Pioneering Neuroscience, 2010, **10**, 43-45

Localization of Glial Cells and Nerve Terminals in Crayfish Abdominal Muscles

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ABSTRACT

Fluorescent dyes and confocal fluorescence microscopy were used to localize nerve terminals and adjacent cells in crayfish abdominal muscles. Nerve terminals were stained with 4-di-2-aspm(4-diethylaminostryly-N-methylpyridinium iodide) and nuclei of adjacent cells were stained with SYTO 61 red fluorescent nucleic acid stain. It was observed that some nerve terminals were closely associated with large nucleic bodies tentatively identified as glial cells. This association was not consistent to all nerve terminals, leading to speculation of different populations of nerve terminals, some of which interact with glial cells and some of which do not. Neither a complete structure nor function has been discovered for these cells.

INTRODUCTION

The discovery of bidirectional signaling in vertebrates between neurons and glia has been a major breakthrough for understanding the role of glial cells in synaptic transmission. It has been revealed that neurotransmitters released from pre-synaptic nerve terminals are able to activate receptors on adjacent astrocytes, resulting in the release of glial transmitters (Allen & Barres, 2005). These glial transmitters (most commonly: glutamate, ATP, and D-serine) are then able to act as enhancers or suppressors of nearby synaptic activity (Newman, 2003). Even though the effects of these glial transmitters have been explored, the mechanism of their release is still unclear.

Much less research has been done on the role of glia in the modulation of synaptic transmission in invertebrates. In fact, although it is known that invertebrates such as crayfish do have glia, such basic facts as their structure and location in the organism are still ambiguous. Some cells tenuously identified as crayfish glia include spindle-shaped cells lying parallel to the muscle fibers (Novotova and Uhrik, 1992) and round, flat cells with elongated processes stretching down to nearby nerve terminals (Harrington and Atwood, 1995). Unfortunately, no further research has been done on either function or morphology of either of these cell types.

In the research described here, fluorescent dyes were used to stain nerve terminals and nuclei of adjacent cells in the crayfish abdominal muscles. It was hypothesized that glial cells would be found in close proximity to nerve terminals due to their role in the modulation of synaptic transmission. Images were obtained with a confocal microscope, and analyzed to show at least one population of cells which we tentatively identified as glial.

MATERIALS AND METHODS

Preparation

A population of four freshwater crayfish was used in this experiment. Each crayfish had its abdomen removed just anterior to the first set of swimmerets. The ventral carapace and flexor muscles were then dissected out to expose the dorsal extensors and deep dorsal extensors. Next, the telson and uropods were removed and the remaining abdominal section cut down the midline. The two resulting pieces were cut across the middle of the third segment, producing four preparations per crayfish (16 total) which were kept in saline until used. The saline solution used in this experiment was a standard 7.4 pH Ringer's solution composed of 5.4mM KCl, 196mM NaCl, 2.6mM $MgCl_2$, 13.5mM $CaCl_2$, and 10mM N-2-hydroxy-ethylpiperzine-n'-2-ethanesulfonic acid (HEPES) buffer.

The two fluorescent dyes used were 4-di-2-asp (4-diethylaminostryly-N-nethylpyridinium iodide) and SYTO 61 red fluorescent nucleic acid stain, both purchased from Molecular Probes, Eugene, OR. The procedures and specifications of both dyes are listed in Table 1. Incubation of dyes was performed by immersion, and each preparation was rinsed with Ringer's solution three times for three minutes before imaging.

Microscopy

All images were obtained using the 40x water-immersion objective of an Olympus confocal, spinning disc microscope with photomicrographic attachment. A FITC filter was used to view structures stained by 4-di-2-asp, while structures stained by SYTO 61 were best seen through a TRITC filter. Images were taken in all three X, Y, and Z planes at 0.99 micron increments.

Table 1. Application of fluorescent dyes to crayfish abdominal muscle preparations.

Fluorescent Dye	Source	Stock Solution	Final Solution	Wavelength	Treatment	Staining Properties
4-di-2-asp (4-diethylaminostryly-N-nethylpyridinium iodide)	Molecular Probes D289	1 mM in H_2O	5µM in Ringer's solution	Ex: 485nm Em: 607nm	Incubate 6-8 min; rinse with Ringer's 3 times for 3 min	Stains mitochondria of nerve terminals; some muscle fiber staining
SYTO 61 red fluorescent nucleic acid stain	Molecular Probes S11343	5 mM in DMSO	1µM in Ringer's solution	Ex: 628nm Em: 645nm	Incubate 1 min; rinse with Ringer's 3 times for 3 min	Nucleic acid stain

RESULTS

The work reported here attempted to localize glial cells and nerve terminals in crayfish abdominal muscles through the use of fluorescence microscopy. Specifically, nerve terminals were stained with a green fluorescent dye (4-di-2-asp), while nucleic acids were stained with a red fluorescent dye (SYTO 61).

