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The Response of Satellite Glial Cells to P2X7 Receptor Activation

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THE RESPONSE OF SATELLITE GLIAL CELLS TO P2X7 RECEPTOR ACTIVATION

by

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ABSTRACT

Satellite glial cells (SGCs) surround the cell bodies of neurons of the peripheral nervous system, including those of the sensory ganglia. Their close apposition to the neuronal soma allows for bi-directional communication between neurons and SGCs, which are thought to regulate neuronal activity. After nerve injury, SGCs in the dorsal root ganglia contribute to neuropathic pain. Although the mechanisms are not fully understood, SGCs show increased coupling via gap junctions, and communicate with the neuron via bi-directional purinergic signaling after nerve injury. The increased coupling between SGCs and neurons may have implications for chronic pain following peripheral nerve injury. *In vivo* studies suggest that injury through the administration of capsaicin to the sensory nerve endings causes SGCs to be activated and proliferate. We have shown that capsaicin treatment in an *in vitro* co-culture of sensory neurons and SGCs increased the expression of the proliferation marker, Ki-67 in the glia. Here, we examine whether purinergic signaling plays a role in the promotion of SGC proliferation.

DEDICATION

For my extremely supportive family, especially my mother, who is my role model and never once doubted my dream of becoming a physician.

For all of my teachers and mentors, from elementary school to college, who have challenged me, encouraged me, and shaped me into the student I am today.

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ABBREVIATIONS

SGC(s): Satellite glial cell(s)

PNS: Peripheral nervous system

CNS: Central nervous system

DRG(s): Dorsal root ganglion (ganglia)

BzATP: 2’(3’)-O-(4-Benzoylbenzoyl)adenosine 5’-triphosphate triethylammonium salt

Ki67+: Ki67 positive

Ki67-: Ki67 negative

ICC: Immunocyctochemistry

Cx43: Connexin-43

SEM: Standard error of the mean

INTRODUCTION

Satellite Glial Cells

Dorsal root ganglia (DRG) are formed by neuronal cell bodies of the peripheral nervous system (PNS), and their axons carry sensory signals from the body to the central nervous system (CNS), including pain signals. Satellite glial cells form a sheath around these neuronal cell bodies (reviewed by Hannini, 2005). SGCs have been compared to astrocytes in the CNS due to their ability to control the microenvironment around the cell body (reviewed by Hannini, 2005). Regulation of the microenvironment can affect neuronal threshold, and play a role in neuropathic pain, where the firing threshold is lowered. SGCs' pluripotency is also demonstrated; SGCs from DRGs were shown to turn into oligodendrocytes, Schwann cells, and astrocytes *in vitro* (Svennigsen et al, 2004). SGCs do not have chemical synapses or dendrites, so paracrine signaling is very important in these cells. ATP is involved in inflammation and its activity has important implications in pathological conditions. Furthermore, SGCs have purinergic receptors, and ATP is believed to be an important mediator in their activity (reviewed by Hannini, 2005).

Purinergic Signaling in SGCs

It is important to understand the mechanisms responsible for pain in order to design therapeutic targets. According to Costa et al (2015), an increase in neuronal sensitivity to ATP, changes in transmitter expression, and increased firing activity of un-injured dorsal root ganglion (DRG) neurons are among the mechanisms proposed to produce chronic pain (Costa et al. 2015). The interaction between SGCs and neurons is thought to affect neuronal activity, due to the

intimate relation between these two cell types. SGCs communicate with neurons and affect neuronal excitability, particularly by decreasing the activation threshold and causing hyper-excitability (reviewed by Takeda et al, 2009). Additionally, the increase in purinergic receptors on SGCs after nerve damage is speculated to heighten sensitivity to ATP, resulting in activation and proliferation (reviewed in Costa et al, 2015).

Response of SGCs to peripheral nerve injury includes morphological changes and release of glial mediators, such as ATP. Activation of SGCs is characterized by increased gap junction formation, increased intracellular calcium concentration, increased levels of GFAP, and increased calcium signaling between cells. Activation of signaling mechanisms between neurons and SGCs seems to be triggered by increased neuronal firing due to nerve injury. The main outcomes of SGC activation due to injury include: increased expression of GFAP, decreased expression and sensitivity of potassium channels, increased SGC coupling by gap junctions, increased sensitivity to ATP, altered expression of purinergic receptors, and release of ATP and cytokines. These outcomes may contribute to chronic pain (Hannani, 2005 and 2012; Takeda et al, 2009; Ohara et al, 2009).

SGCs communicate with each other via gap junctions, which are channels between cells that allow ions and small molecules to be transferred from cell to cell. A molecular component of gap junctions is connexin-43 (Cx43), which can be used to study the formation of gap junctions in SGCs. Gap junctions are seen between adjacent SGCs of a single somata, and between SGCs and their enclosed neuron. After nerve injury, SGCs contact neighboring SGCs surrounding different neurons with the concomitant formation of new gap junctions, which possibly leads to the recruitment of more neurons to the pain response (Hannini et al, 2002; Pannese et al, 2003).

