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Brief trains of high-frequency nerve stimulation lead to variable changes in Ca^{2+} levels in PSCs at the crayfish NMJ.

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ABSTRACT

Our objective was to extend knowledge about the role of glia, specifically perisynaptic Schwann cells (PSCs), in synaptic transmission at the crayfish neuromuscular junction (NMJ). Though it previously was assumed that glial cells play only a passive role in synaptic functions, glial cells recently have been found to play an active role in synaptic transmission in some organisms. We used epifluorescent microscopy with Syto-61, a dye used to distinguish PSCs from nerve cells, and Fluo-4, AM, a calcium (Ca^{2+}) chelator that fluoresces in the presence of calcium ions, to detect changes in calcium concentration within PSCs. An increase in Ca^{2+} concentrations within PSCs after stimulation would support the hypothesis that PSCs actively participate in synaptic transmission. In this study, we were not able to ascertain any definitive conclusions about the role of PSCs in synaptic transmission; in one trial the calcium ion concentration increased after stimulation while in other trials the calcium concentration decreased, possibly as a result of complications in experimental procedure.

INTRODUCTION

Glial cells are non-neuronal cells located in the nervous system that support neurons and their functions (Freeman 2005). Early ideas about glial cells indicated that they were passive participants in synaptic function, providing metabolic and structural support to nerve cells (Newman 2003). Current research, however, shows that glial cells have a more active role in neuronal functioning than previously speculated. Glia directly and indirectly modulate synaptic transmission by performing functions such as releasing gliotransmitters, taking up glutamate, and releasing chemical cofactors (Bonvento *et a.l* 2002, Newman 2003, Robitaille 1998). Perisynaptic Schwann cells (PSCs) are specialized, non-myelinating glia that surround the neuromuscular junction in the peripheral nervous system, and have been shown by Robitaille (1998) to regulate levels of synaptic depression at the frog NMJ. More specifically, Jahromi et al. (1992) have shown that this regulation may be due to an increase in Ca^{2+} concentration in the glial cells after transmitter release. These experiments have provided the most convincing evidence for glial regulation of synaptic transmission.

Nerves send electrical signals to other nerves and surrounding cells via action potentials. An action potential occurs when the membrane potential of a neuron rapidly depolarizes and then hyperpolarizes in a propagating wave down the length of an axon. As an action potential arrives at the terminal of the presynaptic neuron, it triggers the entry of Ca^{2+}. This Ca^{2+} induces the binding of synaptic vesicles with the presynaptic membrane, which causes exocytosis. The molecules of neurotransmitter are thereby released into the synaptic cleft, allowing them to bind to the postsynaptic membrane and change the postsynaptic cell potential. This change in membrane potential can initiate or hinder the generation of an action potential in the postsynaptic neuron (Freeman 2005).

Researchers have found an increasing number of glial cell functions in recent studies, especially at the frog NMJ (Robitaille 1998, Araque *et al*. 1999). We sought to extend their findings to the crayfish NMJ to learn more about the active role of glia in synaptic transmission. The crayfish NMJ is a good model for studying the role of glial cells in synaptic transmission not only because of its simple dissection and large nerves, but also because the basic neurological processes are very similar to those found in more complex organisms where these specific experiments are not possible.

We tested for PSC involvement in synaptic activity at the crayfish neuromuscular junction by measuring changes in Ca^{2+} concentration. The concentration was indicated by fluorescence of Ca^{2+} chelators. Based on previous studies cited above, we expected to see an increase in Ca^{2+} concentration in PSCs after stimulation, signifying an active glial role in synaptic transmission. We report here that there was no consistent increase in Ca^{2+} concentration after stimulation among three trials; our data does not indicate a specific glial role in synaptic transmission at the crayfish NMJ.

MATERIALS AND METHODS

We used epifluorescent microscopy in order to ascertain the location of PSCs and calcium concentration within these cells. Syto-61 is a fluorescent dye that is used to distinguish between PSCs and neurons; it binds to RNA and DNA, which are present in PSCs but not in the axons of neurons. Fluo-4 is a fluorescent dye that binds to calcium and can be used to determine calcium concentrations within cells. Fluo-4 is a polar molecule and consequently cannot diffuse across the cell membrane by itself. A nonpolar AM group is thus attached to Fluo-4 molecules by ester bonds, making the resultant product, Fluo-4 AM, moderately lipophilic and therefore membrane permeable. Once the molecule is inside the cell, non-specific esterases cleave AM groups from the Fluo-4 portion of the molecule and consequently trap the Fluo-4 molecule inside the cell. A difficulty in using Fluo-4 is its low solubility in water; the dye is therefore dissolved in DMSO, a detergent, before being diluted in crayfish saline.

