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An Immunofluorescence Study of N-Acetylaspartylglutamate (NAAG) Localization at the Crayfish Neuromuscular Junction

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ABSTRACT

N-acetylaspartylglutamate (NAAG) is the most abundant neurotransmitter in the mammalian central nervous system (Neale et al., 2003). Recent studies have investigated the potential role of NAAG as a neurotransmitter in a variety of vertebrate organisms (Wroblewska, 2006), and have located it in nerves of invertebrates such as crayfish (Gafurov et. al. 2001). However, no research has been undertaken to locate NAAG specifically to the crayfish neuromuscular junction (NMJ). The crayfish NMJ serves as a simple glutamatergic model synapse that can be used to study the complex glutamatergic synapses in the human brain. Using indirect immunofluorescence, this study aimed to determine the presence and location of NAAG in the crayfish NMJ. Through the use of anti-NAAG and anti-synaptotagmin (a SNARE protein associated with neurotransmitter vesicles in the presynaptic terminal of an axon) antibodies (Cooper, 1995), we hypothesized that NAAG would be present in the pre-synaptic terminal in the NMJ. Inconsistent primary and secondary controls and synaptotagmin staining rendered our results inconclusive.

INTRODUCTION

An important characteristic of neurons is their ability to control synaptic activity through neuromodulators. One such neuromodulator is N-acetylaspartyl glutamate (NAAG), the most abundant and widely distributed peptide neurotransmitter in the mammalian central nervous system (Neale, 2005).

NAAG is released in a Ca^{2+} dependent process and chemically binds to metabotropic glutamate receptors (mGluR3) and NMDA receptors, which ultimately alters the production of excitatory post-synaptic potentials (EPSPs) (Raven et al., 2011). The activation of $mGluR_3$ receptors in a pre-synaptic cell reduces cyclic-AMP (cAMP) levels, which in turn decreases the amount of protein kinase A (PKA) that is activated (Raven et al., 2011). This is thought to reduce the amount of phosphorylation of ion channels, which decreases the amount of glutamate release, causing smaller EPSPs (Berent-Spillson, 2004). Alternatively, high concentrations of NAAG reveal its probable antagonistic properties by preventing glutamate from binding to NMDA receptors, also resulting in smaller EPSPs (Berger, 1995; Bergeron, 2007; Westbrook, 1986). Studies have shown that NAAG's role in this process moderates schizophrenia-like symptoms, influences the spinal signaling of pain and inflammation, and is involved in stroke and diabetic neuropathy (Neale et al., 2005; Carpenter et al., 2003).

The characteristics of crayfish neuromuscular junctions (NMJs) make it an ideal candidate for study. They have large post-synaptic cells, are easily accessible, and have simple yet important neuronal responses. Understanding the function of NAAG in crayfish is important because the NMJ serves as a good model system for studying human brain synapses, as its simple glutamatergic synapses mirror those of complex human brain glutamatergic synapses. Thus, studying the role of NAAG in crayfish will broaden the scope of knowledge of synaptic transmission in both arthropods and mammals, and may ultimately help explain causes of certain neurological conditions such as schizophrenia and pain, aiding research into possible treatments.

Relatively little research has been done on the possible function of NAAG in crayfish. There is evidence to suggest that it is present in the medial giant axon of crayfish (Gafurov et. al. 2001); however, no research has been done to localize NAAG to the crayfish NMJ. This investigation aimed to confirm the presence of NAAG and specifically locate it to the crayfish NMJ using indirect immunofluorescence.

Recent research has suggested that NAAG acts as a neurotransmitter in invertebrates (Wroblewska, 2006). Neurotransmitters are packaged in vesicles that are anchored to pre-synaptic membranes with SNARE proteins such as synaptotagmin. Research using immunofluorescence conducted by Cooper et al. (1995) confirmed the presence of synaptotagmin in the motor nerve terminals of crayfish. Therefore, we hypothesized that NAAG would be present in the crayfish NMJ and co-localized with synaptotagmin in the pre-synaptic boutons of axons.