Supporting this assumption, gap junction blockers reduce the spontaneous firing of neurons in injured DRGs, which is associated with a reduction in pain (reviewed by Huang et al, 2010). Some results suggest that amplification of pain could be caused by increased gap junctions that enable more diffusion of allogeneic substances such as ATP or Ca^{2+} from the site of injury to adjacent areas (reviewed by Huang et al, 2010).

ATP is the main mediator of interactions between SGCs and neurons (Gu et al, 2010; Suadicani et al. 2010). The ionotropic P2X receptor that is expressed in SGCs is P2X7; this is not expressed in DRG neurons (Chen et al, 2008). ATP released by vesicles in neurons can activate the P2X7 receptor of SGCs, leading to the release of cytokines, such as TNF*a*. It has also been shown that release of ATP by the neuronal cell body acts on the P2X7 receptor by increasing the intracellular concentration of Ca^{2+} in surrounding SGCs (Scemes et al, 2006). It is proposed that the waves of calcium are used as a mechanism of communication, mediated by ATP and gap junctions. Overall, electrical or mechanical stimulation of a single cell body led to a propagation in calcium in neurons and surrounding SGCs; this was mediated by P2 receptors and gap junctions (Suadicani et al, 2010). **Figure 1** shows this proposed model by Costa of neuron/SGCs interaction via gap junctions, ATP, and calcium waves (Costa, 2015). ATP is released from neurons into SGCs in response to peripheral injury. Intracellular Ca^{2+} levels then increase in SGCs, where some calcium ions pass through gap junctions from SGC to SGC.

SGCs are not electrically excitable; they respond to electrical or mechanical stimuli by changes in Ca^{2+} through the opening of receptors, specifically the iontropic purinergic P2X receptors. Injury in sensory neurons increases ATP release, which can activate P2X7 receptors. Neurotransmitters such as glutamate, GABA, and ATP may then be released from SGCs

following the activation of P2X7 receptors. A study showed that activation of P2X7 receptors was effective in curbing the inflammation and nerve injury induced hyperalgesia. P2X7 receptors have also been shown to be upregulated in SGCs of injured DRGs isolated from chronic neuropathic pain patients (Gu et al, 2010).

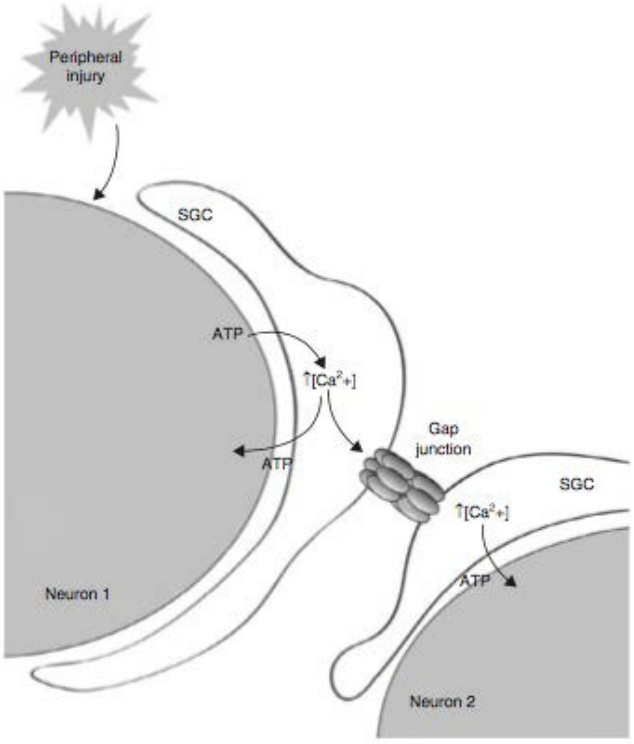

Figure 1: Neuron/SGC interaction Model (Costa, 2015)

Preliminary Data

BzATP was used as a P2X7 purinergic receptor agonist for this research. It is more potent (has a higher affinity) than ATP at P2X7 receptors, which are present in SGCs. A concentration of 100µM was added to the neuron/SGC co-cultures. This concentration was chosen based on an experiment performed by Zhang et al, where *in vitro* dissociated neurons and non-neuronal cells

from DRGs were characterized via the patch clamp technique. After the addition of 100μM BzATP, an inward current was evoked that was significantly greater than ATP in non-neuronal cells (Zhang et al, 2005). Extracellular ATP has a short half-life of less than 60 minutes (Conigrave et al, 2000), but BzATP has a longer half-life, since enzymes do not as readily hydrolyze it. Therefore, the goal of using BzATP was potent and extended activation of P2X7 receptors, triggering the release of neurotransmitters.

