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Identifying CB_1 Receptors in the crayfish neuromuscular junction through immunofluorescence staining

MATTI H. SAHEL, LAURA M. GARCIA, NGOC M. TRUONG
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Cannabinoid receptors (CB_1) are present on presynaptic nerve terminals and alter release of neurotransmitters. Previous research asserts that CB_1 receptors are expressed in the majority of the animal kingdom, yet its presence in invertebrates have led to contradictory evidence supporting and disproving its existence (McPartland et al. 2006). Earlier, a study has found CB_1 receptors in the lizard nerve terminal (Newman et al. 2007). Our goal is to further this discussion by identifying whether CB_1 receptors are present in the crayfish extensor muscle's presynaptic terminals. Our research design centers around the application of immunofluorescence staining through the use of two different antibodies that will result in two different stains (one for CB_1 receptors and the other for the presynaptic terminal). Our major results were the colocalization of anti-SNAP-25 and CB_1. This colocalization suggests that CB_1 receptors, first of all, do exist in the crayfish neuromuscular junction (NMJ), where endocannabinoids may play a functional role.

INTRODUCTION

CB_1 receptors are a type of protein coupled receptor with phylogenic roots evolving from the last common ancestor of bilaterians (McPartland et al., 2006). Endocannabinoids (eCBs) are the signaling molecules that bind to CB_1 receptors and are usually released from the postsynaptic element of the synapse. The activation of CB_1 receptors, which are usually present on the presynaptic terminal, reduces neurotransmitter release (Viveros, 2007).

The experimental question was whether, like lizards and other invertebrates (Newman, 2007), CB_1 receptors would be in the presynaptic terminal of the NMJ. In our experiment we utilized antibody-selective immunofluorescence staining and microscopy in order to identify CB_1 receptors and presynaptic terminals. Our study of the crayfish NMJ shows the colocalization of CB_1 receptors in the presynaptic terminal. Our findings also suggest eCBs may modulate synaptic transmission at the crayfish NMJ.

MATERIALS AND METHODS

Immunofluorescence Staining

A segment of the crayfish tail was removed in order to stain a small portion of the extensor muscle. Ringer solution (RS) was used as a blocking solution, anti-SNAP-25 was used to specifically bind to presynaptic terminals, while the CB_1 antibody to the CB1 receptors. The muscle was fixed in 3% paraformaldhyde at 4 ^oC for one hour, then permeabilized with .3% Triton X-100 at 37 ^oC for 30 minutes, rinsed for fifteen minutes in RS, incubated with the first anti-body (10 uL of anti-human CB_1 and 10 uL of anti-SNAP-25 of concentration .001 mol) at room temperature (RT) for four hours, and then placed at 4 ^oC for twelve hours

The preparation was then rinsed in RS for one hour every twenty minutes. A second antibody (5 uL of goat anti-rabbit and 2.5 uL goat anti-mouse of concentration .0005 mol) was then mixed with a .99 mL RS was added to the muscle, which is kept at 37 ^oC for two hours, rinsed in RS for 30 minutes every ten minutes.

In our previous trials our group experimented with 10 uL of the primary antibody, while using 5 uL of the secondary antibody, as well as 10 uL of the primary antibody and 2.5 uL of the secondary antibody. Neither of these two procedures worked successfully.

Microscopy

The fixed and stained extensor muscle was then removed from the segment of the crayfish tail and placed onto a slide along with an anti-fade reagent, which was then left in the dark at RT for twenty-four hours. After twenty-four hours, the slides were positioned onto the microscope and viewed with a 100x objective. Pictures were then collected when the CB_1

receptors were excited with different wavelengths of light and of synaptic terminals. Our group then fused the photos of the green and red straining to show the colocalization of presynaptic terminals and CB_1 receptors.

RESULTS

In our investigation we attempted to find if there were CB_1 receptors at the crayfish extensor muscle, finding proof of them, we then verified their colocalization with the presynaptic terminal. We did this by applying the immunofluorescence technique where we used a CB_1 specific antibody and a presynaptic terminal antibody in order to stain the two and localized the two using secondary anti-mouse and anti-rabbit antibodies conjugated to the a green and red fluorophores, respectively.

In our experiment, we excited immunofluorescent staining found at the presynaptic terminals and the CB_1 receptors. Our group, however, was not initially successful in locating the CB_1 receptors at the presynaptic terminal. We first experimented with the amounts of primary and secondary antibodies, which created random staining where no specific area could be identified. In our third trial we succeeded in identifying CB_1 receptors, due to overlapping of the presynaptic terminals and CB_1 receptors.

As seen in Figure 1, the green represents CB_1 receptors, red represents the synaptic terminals and the yellow is the overlapping of the red and green staining, indicating colocalization of anti-SNAP-25 and the CB_1 receptors. Figure 2 is a closer view of where this colocalization occurs and the CB_1 and anti-SNAP-25 are able to be seen together.

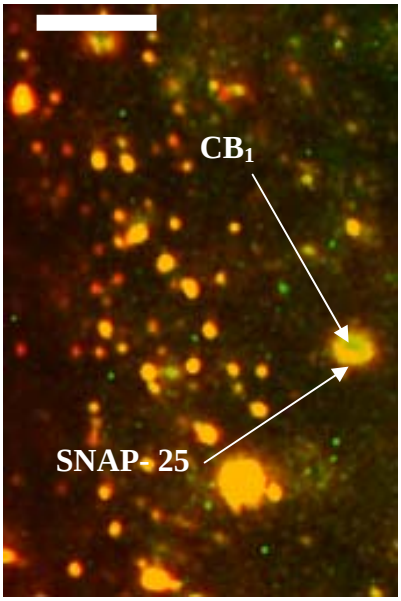

Figure 1. Immunofluorescence staining: the CB_1 receptors are the green stains; red stains are the ANTI-SNAP-25 at presynaptic terminals, and the yellow stains are the overlapping of the two, otherwise known as colocalization . Callibration bar, 10um.

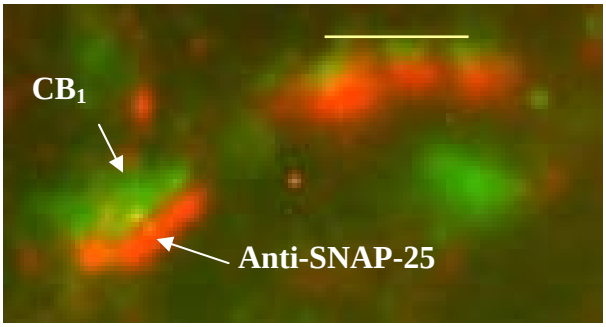

Figure 2. Immunofluorescence colocalization of CB_1 receptors and SNAP-25 in crayfish extensor muscle. The CB_1 receptors are the green and red are the SNAP-25, which localize. Calibration bar 2.5 um.

DISCUSSION

Much like what Newman et al. (2007) found in the lizard NMJ, our experiment also suggested the existence of CB_1 receptors at the presynaptic terminal.

In our first two trials, the overall muscle staining might have been due to too much of the CB_1 antibody, causing the antibody to bind non-specifically to all of the muscles and not just the CB_1 receptors. This is why we reduced the

amount of the CB_1 antibody from 5uL to 2.5uL in our third trial. As we have alluded to in figures 2 and 3, less CB_1 antibodies effectively show the appearance of CB_1 receptors at the presynaptic terminal in contrast to scattered CB_1 receptor stains.

Our experiment initiates further studies into the involvements of CB_1 receptors in the modulation of synaptic transmission.

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