An indirect immunofluorescence protocol of anti-NAAG and anti-synaptotagmin co-stains could show a corresponding location between NAAG and synaptotagmin. Control experiments attempted to account for non-specific binding of the primary and secondary antibodies: for a secondary antibody control we eliminated the primary antibody, and for an attempted primary control (if the secondary control method functioned properly) we used an exocytosis procedure to ensure that the primary antibody bound only to NAAG. Our results remained inconclusive as to the location of NAAG due to inconsistent NAAG antibody controls and anti-synaptotagmin staining.

MATERIALS AND METHODS

Tissue Preparation

Procambrius clarkii (crayfish) were obtained from Carolina Biological Supply (North Carolina, USA). The crayfish were submerged in ice to anesthetize them and the tails were removed. To expose the dorsal extensor muscles, the ventral exoskeleton and viscera were removed by cutting down the lateral ridges of the exoskeleton. The remaining dorsal exoskeleton with the extensor muscles was pinned to a dissecting dish without saline solution and placed under a microscope (ZOOM 2000, Leica) to aid in visibility of the extensor muscle bundles. Using small scissors, forceps, and picks, the four extensor muscle bundles were completely removed from the exoskeleton and placed in a dish made of sylgard filled with 500 microliters of Ringer solution (Figure 1).

Immunofluorescence Protocol

NAAG pre-fix solution incubated for 30 minutes at 30° C. Paraformaldehyde solution was applied for one hour at 4° C. After the tissues were rinsed with Ringers, the application of 0.3% Triton- X 100 solution for 30 minutes at 37° C permeabilized the membrane. Then, the tissues incubated in blocking solution for 1 hour at room temperature. The primary antibody was applied overnight at 4° C and rinsed, followed by secondary antibody incubation for two hours at room temperature.

Synaptotagmin Stain

After completing the immunofluorescence protocol, the tissue was stained for synaptotagmin. This used the same primary and secondary incubation times as the immunofluorescence protocol.

Solutions

Solution	Components
Regular saline solution (Ringers)	5.4 mM KCl 196 mM NaCl 2.6 mM $MgCl_2 \bullet 6H_2O$ 10 mM Sodium Hepes Buffer 13.5 mM $CaCl_2 \bullet 2H_2O$ pH 7.4
60 mM KCL Ringers	60 mM KCl 141 mM NaCl 2.6 mM $MgCl_2 \bullet 6H_2O$ 10 mM Sodium Hepes Buffer 13.5 mM $CaCl_2 \bullet 2H_2O$ pH 7.4
NAAG pre-fixation solution	6% EDAC and 4% NHS dissolved in DMSO and diluted in Ringers
Fixation Solution	3% paraformaldehyde in Ringers
Permeabilizing solution (0.3% Triton-X 100 solution)	25 mL ringer 75 µL Triton-X 100 (Sigma)
Blocking solution	100 mL ringer 2 g Bovine Serum Albumin (Fisher) 10 µL Triton-X 100
NAAG primary antibody	1:100 (gift from Dr. Joseph Neale, Georgetown) diluted in blocking solution
NAAG secondary antibody	1:200 Goat anti Rabbit IgG FITC (American Qualex Antibodies) diluted in blocking solution
Synaptotagmin primary antibody	1:50 3H2 2D7-c (Developmental Studies Hybridoma Bank, Iowa City, Iowa) diluted in blocking solution
Synpatotagmin secondary antibody	1:200 Goat anti Mouse TRITC (American Qualex Antibodies) diluted in blocking solution
Mounting solution	ProLong® Gold antifade reagent without DAPI sensitivity (Invitrogen)

Secondary NAAG Antibody Control

To test for the possibility of non-discriminant binding of the NAAG secondary antibody, the immunofluorescence protocol was conducted without primary NAAG antibody. Tissues were also subjected to a complete synaptotagmin stain.