While sensory neurons have receptors for ATP and capsaicin, SGCs are thought to have receptors for ATP, specifically the P2X7 receptor (reviewed by Hannini, 2005). Capsaicin is a neurotoxin that causes neuronal cell death, and is used to model nerve injury. The mechanism of nerve destruction is mediated by excessive calcium influx (Hiura 2000). *In vivo* studies have suggested that injury through the administration of capsaicin to the sensory nerve endings causes SGCs to be activated and proliferate. After capsaicin-induced death, regeneration of neurons was seen 60 days post-capsaicin treatment (Czaja et al, 2008). These results suggest that adult rats had precursor cells capable of neural plasticity through DNA synthesis and cell division after neuronal destruction (Czaja et al, 2008). **Figure 3** and **Table 1** show that capsaicin mediated a 2-fold increase in the percentage of Ki67+ neuronal associated cells when added to a neuron/SGC co-culture. There was no significant statistical difference between the non-neuronal associated cells (Dale George, unpublished results). We did not expect to see a significant difference in the non-neuronal associated cells because SGCs do not have the receptor for capsaicin. Therefore, the capsaicin must affect the neurons, which then affect SGC proliferation through a cascade of events. However, the mechanism causing the increase in SGC proliferation remains unknown.

Figure 2 shows the preliminary results of immunocytochemistry (ICC) staining with P2X7 and Cx43 primary antibodies after 1 day *in vitro*. Immunofluorescent images on the confocal microscope were taken. Both P2X7 and Cx43 appear to be present between neurons and SGCs, and between SGCs themselves, as expected. Further quantitative studies could be done to detect potential up regulation of gap junctions or purinergic receptors after the addition of BzATP and capsaicin. If similar results were found for both BzATP and capsaicin, it would support the idea that purinergic signaling and gap junctions are important in regulating the proliferation of neuronal associated SGCs.

Capsaicin causes sensory neuron loss through stimulation of sensory nerve endings. Capsaicin is used to induce damage on these cells to model a peripheral nerve injury state. After loss, regeneration is observed after 60 days, proposing that SGCs may represent the source of the new neurons, supporting the idea of SGC plasticity (Czaja et al, 2008). We have recently developed an *in vitro* preparation of DRGs that includes their sheath of SGCs (George et al, under review). My preliminary characterization of the preparation reveals the presence of both gap junctions as evidenced by Cx43 immunocytochemical staining, and P2X7 receptors (**Figure 2**). Treatment of that *in vitro* preparation with capsaicin reveals an increase in the number of proliferative cells associated with the neuron, as evidenced by immunocytochemical staining with the proliferative antigen, Ki-67 (Dale George, unpublished results) We hypothesize that this increased proliferation results from purinergic signaling between the neuron and the SGC, and will test this hypothesis in this thesis.

Hypothesis

In response to P2X7 receptor activation, proliferation of SGCs surrounding neurons is increased.

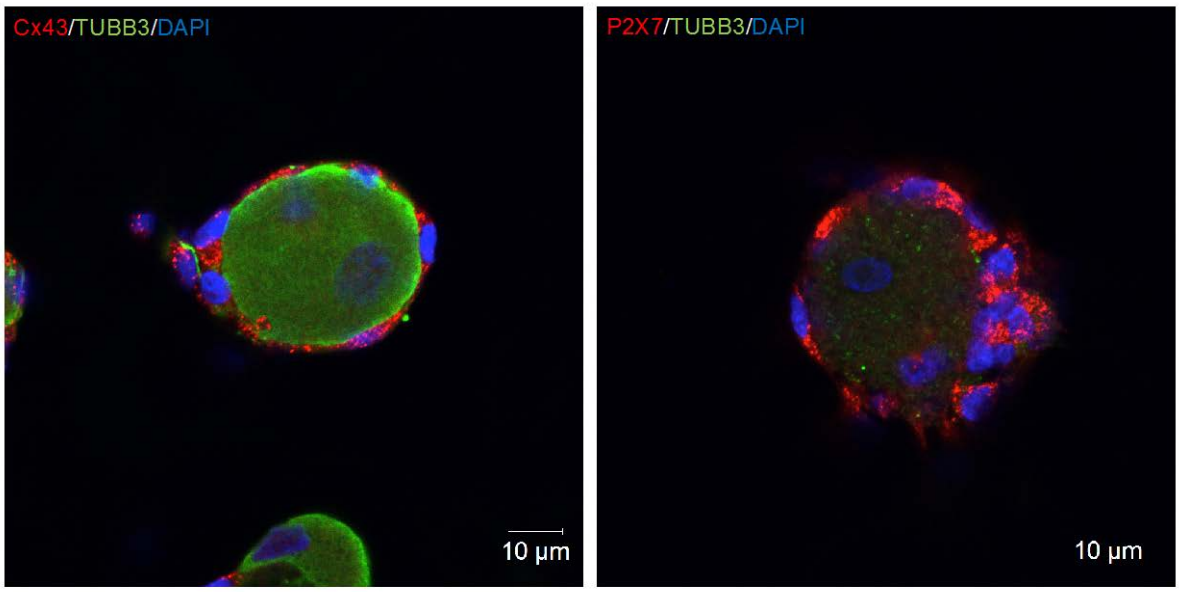

Figure 2: Cx43 and P2X7 ICC Staining: Cells were prepared as described in Materials and Methods. These cells were fixed after 1 day *in vitro*, and stained with the Cx-43 primary antibody (left) to show the presence of gap junctions, and the P2X7 primary antibody (right) to show the presence of this purinergic receptor.

