Evidence That Synapses Do Not Form in Embryonic Chick Neuronal Cultures As Measured By Syntaxin Staining Compared With Tubulin Staining

Allyssa Lumbert
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Introduction

The formation of synapses help the brain to carry out its everyday functions and have a major role in the normal functioning of the brain. A synapse is the gap between two neurons in which the neurons communicate through the release of neurotransmitters from one neuron to the other. Many neurological disorders, such as autism, are thought to occur as a result of abnormalities in synapse formation. For example, genes that are mutated in autism have been found to influence pruning (Huber & Cowan 2014). The process of pruning eliminates unnecessary synapses, resulting in an abundance of synapses in brains that have a defective gene that regulates pruning (Huber & Cowan 2014). The SNARE complex is a group of trans-membrane vesicle proteins that are involved in the fusion of the vesicle membrane with the plasma membrane and the exocytosis of neurotransmitters. Neurotransmitters are contained in and released from synaptic vesicles, which contain proteins that determine the destination and function of the vesicle (Sudhoff 2004). Syntaxin-6 is a member of the SNARE complex that is mainly found in the trans-Golgi network, but can also be located in many compartments in the cell as it has the ability to associate with many SNARE partners (Wendler & Tooze 2001).

I collaborated with Rachel Kirk in this experiment. We carried out our experiment by fixing dorsal root ganglia and sympathetic chain neurons from *Gallus gallus* embryos and performing immunocytochemistry. We used chick embryos because they are easily accessible, affordable, and can be dissected with ease and precision. Although seemingly very different species, humans share 60% of their DNA with chickens, suggesting that neuronal behavior in cultures of *Gallus gallus* cells can provide information about neuronal behavior in humans (Purdy 2004). Rachel and I stained the neurons using an antibody against Syntaxin-6. In our analysis, we defined a puncta as a small circle of a visibly greater brightness than the surrounding axons. We define an en passent synapse as a puncta at a junction point of two axons revealing a greater difference in brightness between the puncta and surrounding axons in red fluorescence than with green fluorescence. We used a DM1A antibody against alpha-tubulin, which stains green and we used primary and secondary antibodies to stain for syntaxin-6, which stains red. The staining of syntaxin-6 should reveal the location of synaptic vesicles and the alpha-tubulin should reveal the location of the axons, so if synapses are present, the ratio of puncta to surrounding axon brightness should be greater in red fluorescence than in green fluorescence.

I hypothesized that synapses form in culture as measured by a higher puncta to axon ratio in syntaxin-6 staining than in tubulin staining. I was led to this hypothesis as the buffer we created and added to the cells is designed to mimic the extracellular fluid of the cells in their natural state. The sucrose in the fixation/permeabilization buffer keeps the cells from breaking apart and acts as a stabilizing agent. The EGTA prevents proteolysis so I predicted that all of the proteins necessary for synapse formation, including cell adhesion molecules and trans-membrane proteins, would still carry out their functions in synaptogenesis. Previous studies have revealed that synapses form in cell culture. For example, a study revealed that neurons from the abdominal ganglia of *Aplysia californica* form specific synapses and avoid forming unspecific synapses in cell culture (Camardo, Proshansky, & Schacher 1983). Taking the evidence that synapses form in cell culture in invertebrates, I hypothesized that synapses will form when dorsal root ganglia and sympathetic nerve chains are cultured in the *Gallus gallus*, which, as vertebrates, have a more complex nervous system than *Aplysia californica*. .Another study revealed leech CNS neurons to form specific synapses in cell culture, which further led me to believe that *Gallus gallus* neurons will form synapses in cell culture (Fernandez de Miguel & Drapeau, 1995).

With the knowledge that neurons do not need to be present in living organisms in order to form synapses, but can form in culture, researchers might be able to better test the role of synaptic formation in neurological disorders or could possibly test different drugs and their roles in synapse formation in cell culture.