Both Fluo-4 AM and Syto-61dyes are excited by light at specific wavelengths; 488nm and 628nm, respectively. By using an Epifluorescent microscope, light at these specific wavelengths can be selected and used to excite the electrons of fluorescent dyes to a higher energy level and then emit light as the electrons fall to their resting state (Marshall *et al*. 2002).

Crayfish Preparation

The fast extensor muscle group located in the tail of the crayfish, *Procambarus clarkii* was used as the model for our experiment. Prior to experimentation, the crayfish was placed in an ice bath for approximately 20 minutes to slow down the corporal functioning of the animal; the tail was then clipped from its body. The ventral portion of the exoskeleton along with unneeded muscle and digestive tissue was removed to expose the lateral and medial tail extensor muscles.

After dissection, the tail was immersed in a mixture containing 5 mL of crayfish Ringers solution (KCl, 5.4 mM; NaCl, 204 mM; $MgCl_2$ * $6H_2O$, 2.3 mM; $NaHCO_3$, 2.3 mM; Dextrose, 2.0 mM; $CaCl_2$ * $2H_2O$, 13.5 mM: pH 7.42) and 2 µL Fluo-4, AM (200µg/µL, Molecular Probes) in DMSO in a light deprived container at 33°C for an incubation period of 30 minutes. The tail was then rinsed with fresh crayfish Ringers solution. 5 µL Syto-61 (5mM; Molecular Probes) in 5 mL of crayfish Ringers solution was applied to the tail; the tail was incubated in a light deprived container at room temperature for 5 minutes. The tail was rinsed again, pinned in an observation dish, and bathed with crayfish Ringer's solution to increase longevity of the preparation.

Three crayfish were used as subjects in this experiment, and each of the three trials indicated refers to a different crayfish.

Preliminary Experimentation

To acquaint ourselves with the microscope and dye application, we first practiced with the application of Syto-61and 4-Di-2-Asp (10mM; 10 µL in 10 mL; Molecular Probes). We observed the relative location of nerve cells and glial cells, as indicated in Fig. 1.

Stimulation

Before stimulation, the dyed tail was observed under a Leica ZOOM 2000 microscope to select a clearly visible, intact muscle group. Stimulation wires were fixed to either side of the dorsal ridge. A stimulus of 14 V at 50 Hz was applied for a duration of 5s (GRASS SD9 Stimulator). The voltage applied was selected to generate an action potential without excess fatigue to the muscle.

Epifluorescent Microscopy and Imaging

We used an Olympus BX51 fluorescent microscope with the 60X water immersion objective to observe the dyed crayfish neuromuscular junction, focusing on the group of muscle cells adjacent to the stimulation wires. A wavelength of 488nm was used to excite the Fluo-4 dye; 628nm was used for the Syto-61 dye. The QCapture v1.1.2 software program was used to receive and store all digital photographs taken in our experiments. Prior to stimulation, pictures were taken with a QImaging digital camera attached to the microscope to determine location of glial cells. Pictures were taken every 10s for 200s; stimulation was applied after 32s. Average exposure times for pictures of the Fluo-4 AM dye were 750-1000ms; average exposure times for the Syto-61 dye were 40-60ms. See Fig 2-6.

Data Analysis

Metamorph software was used to quantify the intensity of the fluorescence. Regions were formed using the software around areas of fluorescence; region statistics indicating average fluorescence of each pixel were then displayed and recorded for each picture. See fig. 3 for an example of a region drawn. Regions were moved with areas of fluorescence when it was obvious that the stimulus caused the muscle to move. Average fluorescence of an area of background was also recorded to account for fading of fluorescence due to dye overexposure. Average net fluorescence was then plotted against time (Fig. 9-11) To enable data from different days to be accurately compared, the change in average net fluorescence, ΔF, between each picture was recorded and then divided by initial fluorescence, $F°$. These $\Delta F/F°$ values were averaged over three days of experiments and

then plotted against time (Fig 12). A Student's t-test was then performed on the ΔF/F° values immediately before and after stimulation.

RESULTS

We were initially successful in locating glial cells and determining their spatial relation to nerve cells (Fig. 1). We also found that calcium ions could be detected within glial cells (Fig. 2). The slight shift in the two images used to make the overlay resulted from muscle contraction.