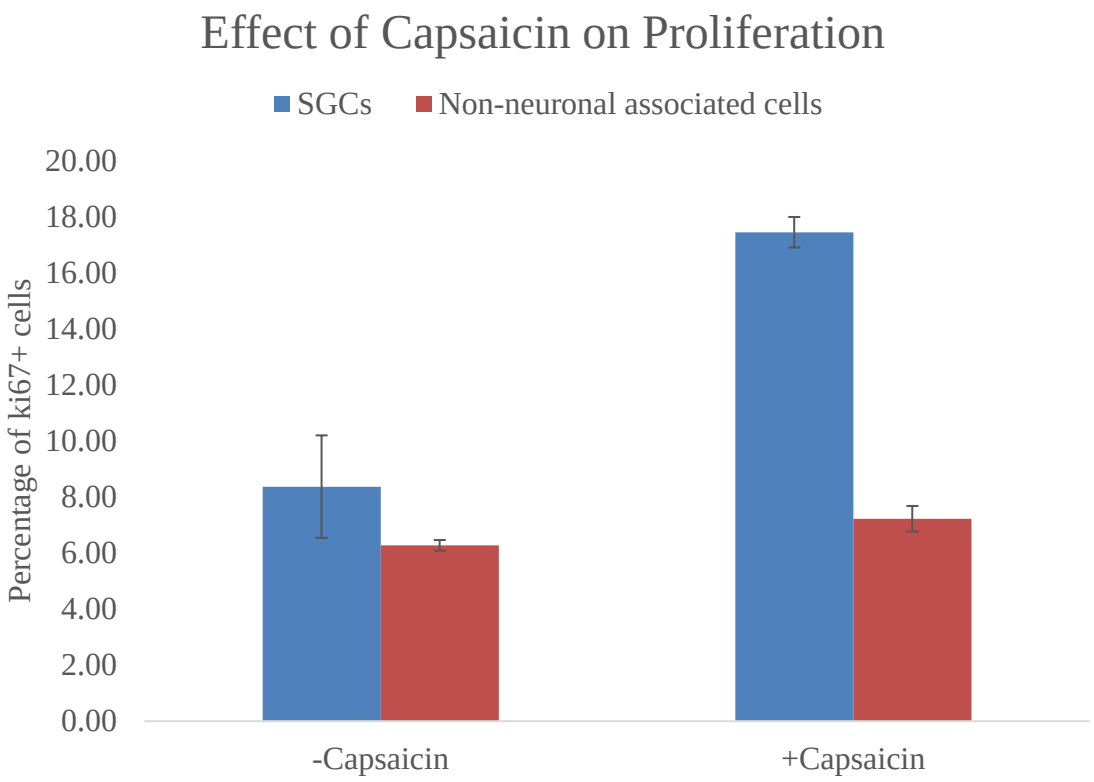

Figure 3: Effect of Capsaicin on Proliferation of SGCs in a Neuron/SGC Co-culture System (Dale George): Cells were prepared as described in Materials and Methods. 1µM capsaicin was added 24 hours after plating to half of the co-cultures. These cells were then fixed, stained, and imaged after 2 days *in vitro* for quantification of percentage of Ki67+ cells. There was a significant difference between the control (left) and experimental (right) groups for neuronal associated cells (blue bars). Experiment performed by and data obtained from Dale George.

Table 1: Effect of Capsaicin on Proliferation (Dale George)

	Neuronal associated cells	Non-neuronal associated cells
-Capsaicin	8.37	6.28
+Capsaicin	17.45	7.23

MATERIALS AND METHODS

Preparation of Neuron/SGC Co-cultures

Dorsal root ganglia (DRGs) from adult female Sprague Dawley rats were isolated under aseptic conditions. The DRGs were trimmed under a dissecting microscope to remove sheaths and nerve roots attached to the ganglion. They were transferred to a conical tube containing 5mg/ml collagenase, where they incubated for an hour in a water bath. Every 15 minutes, vortexing of the solution was performed. A fetal bovine serum (FBS)- coated small-bore glass pipette was used to further break down the DRGs by pipetting up and down. This solution was centrifuged at 1500 rpm for 5 minutes at 4°C. After the spin, the supernatant (collagenase) was removed. The cells in the pellet were re-suspended in 2ml of neurobasal media by pipetting up and down. A bovine serum albumin (BSA) cushion containing equal amounts of 10% and 5% BSA was prepared before layering the re-suspended cell solution on top. The cushion with cells was centrifuged at 115g for 4 minutes with a slow break. Schwann cells and debris will be seen between the 10% and 5% BSA layers; the pellet contains DRGs with SGCs. The supernatant (containing BSA, Schwann cells, and debris) was discarded, and the pellet was re-suspended in neurobasal media. Another BSA cushion was prepared, and subsequent layering of cells followed by centrifugation was repeated. Fraction 1 refers to the pellet obtained after two rounds of centrifugation with the BSA cushion. The Fraction 1 pellet was re-suspended in the appropriate calculated amount of neurobasal media to obtain a density of 3,500 cells/ coverslip.

The cell solution was seeded onto 12mm German glass coverslips coated with 0.01% poly-L-ornithine (Sigma) and 50µg/ml laminin.