Materials and Methods:

Dissection

Dorsal root ganglia and sympathetic chains were dissected from chick, *Gallus gallus*, embryos and sea urchins, *Pluteus larvae*, using the protocol *Primary culture of chick embryonic peripheral neurons 1: Dissection* designed by Robert Morris (Morris 2014a). DMEM was used instead of the HBSS called for in the protocol. The cells used were also on coverslips instead of in suspension.

Fixation, Rehydration, and Staining:

The protocol *Immunoflour staining of SU embryos – MeOH fixation* was followed, with some adjustments (Morris, 2013). The MeOH fix buffer in step 1 was made using 3ml 250 mM EGTA to 150 ml MeOH. *Pluteus larvae*, embryos were used for the positive controls and *Gallus gallus* embryos were used for the experimental and negative controls. Some of the cells from each species were fixed with a 1:50 solution of 250 mM EGTA in MeOH and some were fixed with a formaldehyde/gluteraldehyde (F/G) solution. The cells were then rehydrated, mounted onto coverslips treated with poly-K and laminin, and blocked (Morris, 2013). The sea urchin slides (one MeOH-fixed and one F/G-fixed) were used as our positive controls to indicate the effectiveness of the antibodies used with the assumption that the antibodies would fluoresce in the sea urchin cells. One F/G-fixed slide was used as a negative control and one MeOH-fixed slide was used as a negative control. The negative controls tested for autoflourescence and used the secondary antibody (RGAR), but not the primary antibody. One F/G-fixed slide was used as an experimental slide and two MeOH-fixed slides were used as experimental slides. Syntaxin-6 and DM1A were used as the primary

antibodies. DM1A was used as a 1:50 concentration while we used both a 1:50 concentration and a 1:100 concentration of the syntaxin-6 antibody to observe whether one concentration of solution stains better than the other. The 1:50 syntaxin-6 solution was added to one of the MeOH experimental slides and the 1:100 syntaxin-6 solution was added to the other MeOH experimental slide, both positive control slides, and the experimental formaldehyde-fixed slide. We used Tetramethyl Rhodamine (RGAR) as our secondary antibody at a concentration of 1:100.

The coverslips were mounted on slides with slide 1 containing both positive control CSs, slide 2 containing both negative controls, slide 3 containing the F/G-fixed CS with1:100 syntaxin-6 and the MeOH-fixed CS with 1:100 syntaxin-6 and slide 4 containing the MeOH-fixed 1:50 concentration CS.

Imaging and Analysis

The slides were imaged using a Nikon Eclipse E400 microscope and the Capricorn computer using the protocol *Imaging Fluorescence on Gemini and Pisces computers in ICUC* (Morris, 2014b).Each coverslip was imaged under brightfield, blue, green, and red fluorescence at a magnification of 40x. The brightness of the MeOH-fixed CS on slide 3 was measured in lumens using the protocol *Image Analysis for Independent Research 2014* (Morris, 2014c). Puncta located at the point of axon crossings were selected and average brightness was measured in the red fluorescence image and then an area of the axon leading to the puncta was selected and measured. The corresponding regions were selected and measured on the green fluorescence image using the same size region. The ratio of brightness of puncta to surrounding axons was compared between red fluorescence and green fluorescence. The arrows were drawn using the Image J software.