Qualitative Results

Qualitative results included noticeable visual differences in green fluorescence intensity, which indicated presence of calcium ions, before and after stimulation. In trial 1, fluorescing areas brightened after stimulation (Figs. 3-4). In trials 2 and 3, however, fluorescing areas dimmed after stimulation (Figs. 5-8). All trials showed muscle movement and a slight, if not severe, loss of focus after stimulation.

Quantitative Results

In trial 1, net fluorescence intensity sharply increased following stimulation, and then gradually decreased over time (Fig 9). In trials 2 and 3, net fluorescence intensity decreased following stimulation (Figs 10-11). An average of ΔF/F° values, showing relative change in fluorescence intensity, showed a large amount of variation. With the exception of the point immediately after stimulation, approximately 32s, where ΔF/F° sharply increases, the values generally remained between -0.1 and 0.0 (Fig 12).

A t-test performed on average net intensity values immediately before and after stimulation (30s and 40s) yielded a P-value of 0.25, indicating the values are not significantly different.

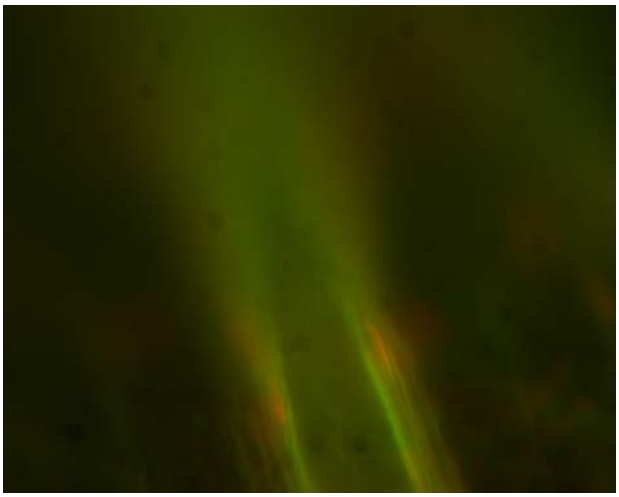

Figure 1. Perisynaptic Schwann Cells (PSCs), indicated in red by fluorescent Syto-61 dye, ensheathe nerve cells, indicated in green by fluorescent 4-Di-2-Asp dye.

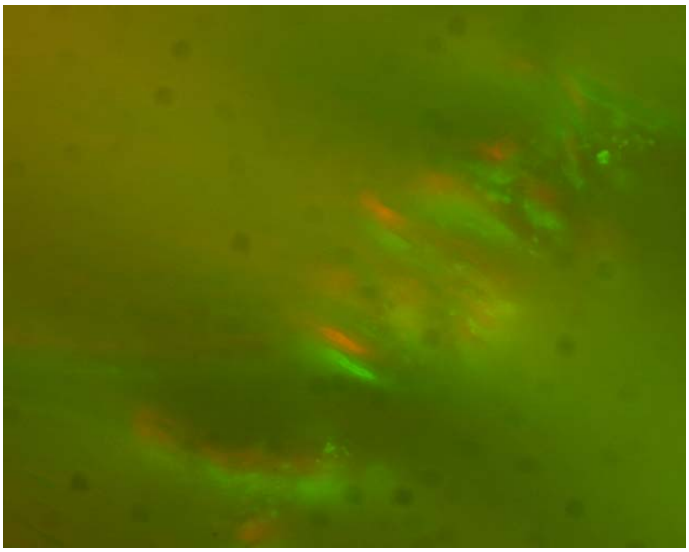

Figure 2. Calcium ions, displayed here by the green fluorescent regions through application of Fluo 4-AM dye, are present near Perisynaptic Schwann Cells (PSCs), indicated in red by fluorescent Syto-61dye, prior to stimulation.

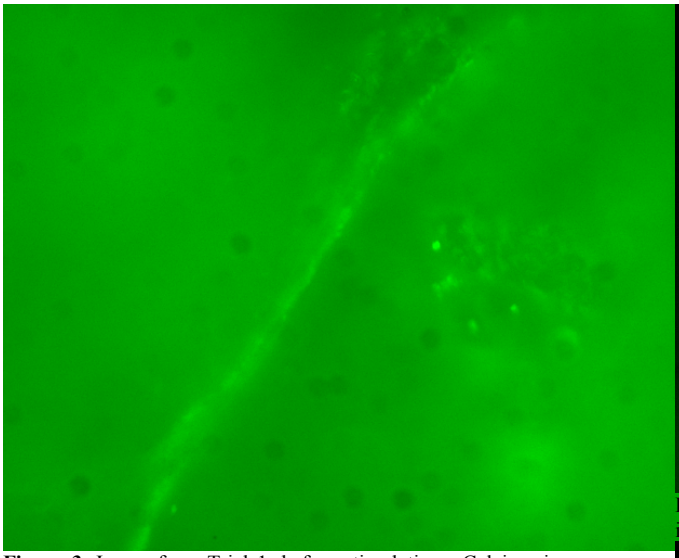

Figure 3. Image from Trial 1, before stimulation. Calcium ions are present in the green fluoresced areas.