BzATP Addition

24 hours after plating the neuron/SGC co-culture onto the coverslips, BzATP (Sigma) was added to select coverslips (+BzATP). The BzATP stock was prepared under aseptic conditions by the addition of deionized water. BzATP was added at a concentration of 100µM. A comparable amount of sterile deionized water was added to control coverslips (-BzATP). The cells incubated for another 24 hours after the addition of BzATP. The cell cultures incubated for a total of 48 hours after plating before they were fixed with paraformaldehyde. This time point was chosen because capsaicin-induced neuronal damage occurs within 12-24 hours after capsaicin treatment (Czaja 2008). After 24 hours, the neurons and SGCs undergo changes to repair damage. Therefore, we expected regeneration mechanisms of SGCs to occur within about 24 hours after inducing their response to increased ATP production.

Immunocytochemistry

The neuron/SGC co-culture coverslips were fixed in 4% paraformaldehyde for 10 minutes, followed by permeabilization with 0.3% triton X in 4% paraformaldehyde for 10 minutes on ice. The cultures were then washed with PBS and blocked for 30 minutes in 10% normal goat serum (NGS) and incubated overnight at 4°C with primary antibodies. The primary antibodies were: mouse TUBB3 (1:300), and rabbit Ki67 (1:50). The cultures were washed with 10% NGS to remove the primary antibodies. The cultures were then incubated for 45 minutes at room temperature in the dark with secondary antibodies. The secondary antibodies used were:

anti-rabbit Alexa-Fluor 568 (1:1000); anti-mouse Alexa-Fluor 488 (1:1000); DAPI (1:200). After washing off the secondary antibodies, the coverslips were mounted to microscope slides with Fluorogel. Images were acquired with Zeiss LSM710 confocal microscope.

Quantification

20 images for each condition (-BzATP and +BzATP) were acquired at 20X magnification on the Zeiss LSM710 confocal microscope. These images were obtained by capturing different frames from one edge of the coverslip to the next, in both horizontal and vertical directions. Images that showed an overlap of the same area of the coverslip upon analysis were not quantified. After 40 images in total were obtained, the Volocity software was used to find the percentages of Ki67 positive (Ki67+) SGCs for each condition. For each individual image, the number of neurons, number of cells around the neurons, number of non-neuronal associated cells, number of Ki67+ cells around the neurons, and number of Ki67+ non-neuronal associated cells were hand counted, with the aid of Volocity, based on morphology, location, and fluorescence. Neurons exhibited beta-3-tubulin fluorescent staining (green color), and majority were round in shape with processes extending from the soma. Neuronal associated SGCs displayed DAPI fluorescent staining (blue color); they either were touching the neurons, or it could be inferred that the SGCs recently migrated away from soma. Non-neuronal associated SGCs also displayed DAPI fluorescent staining (blue color), but had no clear connections to neurons. Ki67+ cells were identified for both neuronal associated and non-neuronal associated cells based on intensity of Ki67 staining (red color). Ki67 is concentrated in the nuclei of dividing cells; therefore, Ki67+ cells had a red stained area overlapping with a DAPI (DNA/ nuclear marker) blue stained area. Ki67+ cells appeared as bright pink, roundly stained cells. The

percentage of Ki67+ cells around the neurons was calculated by dividing the number of Ki67+ cells around the neurons by the total number of cells around the neurons. The percentage of Ki67+ not around the neurons was calculated by dividing the number of Ki67+ non-neuronal associated cells by the total number of non-neuronal cells. The percentages of the 20 images for each condition were averaged to find the mean percentage of Ki67+ cells for both neuronal associated cells and non-neuronal associated cells.

The entire experiment was repeated twice (using DRGs from different rats, with experiments performed independently of each other). n=1 refers to the first time the experiment was performed, and n=2 refers to the second time the experiment was performed. The overall average percentages of Ki67+ cells (**Table 2**) were obtained by averaging the mean percentages of both experiments (n=1 + n=2). The GraphPad Prism software was used for statistical analysis, utilizing the 2-way ANOVA followed by Tukey's multiple comparisons test. This calculator function provided comparisons between –BzATP: neuronal associated cells and non-neuronal associated cells, and +BzATP: neuronal associated cells and non-neuronal associated cells conditions, along with the P value, and statistical significance between each condition. Standard error of the mean (SEM) values were used for the creation of error bars on **Figure 4.**