Results

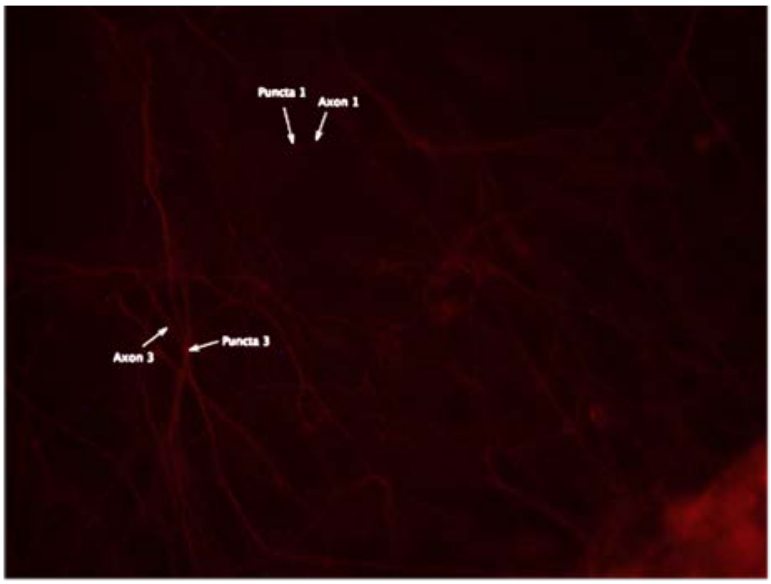

Figure 1: Red fluorescence of MeOH-fixed 1:100 syntaxin-6 experimental (slide 3). Notice that the difference in brightness between puncta 1 and axon 1 appears slightly lower than the difference in brightness between puncta 2 an axon 2 in Figure 2. Also notice that the difference in brightness between puncta 3 and axon 3 appears about the same as the difference in brightness between puncta 4 and axon 4 in Figure 2.

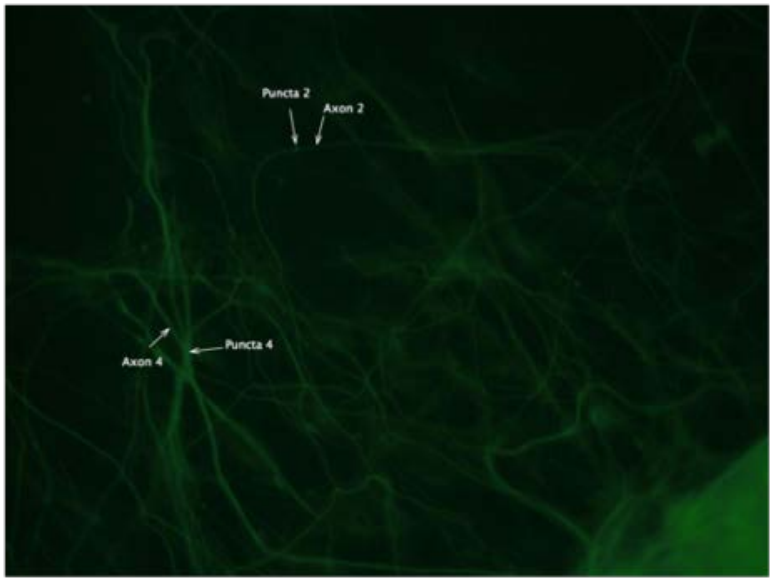

Figure 2: Green fluorescence of MeOH-fixed 1:100 syntaxin-6 experimental (slide 3)

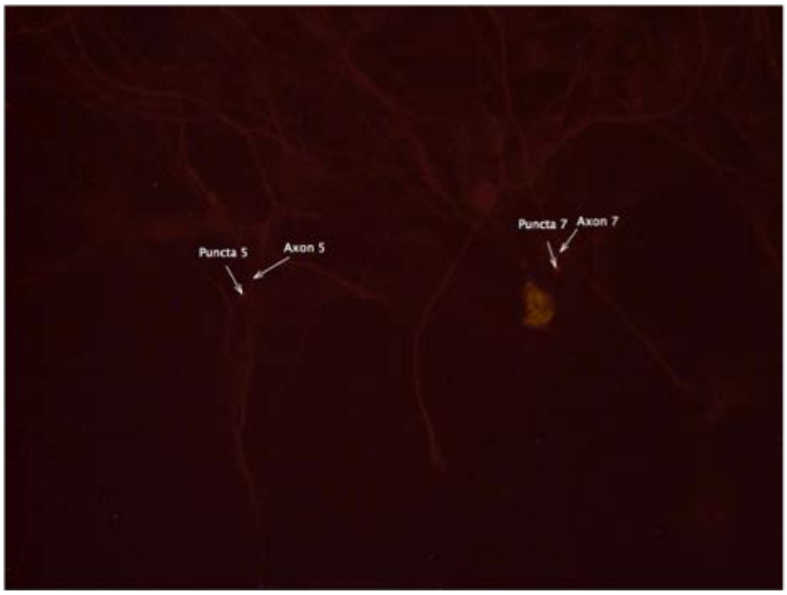

Figure 3: Red fluorescence of MeOH-fixed 1:100 syntaxin-6 experimental (slide 3). Notice that Puncta 5 stained brighter than axon 5, whereas the corresponding puncta 6 in Figure 4 stained less brightly than axon 6. Also notice that the ratio of puncta to axon brightness between puncta 7 and axon 7 is greater than the ratio of puncta to axon brightness between puncta 8 and axon 8 in Figure 4.