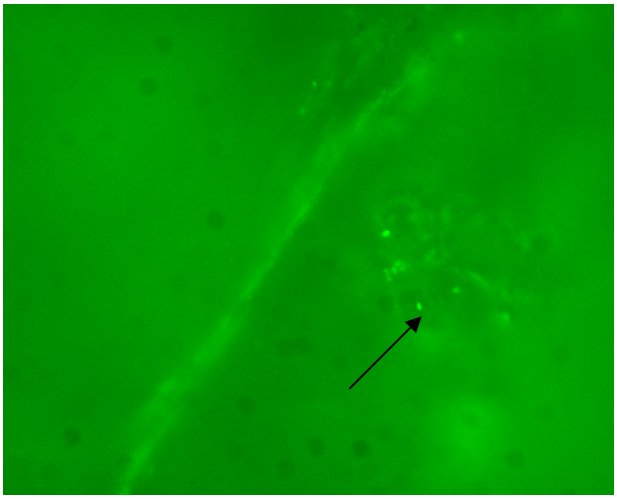

Figure 4. Image from Trial 2 after stimulation. The indicated area of fluorescence brightened after stimulation, indicating an increased presence of calcium ions.

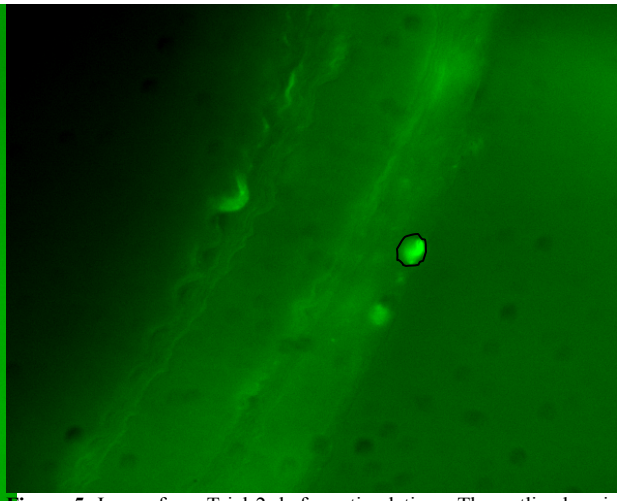

Figure 5 . Image from Trial 2, before stimulation. The outlined region indicates a sample of the regions used to average fluorescence.

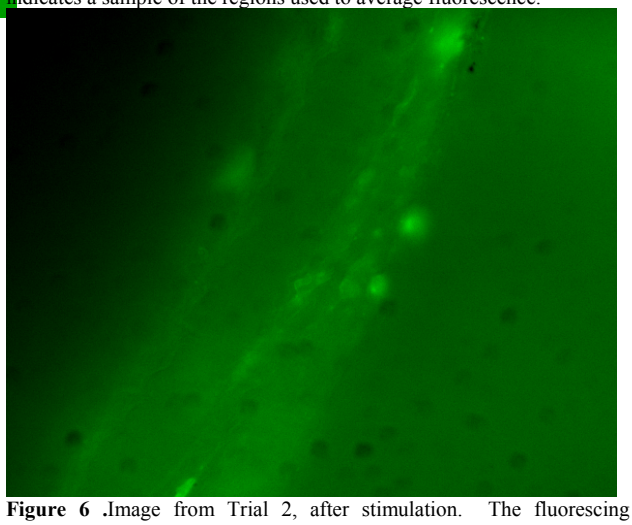

Figure 6 . Image from Trial 2, after stimulation. The fluorescing regions, indicating calcium ion concentration have shifted due to the stimulation impulse.