RESULTS

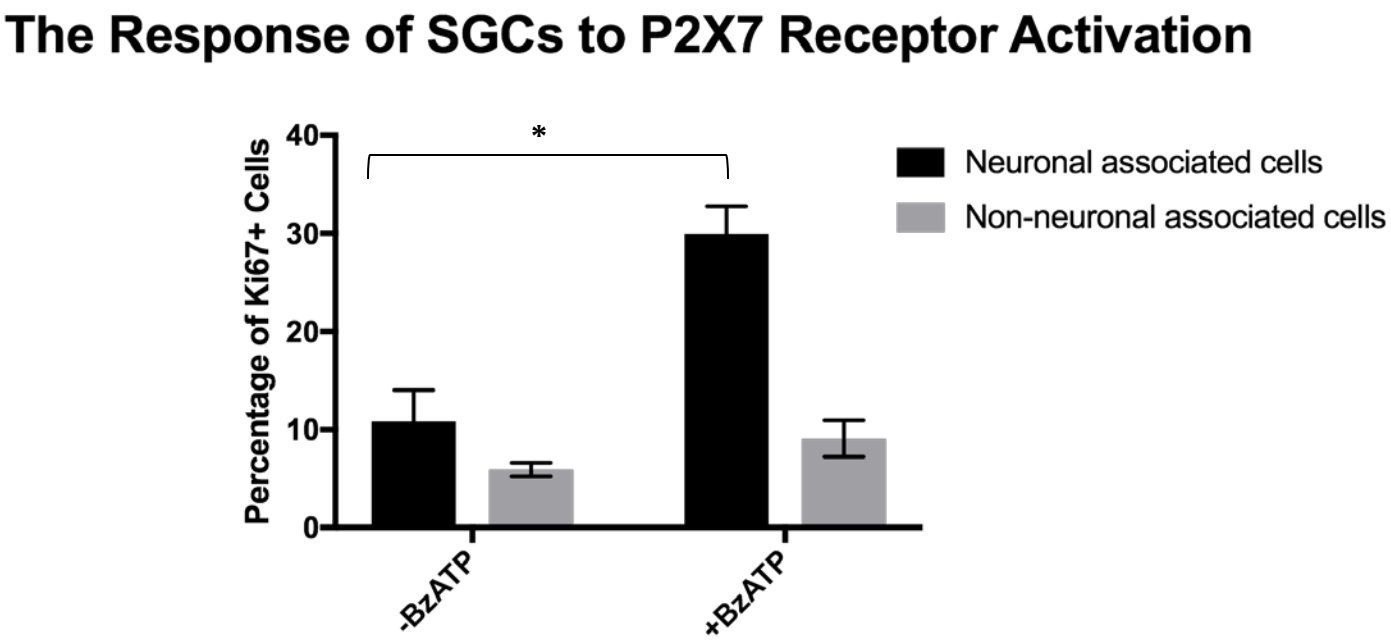

Figure 4: The Response of SGCs to P2X7 Receptor Activation: Cells were prepared as described in Materials and Methods. 100µM BzATP was added 24 hours after plating to half of the co-cultures. These cells were then fixed, stained, and imaged after 2 days *in vitro* for quantification of percentage of Ki67+ cells. A 2-way ANOVA followed by Tukey's multiple comparison test was performed; *p= 0.0154.

Table 2: Statistical Analysis

Group	**-BzATP**		**+BzATP**	
	Neuronal	**Non-Neuronal**	**Neuronal**	**Non-Neuronal**
Mean % of Ki67+ Cells	10.81	5.92	29.94	9.06
SEM	3.22	0.70	2.80	1.84
N= 2				

Table 2 and **Figure** 4 show a 2.8-fold increase in proliferation of neuronal associated SGCs in response to P2X7 receptor activation by BzATP. This difference is considered statistically significant (P Value=0.0154) by the 2-way ANOVA followed by Tukey's multiple comparison tests. No significant difference (P Value= 0.7854) was observed in non-neuronal associated SGCs in response to BzATP. Furthermore, the 2-way ANOVA followed by Tukey's multiple comparison tests showed no significant difference between neuronal associated and non-neuronal associated cells for the control condition. However, there was a significant difference between neuronal associated and non-neuronal associated cells for the experimental condition. These results show that there is no difference in the proliferation percentages of SGCs in relation to the neurons until the P2X7 receptors are activated. There are P2X7 receptors on both neurons and SGCs whether they are associated with neurons or not. Therefore, we expected the same effect on proliferation for both neuronal and non-neuronal associated cells, in response to P2X7 activation by BzATP which we did not find. This demonstrates that another mechanism in addition to, or in response to P2X7 receptor activation is at work.

