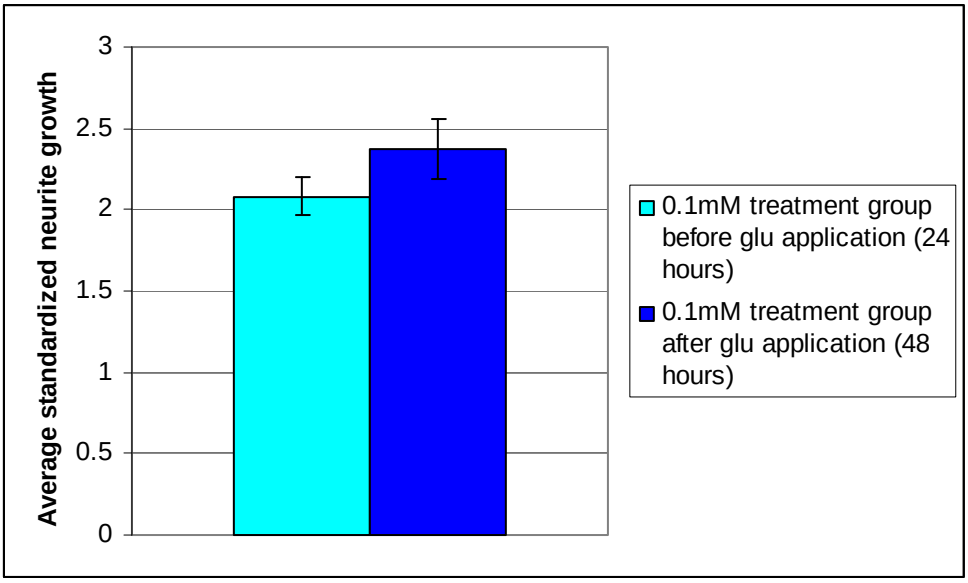

Figure 4. Mean neurite growth of cells, standardized for differing cell body sizes, in the 0.1 mM glutamate treatment group after 24 and 48 hours of growth, before and after glutamate application respectively (+/- *SE*).

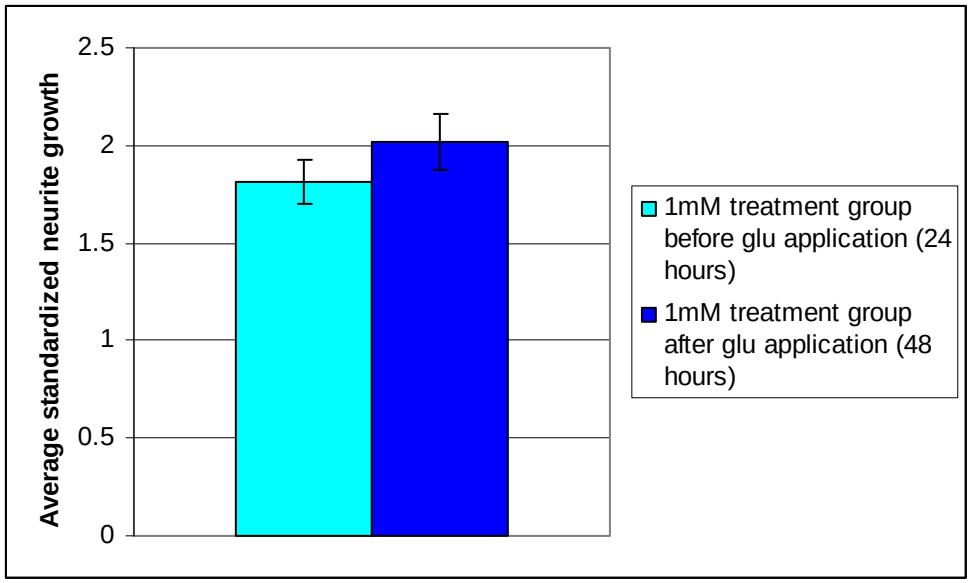

Figure 5. Mean neurite growth of cells, standardized for differing cell body sizes, in the 1 mM glutamate treatment group after 24 and 48 hours of growth, before and after glutamate application respectively (+/- *SE*).