Exocytosis Protocol

To control for primary antibody non-discriminant binding, tissues were exposed to the 60 mM KCl Ringers for 15 minutes and then processed using the complete immunofluorescence protocol. This depolarized the cell and should theoretically cause neurotransmitter depletion. In the 60 mM KCl Ringer, NaCl must be reduced as KCl increased in order to ensure that there was no change in osmolarity that would promote a diffusion of water out of cells.

Mounting Fixed Tissues

After the antibody incubation, the muscles were removed from the dish and rinsed thoroughly with normal Ringer solution. Using the flat bottom of a clean petri dish, the muscles were flattened prior to placing a cover slip on the slide. One drop of mounting solution was placed over the muscle, and was covered using a cover slip and sealed into place using clear nail polish. A cylindrical weight of 50 grams was placed on top of the cover slip to ensure it remained flat. Slides were kept at 4° C in the dark to prevent fading of the fluorophores.

Imaging

Slides were viewed by a confocal microscope **(**Olympus IX81) using a 60x objective. Using SlideBook (Intelligent Imaging Innovations) software, 3-D rendered images were taken of a desired section on the slide through z-stacking and were compiled in MetaMorph (Molecular Devices) software. Photoshop (Adobe) was used to crop images and insert a scale bar.

Figure 1. Dissection of dorsal extensor muscle bundles.

RESULTS

Through an immunofluorescence protocol, we were unable to conclusively determine the precise location of NAAG due to inconsistencies with controls and synaptotagmin stains.

The anti-NAAG secondary antibody fluoresced under FITC wavelength and the anti-synaptotagmin secondary antibody fluoresced under the TRITC wavelength, allowing us to use both antibodies on the same preparation. The NAAG-only stain allowed us to potentially verify that NAAG is present in the crayfish neuromuscular junction (Figure 2A). To identify NAAG's precise location, we co-stained for synaptotagmin, a SNARE protein that associates with neurotransmitter vesicles in the pre-synaptic membrane, to support our hypothesis that NAAG is present in the pre-synaptic cells of synapses (Figure 2B). Since the synaptotagmin stain appears to correspond with what we think is a NAAG stain, we can theorize that NAAG is located in the boutons of axon terminals in the crayfish NMJ (Figure 2C). In addition, the NAAG stain that appears in the axon and does not co-localize with synaptotagmin corresponds with previous results that found NAAG in the medial giant axon of crayfish (Gafurov 2001).

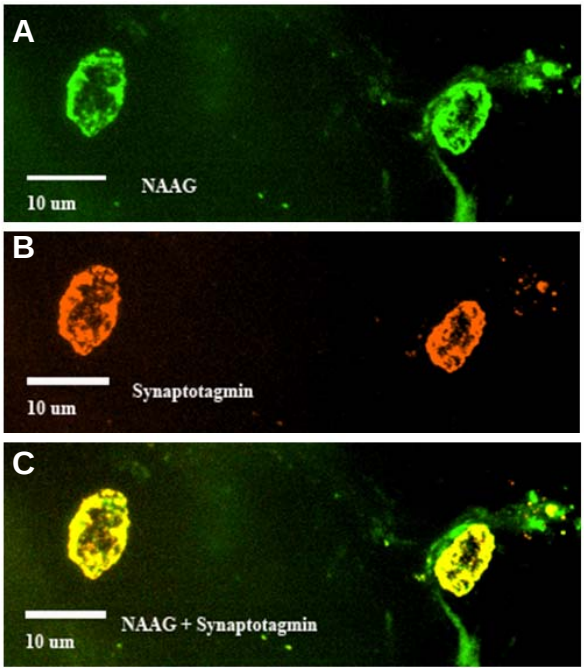

Figure 1. NAAG and synaptotagmin stain showing potential location of NAAG. (A) is the complete NAAG stain. (B) shows the complete synaptotagmin stain. (C) illustrates the merged images of anti-NAAG and anti-synaptotagmin.