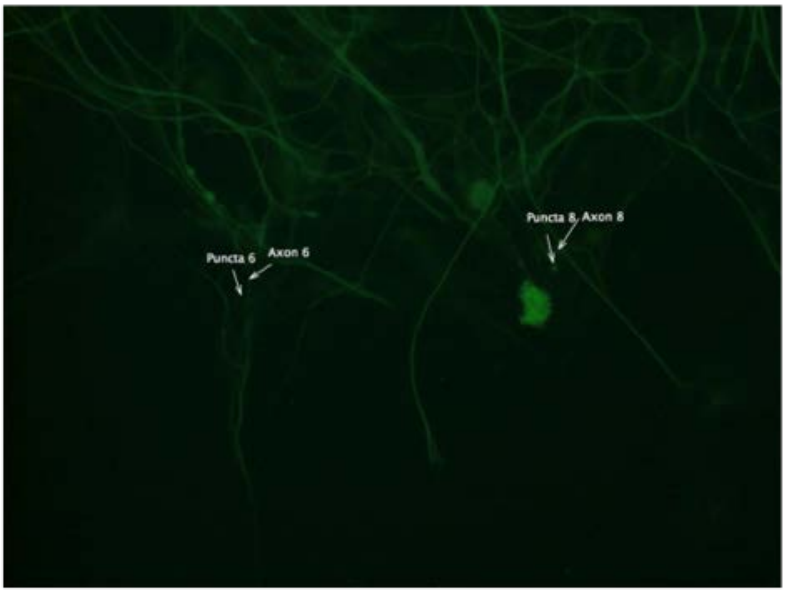

Figure 4: Green fluorescence of MeOH-fixed 1:100 syntaxin-6 experimental (slide 3)

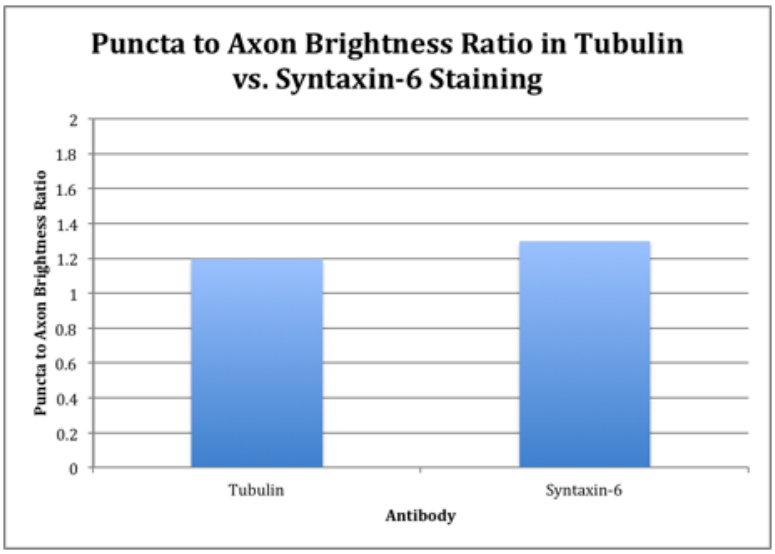

Figure 5. Notice how the ratio of puncta to axon brightness was very slightly higher in syntaxin-6 staining than in tubulin staining.