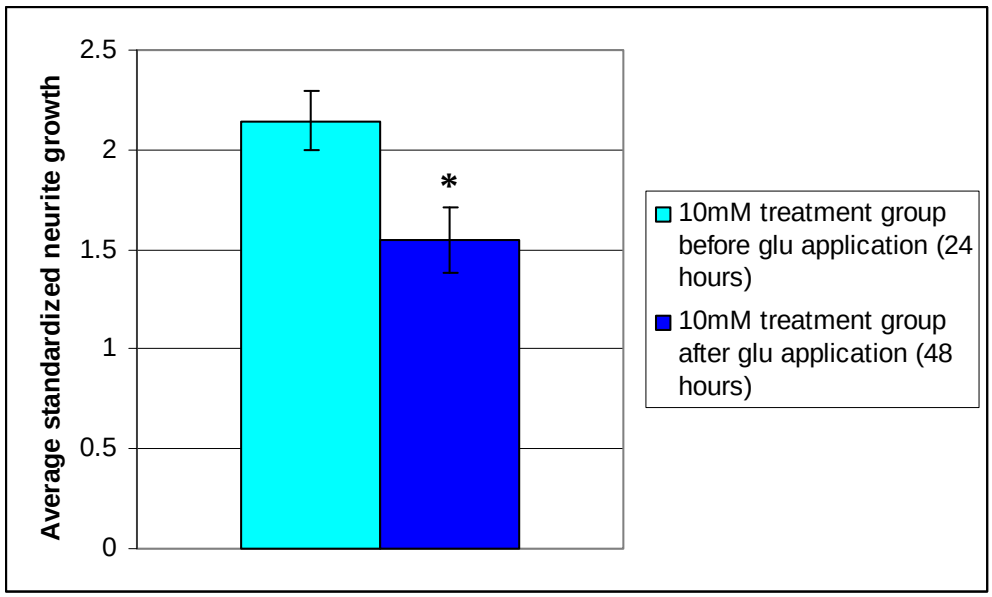

Figure 6. Mean neurite growth of cells, standardized for differing cell body sizes, in the 10 mM glutamate treatment group after 24 and 48 hours of growth, before and after glutamate application respectively (+/- *SE*).

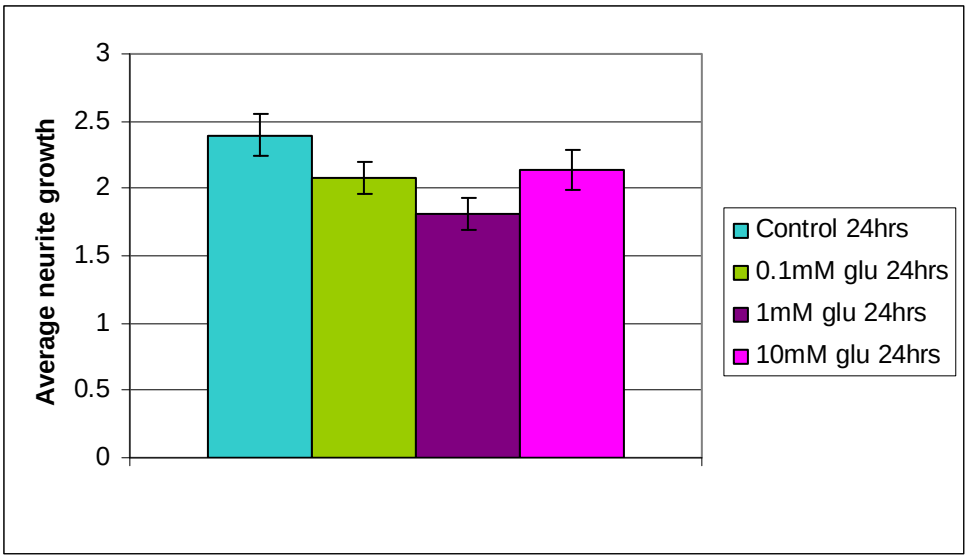

Figure 7. Neurite growth, standardized for differing cell body size, after 24 hours and *before* glutamate application to all treatment groups (+/- *SE*).

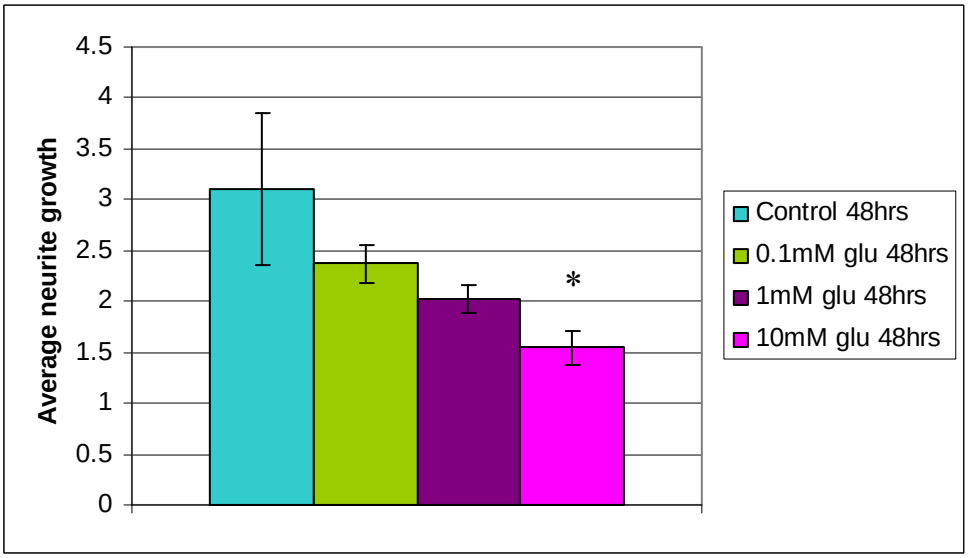

Figure 8. Neurite growth, standardized for differing cell body size, after 48 hours of growth and *after* glutamate application to all treatment groups (+/- *SE*).