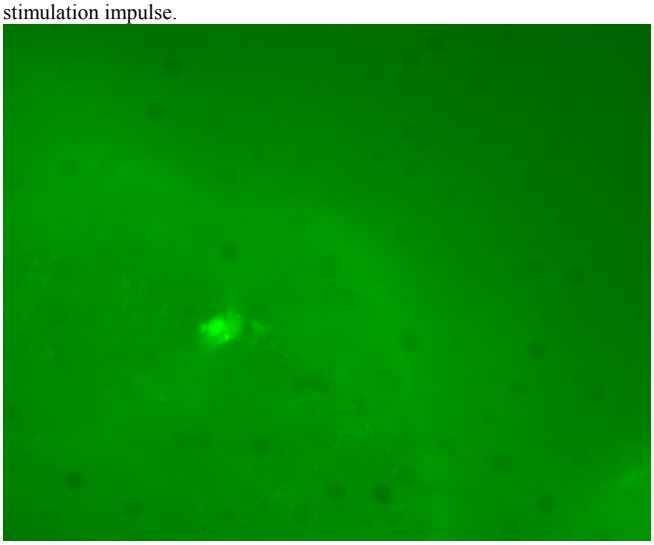

Figure 7. Image from Trial 3, before stimulation. Calcium ions are present in the green fluoresced area.

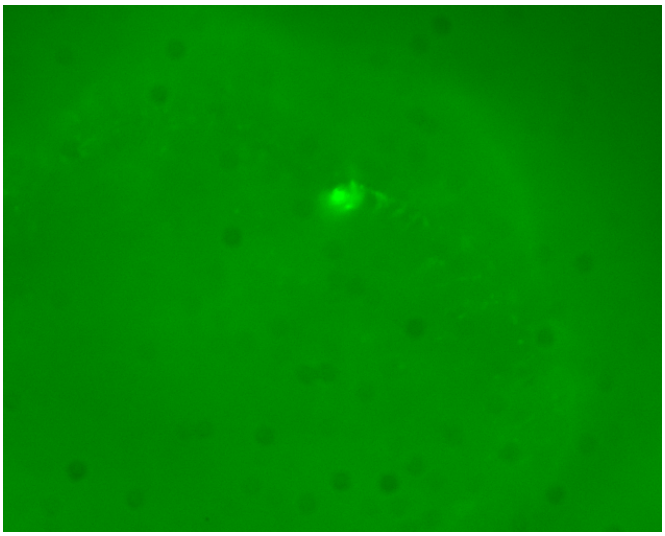

Figure 8. Image from Trial 3, after stimulation. The fluorescent concentration of calcium ions has shifted and gone slightly out of focus due to the impulse.

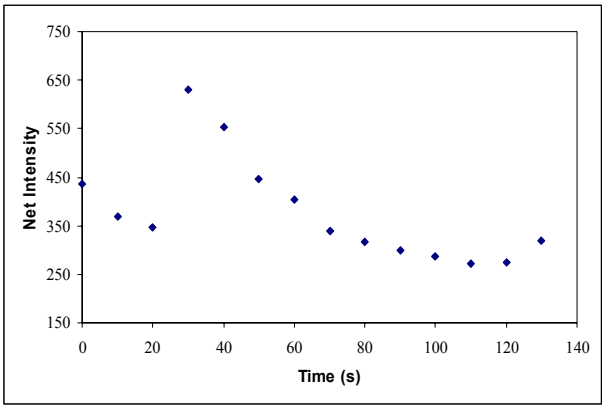

Figure 9. Net fluorescence intensity (intensity of selected fluorescent area – intensity of selected background region) for Trial 1 (Fig. 3-4) increases immediately after stimulation at 32 s and then gradually decreases over time.

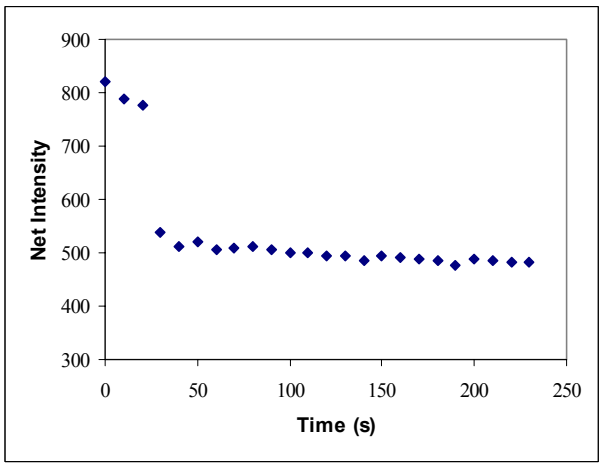

Figure 10. Net fluorescence intensity decreases over time for Trial 2 (Fig. 5-6). Muscle contraction caused by the applied stimulus is responsible for the largest decrease in fluorescence intensity at 32s.