Conclusions

The hypothesis that synapses form in cell culture as measured by puncta to axon ratio in red fluorescence compared with green fluorescence was not supported by the data in this experiment. Although the average ratio of brightness between the puncta and surrounding axons in red fluorescence was greater than in green fluorescence, there .was not a significant difference in ratio of brightness. In addition, exactly half of the puncta and axons measured revealed a greater puncta to axon brightness ratio in green fluorescence than in red fluorescence. However, my collaborator Rachel Kirk found that synapses form as measured by counting synapses that form only in Syntaxin-6 staining but not in DM1A staining (Kirk, 2014). These results could suggest that the puncta I measured were not synapses, but that synapses do form in cell culture, as represented by the puncta observed and counted by Ms. Kirk that were present in syntaxin-6, but not DM1A staining.

If the puncta we measured are synapses, then we conclude that the syntaxin-6 and RGAR successfully stained for syntaxin-6 on synaptic vesicles. The brightness of the axons in green fluorescence were brighter than the axons in red fluorescence, revealing that the alpha-tubulin staining was also successful. The brighter staining of the puncta in green flourescence than in red does not necessarily reveal that the puncta are not synapses because as tubulin provides structural support for neurons, it could be present on synaptic vesicles as well. If the areas in which puncta to axon ratio were higher in green than in red fluorescence were synapses, the high level of brightness of the puncta in green fluorescence could have resulted from a high concentration of alpha-tubulin at those specific synaptic vesicles.

Our negative controls did not reveal red or green fluorescence, which implies that the staining of alpha-tubulin and syntaxin-6 were successful. The ovals stained in blue fluorescence reveals that the cell bodies were stained and that DNA staining was successful, as axons were not stained in blue fluorescence.

One limitation that we had in our study was that we only performed the immunocytochemistry once and therefore had a small amount of data from which to draw conclusions. If we had more time to conduct our experiment, we would have repeated the ICC at least three times to minimize any errors that occurred. One source of error we experienced was that we added Hoechst to our negative controls, which resulted in blue fluorescence in our negative controls. However, we were still able to see that green and red fluorescence were absent in our negative controls. One of our coverslips (formaldehyde-fixed 1:100 concentration) also broke during fixation and we observed bacteria on the slides, which could have been due to unsterile preparation of the slides or failing to completely seal the slides with nailpolish, allowing bacteria to enter under the coverslips. If we repeated the experiment, we would be careful to correct these sources of error.

If synapses were forming in our cultures, the neurons were differentiating and migrating to their destinations. Growth cones were then extending the axons of the neurons and cell recognition was occurring between the recognition molecules on the axons of one cell with the dendrites of another cell (Bean, 2014). This would reveal that the cell adhesion molecules and other proteins, including transport and trafficking proteins involved in synapse formation remain intact in cell culture. ATP must also continue to be available to the cells, as ATP is required for proton pumps to generate electrochemical gradients necessary for neurotransmitter uptake and transport (Bean, 2014).

However, as my results did not support the hypothesis that synapses form in cell culture, there could be various reasons that synapses did not form. The study investigating synapse formation of *Aplysia* in culture revealed that an initial axon was required to be plated with the L10 neuron in order for it to make synapses with target neurons similar to those synapses it makes *in vivo* (Camardo et. al. 1983). Perhaps our cells did not form synapses in culture because we did not plate them with an initial axon. Other explanations could be that the cells failed to recognize their targets or that they failed to release neurotransmitters. If synapses form, as suggested by Ms. Kirk's data, neurotransmitters are also produced in cell culture, as the potential synapses were observed at synaptic vesicles. Fernandez de Miguel and Drapeau observed serotonin (5-HT) as the neurotransmitter released at the synapses they found in leech neurons in culture (Miguel & Drapeau, 1995). Future studies could test the neurotransmitters released in *Gallus gallus* cultures.

As there is existing literature supporting evidence that synapses form in cell culture and my collaborator Rachel Kirk found synapses to form in our culture based on her analysis of the data, it is difficult to conclude that synapses do not form in cell culture. However, an important piece of evidence that my results reveal is that not all of the puncta at junctions between two axons are synapses, suggesting that some axons that come into contact form synapses, while others do not.

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