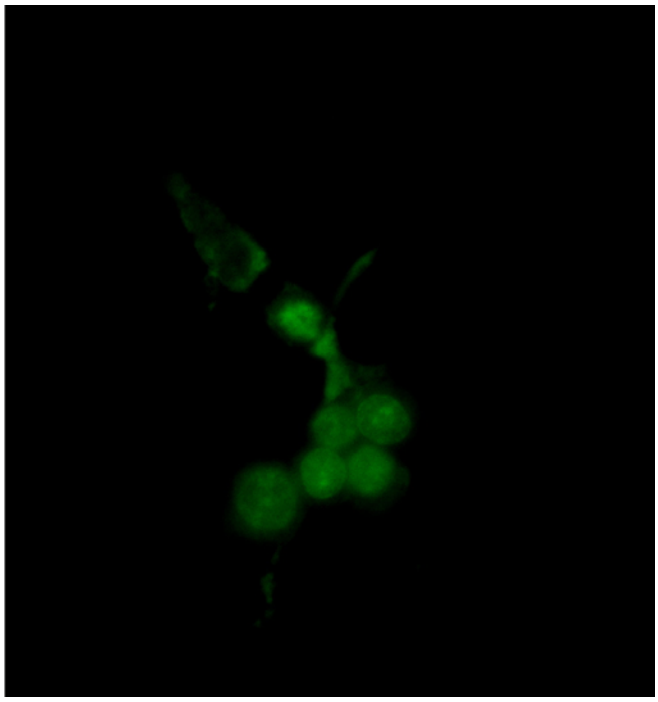

Figure 9. Cluster of six cultured cells labeled with green fluorescence for glutamate receptors (GluR-1, 2, 3, 4).

(a) (b)

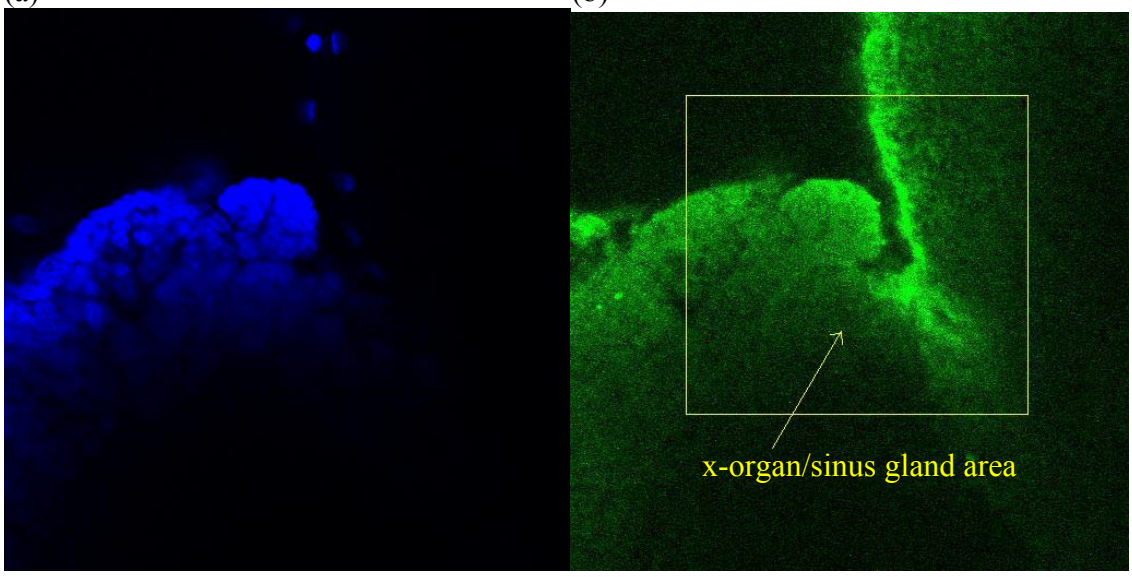

Figure 10. X-organ/sinus gland area of the fiddler crab eyestalk stained for (a) cell nuclei with DAPI blue fluorescence (b) glutamate receptors (GluR-1, 2, 3, 4) with fitc green fluorescence.