However, control stains suggest that the secondary antibody bound to substances other than NAAG, causing structures to fluoresce that may not have been NAAG (Figure 3B). The primary antibody control, the exocytosis protocol which should cause neurotransmitter depletion through depolarization caused by incubation in high KCl solution, appeared to not have depleted NAAG. However, since the secondary antibody control did not function, this may be due to secondary antibodies binding non-discriminately. Further preps also showed that the synaptotagmin antibodies were not visible (Figure 3C). The combination of discrepancies in controls and inconsistent synaptotagmin staining rendered our results inconclusive.

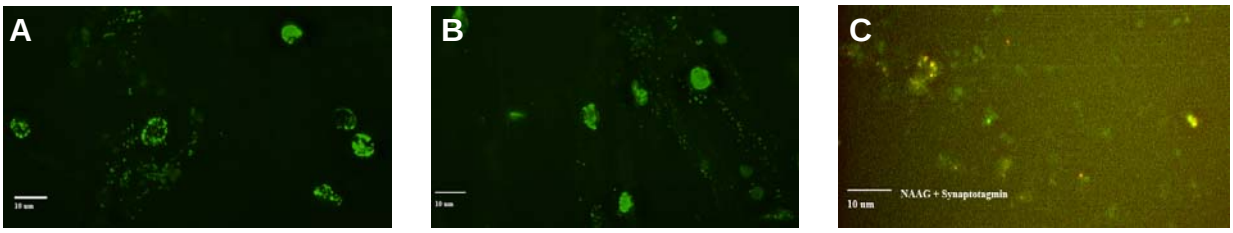

Figure 3. Inconsistencies in control experiments for NAAG and results for synaptotagmin. (A) NAAG primary antibody control. The appearance of bouton-like structures where NAAG should not be expected after neurotransmitter depletion suggest a failure of primary antibody control or depletion protocol. (B) NAAG secondary antibody control. Fluorescence without the presence of a primary antibody suggests non-discriminant binding of NAAG secondary antibodies. However, this prep was cross-contaminated with primary antibody. (C) NAAG secondary antibody control and synaptotagmin stain. This synaptotagmin stain had a 1:25 dilution. The non-visibility of synaptotagmin indicates inconclusive protocol discrepancies.

DISCUSSION

Our results could not determine whether NAAG is present at the crayfish NMJ. The inconclusiveness is mainly due to uncertainties about the secondary antibody control. The secondary antibody appeared to bind to many structures when primary antibodies were excluded from the procedure. Therefore, we cannot be sure the staining is only revealing the presence of NAAG. Possible further experiments include altering the protocol to lower the antibody concentration and rinsing more with blocking solution after applying antibodies. Blocking solution includes proteins that may siphon off extra antibodies, a property that normal Ringer solution does not have.

The exocytosis protocol exposed our tissues to a 60 mM KCl Ringer solution for 15 minutes to cause neurotransmitter depletion. Theoretically, there should not be any fluorescence with this protocol (Figure 3). Results for this control are uncertain for three reasons. First, NAAG may not be depleted at all through depolarization. Second, NAAG may have a re-uptake process that is quicker than the 15-minute incubation time. This lack of knowledge about the re-uptake length requires further protocols with differing incubation times and further research into the re-uptake process of NAAG. Third, since the secondary antibody did not successfully bind to the primary, it is uncertain what areas of fluorescence in Figure 3 were successful secondary antibody stains and what areas were the result of non-discriminant binding.

The anti-synaptotagmin staining requires more preparations with varying dilutions of the primary synaptotagmin antibody to prevent competitive binding. Also, exploration into an alternative co-localization method is needed. Another option is choosing a different protein to stain for, such as other SNARE proteins like synaptobrevin, using a Western Blotting procedure to ensure that synaptobrevin is stained (Ida et al., 1996).

It is also possible that the NAAG and synaptotagmin stain (Figure 2) was successful, but that NAAG is present in glial cells. The presynaptic terminals were expected to be 3-4 um long (Cooper et. al., 1995), so staining in the glial cells would explain the appearance of larger structures (Kondoh, 1986).

While this experiment began as a definitive attempt to identify NAAG's location in the crayfish neuromuscular junction, our research uncovered the need for further study to begin developing a functioning NAAG immunofluorescence protocol.

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