Out of all the preparations examined, a very small number of nerve terminals were accompanied by the large red-stained bodies presumed to be glial cells. In Figure 1, one of these supposed glial cells is found superimposed on top of nerve terminal A, while nerve terminal B is alone.

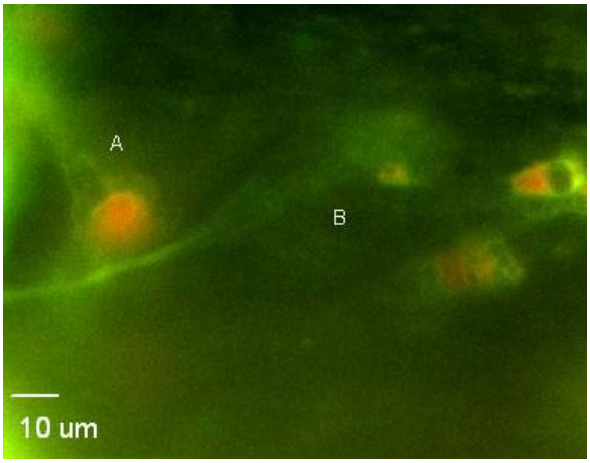

Figure 1. Fluorescent image of nerve terminals stained with 4-di-2-asp (seen through FITC filter), and nucleic acids stained with SYTO 61 (seen through TRITC filter). [**A]** shows a nerve terminal (green) adjacent to a probable glial cell (red). [**B]** shows an independent nerve terminal.

Figure 2 shows a single nerve terminal innervating several smaller muscle nuclei, but no glial cell is present. This figure highlights the fact that even nerve terminals without glial cells were often located in close proximity to muscle nuclei. These results are representative of all the nerve terminal images taken.

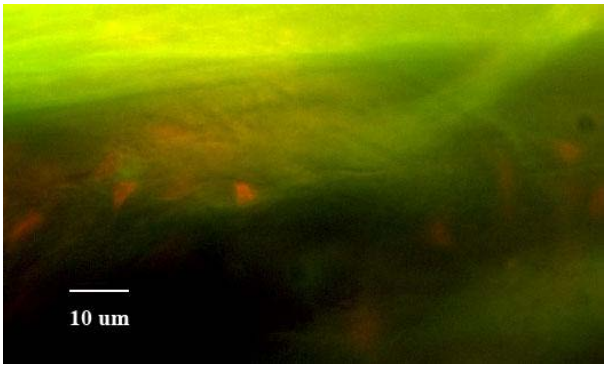

Figure 2. Fluorescent image of a single nerve terminal stained with 4-di-2-asp (seen through FITC filter), and nucleic acids stained with SYTO 61 (seen through TRITC filter). Nucleic acid stain (red) shows muscle nuclei at the nerve terminal (green), but no glial cell.

The data showed that occurrences of nerve terminals in close proximity to glial cells were much less common than those of nerve terminals that appeared alone or close to muscle nuclei.

DISCUSSION

As the nerve terminals unaccompanied by glial cells outnumbered those accompanied by glial cells, the final results neither supported nor disproved the hypothesis that glial cells may be found in close proximity to crayfish abdominal nerve terminals due to their role in the modulation of synaptic transmission. One possible reason for these results is that there may be different populations of nerve terminals, some of which interact with glial cells and some of which do not. It is also possible that the SYTO 61 red fluorescent dye that was used to stain the nucleic acids may not be the best for viewing glial cells in crayfish. No previous research has applied this dye in crayfish, so it was very time consuming to find a specific concentration or duration of incubation that gave favorable results. SYTO 61 also produced a lot of non-specific staining of all nucleic acids, whether glial, muscular, or mitochondrial, which

made it difficult to distinguish the glial nucleic acids from the others. In addition, the muscles had to remain attached to the exoskeleton to prevent balling-up, which made 3-D imaging very complicated due to the cylindrical nature of the carapace.

It was also difficult to distinguish glial cells from other structures due to the fact that very little research has previously been done on crayfish glial cells and so we were unsure what they actually looked like. The satellite cells observed by Novotova and Uhrik (1992) were described as spindle-shaped cells lying parallel to the muscle fibers, however Harrington and Atwood (1995) could find no evidence of these satellite cells. Instead, they identified "muscle surface cells" which were round, flat cells with long processes located near or on top of nerve terminals. The cells identified as glia in this experiment (Figure 1A) most closely resemble these "muscle surface cells" although the dyes used were not able to show any structure beyond nuclei, so any identification is tentative at best.

In future research, it would be beneficial to stain the preparations with different or additional dyes to localize other aspects of these supposed glial cells and create a more complete picture of their overall structure. Having positively identified glia in close proximity to nerve terminals, the next step would be to include staining to localize the mGluRs or NMDARs of the nerve terminals using specific immunofluorescent dyes. This could lead to a better understanding of the role that glial cells play in the modulation of synaptic transmission in invertebrates.

ACKNOWLEDGEMENTS

The authors thank Dr. Clark Lindgren for providing the impetus for this experiment and the Grinnell College Department of Biology for their generous funding.

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