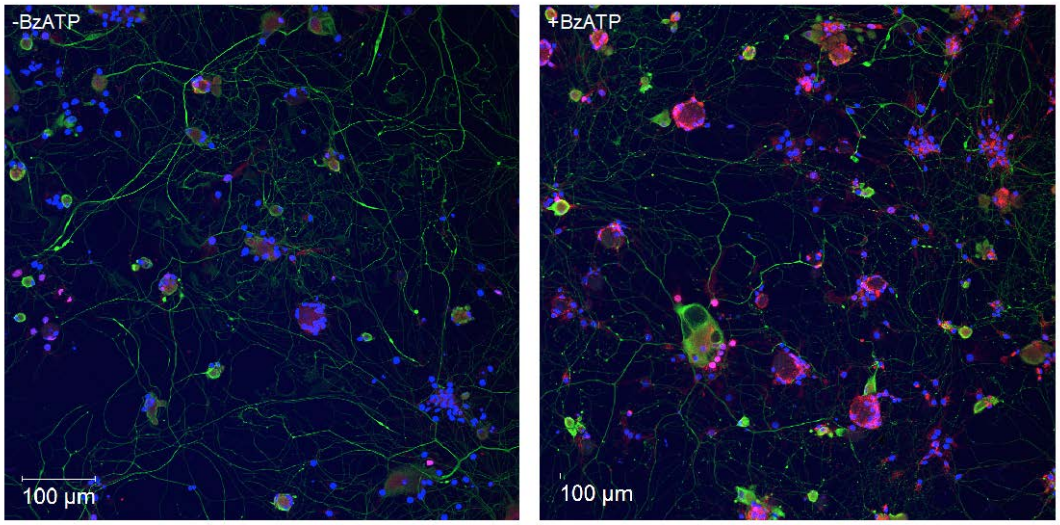

Figure 5: Confocal Image -/+ BzATP Comparison: Cells were prepared as described in Materials and Methods. 100µM BzATP was added 24 hours after plating to the co-culture on the right. These cells were then fixed, stained, and imaged (20x) after 2 days *in vitro*. The Ki67 primary antibody is shown in pink, DAPI in blue, and beta-3-tubublin in green.

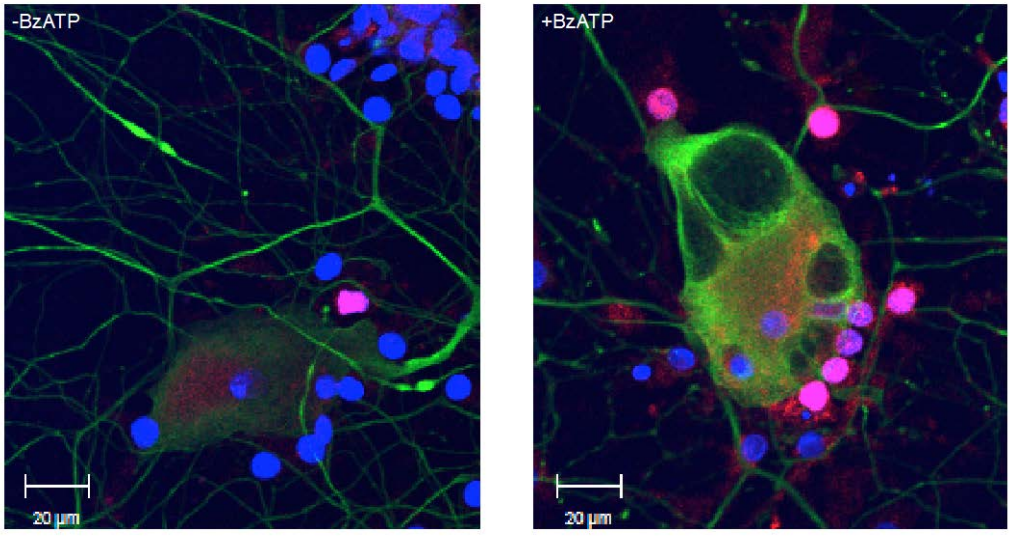

Figure 6: Confocal Image -/+ BzATP Comparison (Zoom): Cells were prepared as described in Materials and Methods. 100µM BzATP was added 24 hours after plating to the co-culture on the right. These cells were then fixed, stained, and imaged (20x) after 2 days *in vitro*. The Ki67 primary antibody is shown in pink, DAPI in blue, and beta-3-tubublin in green.

Table 2 shows the average percentage of Ki67 positive cells (n=1 + n=2), for both –BzATP and +BzATP conditions, and for both neuronal associated cells and non-neuronal associated cells. The final P Value for neuronal associated Ki67+ cells was P= 0.0154, which is considered statistically significant. This represents the increased proliferation of neuronal associated SGCs in response to P2X7 receptor activation. The final P Value for non-neuronal associated Ki67+ cells was P= 0.7854, which is considered not statistically significant; the P2X7 receptor activation had no significant effect on the proliferation of SGCs that were not neuronal associated. Two confocal images taken at 20x (one with BzATP added, and one without BzATP added) that were used for quantification are shown in **Figure 5. Figure 6** shows the same images, zoomed in to show a single neuron with surrounding SGCs. In these images, the Ki67+ SGCs are easily discernable surrounding the soma. The two images have comparable cell densities, and comparable DAPI and beta-3-tubulin staining. However, the quantity and intensity of Ki67 staining is much greater in the +BzATP image, representing the significant difference in percentage of Ki67+ neuronal associated cells after P2X7 receptor activation.

DISCUSSION AND CONCLUSION

This experiment suggests that ATP mediates the proliferation of SGCs. Ki67 is a nuclear protein that is expressed during cell proliferation. The Ki67 antibody was used to find the growth percentages of the neuron/SGC co-cultures, with and without the addition of BzATP. When BzATP was added, more P2X7 receptors were activated, which triggered a cascade of events resulting in an increased percentage of SGCs in active phases of cell cycle. This supports the idea that there is cross-talk between neurons and satellite glial cells with ATP as a mediator. Similar results were seen in another preliminary *in vitro* study; the addition of capsaicin caused an increase in Ki67+ cells in a neuron/SGC culture (Dale George, unpublished results). It has also been demonstrated that SGCs are induced to proliferate after axotomy or minor skin trauma (reviewed by Hannini, 2005). Together, these outcomes suggest that ATP is an important neurotransmitter released by neurons during peripheral nerve injury that aids in increased proliferation of SGCs.