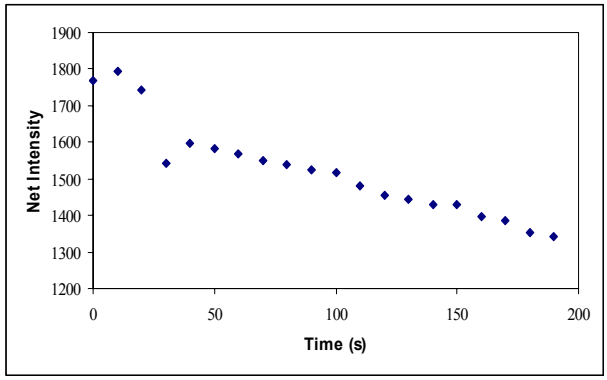

Figure 11. Net fluorescence intensity decreases over time for Trial 3 (Fig. 7-8). Stimulation was applied at 32 s.

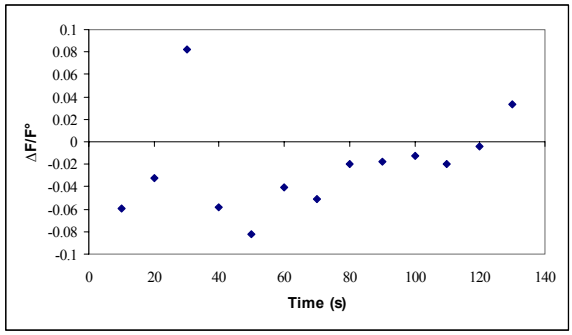

Figure 12. ΔF/F° changes over time. Except for the value immediately after stimulation, ΔF/F remain negative until the last data point.

DISCUSSION

Our research showed that in all trials, stimulation induced a change in calcium concentration in PSCs. One trial showed an increase in fluorescence intensity, indicating an increase in calcium ion presence, while the other two trials showed a decrease in fluorescence intensity. This variation may be due to several factors.

In trial 1, the increase in calcium concentration following stimulation and subsequent gradual decrease aligns with results attained in experiments performed by Jahromi et al. (1992). This increase may indicate that PSCs play an active role in synaptic transmission; because calcium ions trigger the release of neurotransmitters in neurons, an increase in calcium ions in PSCs could have a similar effect. Trial 1 likely provided the most accurate results as shift and loss of focus due to muscle contraction after stimulation were least severe in these pictures.

In trial 2, the qualitative and quantitative decrease in net fluorescence intensity likely resulted from a loss of focus in the picture due to muscle movement during stimulation (Fig. 3,4). A glutamate antagonist could also be used in future studies to minimize muscle contraction and resultant loss of focus; however, one should this weigh this advantage against the possibility that a glutamate antagonist may block neurotransmitter

receptors on glial cells. Also, similar studies on the role of glial cells using the lizard model have been successful in using α-bungarotoxin to limit muscle contraction (Jahromi 1992).

In trial 3, qualitative and quantitative data again show a decrease in net fluorescence intensity. In this case, it is likely due to a shift in fluorescence area after stimulation (Figs 5,6).

Our ΔF/F° values did behave as expected; these values show that fluorescence increased sharply immediately after stimulation as calcium ions entered the PSCs and then gradually decreased as the calcium ions diffused out of the cells. However, as trials gave significantly different results, as evidenced by the Student's t-test, these results need to be replicated with a greater sample size in order to be statistically significant.

Because Syto-61 binds to nucleic acids, it fluoresces the nuclei of both muscle cells and PSCs. Therefore, we could not always differentiate between the two types of cells. If we were looking at calcium fluorescence near muscle cells rather than PSCs, we would not expect to see an increase in fluorescence after stimulation.

While we were unable to statistically support evidence for a net change in Ca^{2+} concentration in PSCs after stimulation, our results do indicate a transfer of Ca^{2+} ions into or out of glial cells during an action potential; this indicates some PSC involvement in transmission at the synapse. Measuring changes in Ca^{2+} concentration may not be the best or only way to demonstrate that glia do influence synaptic transmission. Other ions, such as Na^{+}, K^{+}, and Cl^{-} are the accepted electrochemical forces behind action potential generation in nerves. It may be that these ions also interact within glia to modulate synaptic transmission on a smaller and less explored level. Further research accounting for muscle contraction or exploring effects of stimulation on other ion concentrations within PSCs could further support the case that these cells actively participate in synaptic transmission.

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