Additionally, the final P Value for control versus experimental groups was significant only for neuronal associated Ki67+ cells, not for non-neuronal associated Ki67+ cells. This finding was unexpected; all of the SGCs have purinergic receptors, so we expected to see the same result in both neuronal and non-neuronal associated SGCs. This experiment suggests that ATP communication is localized relatively closely between neurons and their associated satellite glial cells. The neurons must play a role in the increased proliferation of SGCs that we see. We hypothesize that injury catalyzes a cascade of events between neurons and SGCs. **Figure 7** (designed by Dale George) depicts a proposed purinergic-signaling model. Upon injury, an

unknown molecule acts on neurons, resulting in Ca^{2+} wave increase, and then ATP is released from neurons, where it binds to P2X7 receptors on SGCs. SGCs would then proliferate, and connexins would link the proliferating cells. Gap junctions are likely to mediate this cross-communication during neuropathic pain, as shown in the figure. Particular molecules acting specifically upon neurons, but not SGCs, to activate the cascade could explain the unexpected result. Another possibility is the ATP concentration only being high enough in the space between neurons and SGCs to have a significant effect on proliferation of surrounding SGCs. Perhaps the threshold for activation of SGCs is not reached by adding 100µM of extracellular BzATP to the co-culture. The ATP concentration is most likely amplified by cross-talk between closely associated neurons and surrounding SGCs. A dose dependent study of different BzATP concentrations would be helpful in answering this question.

More experiments are needed to determine the fate of the SGCs that were induced to active cell cycling. There is a possibility that the neurons are dying in the experimental condition in response to BzATP addition. Perhaps, this death is causing increased release of ATP between neurons and neuronal associated SGCs. The higher concentration of extracellular ATP between the neurons and neuronal associated SGCs due to this death response could result in the difference that is observed only in neuronal associated SGCs, and not non-neuronal associated SGCs. We could induce neuronal cell death, and see if the same results are observed as in the original experiment. We could also test to see if the neurons are in fact dying in response to BzATP addition.

In conclusion, this thesis suggests that ATP has an important role in neuron-SGC signaling and subsequent proliferation in neuronal associated SGCs. Experiments with ATP

inhibition are also needed to confirm that ATP is a mediator molecule for proliferation of SGCs. Hypothetically, adding apyrase, an enzyme that degrades extracellular ATP, with capsaicin would "cancel out" the effect of the purinergic signaling. If we saw no increased proliferation in the SGCs surrounding the neurons after adding both capsaicin and apyrase, we could confirm that puringeric receptor activation is important in the increase of proliferating neuronal associated cells after capsaicin addition that we originally observed. The possibility of designing therapeutic targets to regulate SGC proliferation through purinergic signaling following injury is raised.

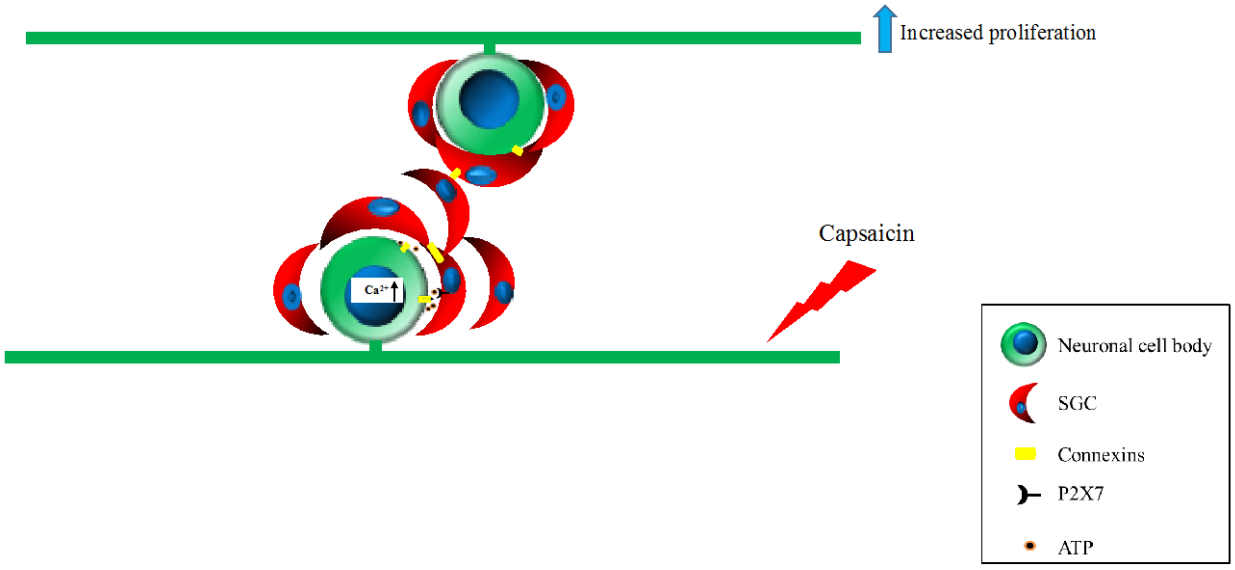

Figure 7: Proposed Model (Dale George)

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