Formation of Synapses in Embryonic Chick Neuronal Cultures

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Introduction:

The purpose of this experiment was to test the hypothesis that neurons form synapses in culture as measured by abundance of puncta (brighter spots) in the secondary antibody stain. Synapses are specialized clefts between neurons at which neuron to neuron communication occurs. Synaptic development and connectivity is crucial in maintaining proper brain function. It is a widely accepted belief that information in the brain is stored as the chemical or structural changes of synapses. It is also thought that the formation of new synapses and elimination of old ones is another mechanism for storing information in the brain. Furthermore, the synapse is a very important part in the development of the nervous system and problems with the neuronal circuits stemming from a variety of causes such as genetics, drugs, aging or viruses can directly effect neuronal communication and manifest in psychiatric and neurological disorders (Van Spronsen & Hoogenraad, 2010).

In this experiment, neurons from the domestic chicken (*Gallus gallus*) embryo were examined. Neuronal cultures of animals could be used as models for the human nervous system. Determining when or if synapses develop in chick embryo cultures could aid scientists in many research endeavors. For example, it is speculated that changes at a synaptic level could make individuals more vulnerable to Autism Spectrum Disorders and a loss of neurons and synapses in certain regions of the brain is seen in individuals with Alzheimer's Disease (Van Spronsen & Hoogenraad, 2010). Examining synapses in culture could lead to conclusions about neurological disorders like Autism, Alzheimer's and many others, which could greatly advance the medical field. Obtaining neurons from animals is much easier than obtaining them from humans and growing and studying neurons in culture is a much more feasible option for scientific experiments such as testing drug and alternative therapies.

The antibody Syntaxin 6 was used in this experiment to label its antigen, the synaptic vesicles, which could indicate whether synapses form. Syntaxin 6 is a member of the SNARE protein family that is localized in the trans-Golgi network and in endosomal structures. Syntaxin 6 is expressed widely within the brain as well as in the lungs and kidneys. Unlike other members of the SNARE family, Syntaxin 6 has a wide variety of other molecules that it interacts with, depending on the type of cell being examined. This suggests that Syntaxin 6 is a part of several membrane-trafficking events ("Syntaxin 6," n.d.). Studies have indicated that some members of the Syntaxin family could function in certain trafficking on the target membranes, such as in synaptic transmission. Other members of this family may be involved in other trafficking steps, such as on the vesicle membrane (Block, Klumperman, Davanger &Scheller, 1997).

In order to test the hypothesis that synapses form in cell cultures as measured by the abundance of puncta, chick neurons were treated with the Syntaxin 6 antibody at two different concentrations, 1:100 and 1:50 in order to determine which concentration yielded the best staining. The Synatxin 6 was used to label the synaptic vesicles, whose presence could show synapse formation. It was predicted that the higher concentration of antibody would enhance the brightness of the stain and could make the areas that would be qualified as synapses more evident. In order to analyze data, images were taken from a coverslip treated with the 1:100 concentration of Syntaxin 6 and from a coverslip treated with the 1:50 concentration of Syntaxin 6. A positive control image was also examined in order to test that the antibodies stained properly.

Materials and Methods:

The neurons used in this experiment were dissected from ten-day *Gallus gallus* eggs using the procedure from *Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION* (Morris, 2014). These neurons were then fixed in methanol following the procedure from *Immunofluor staining of SU embryos- MeOH fixation* (Morris, 2008) with some adjustments.

There were several changes made to this procedure. Sea urchin embryos were used for the positive control condition however; all of the other cells that were used came from chick embryos. This procedure also assumed that the cells were in suspension, but the cells used were on coverslips. Additionally different antibodies and concentrations of antibodies than the procedure called for were used. The primary antibodies used were anti- alpha- tubulin (DM1A) and Syntaxin 6. A solution with a 1:100 concentration of DM1A was used. Two different concentrations of Syntaxin 6 were used in order to examine the potential affects antibody concentration could have on the results. One of the solutions used contained a 1:100 concentration of Syntaxin 6 and the other had a 1:50 concentration. The secondary antibody used in this experiment was Tetramethyl Rhodamine (RGAR). This antibody was used at a 1:100 concentration. The application of the Hoechst stain was the same as the cited procedure called for.

The procedure from *Imaging Fluorescence on Gemini and Pisces computers in ICUC* (Morris, 2014) was followed to obtain images of the cells. Images of the same section were taken from each fluorescence channel in order to examine the differences in each antibody stain. For the purposes of this experiment, a synapse was defined as a puncta anywhere in the image that was exhibited in the red stain (Syntaxin 6) but not in the green stain (DM1A). A positive control was examined initially in order to determine if differential staining between the two antibodies actually occurred. To collect the experimental data twelve total images were analyzed, six Syntaxin 6 images and the exact same DM1A images. Three of these images were taken from the slide treated with a 1:100 concentration of Syntaxin 6 and three were taken from the slide treated with a 1:50 concentration of Syntaxin 6.

An arrow was drawn using ImageJ anytime a puncta was observed in the Syntaxin 6 and not in the DM1A. Puncta were counted if they only appeared in the Syntaxin 6 stain, those that appeared in both the Syntaxin 6 and DM1A were not considered significant in this experiment, as they were often much brighter in the DM1A stain. The average number of puncta in each concentration were obtained and graphed in order to examine the difference between the more and less concentrated solution of secondary antibody.

Results:

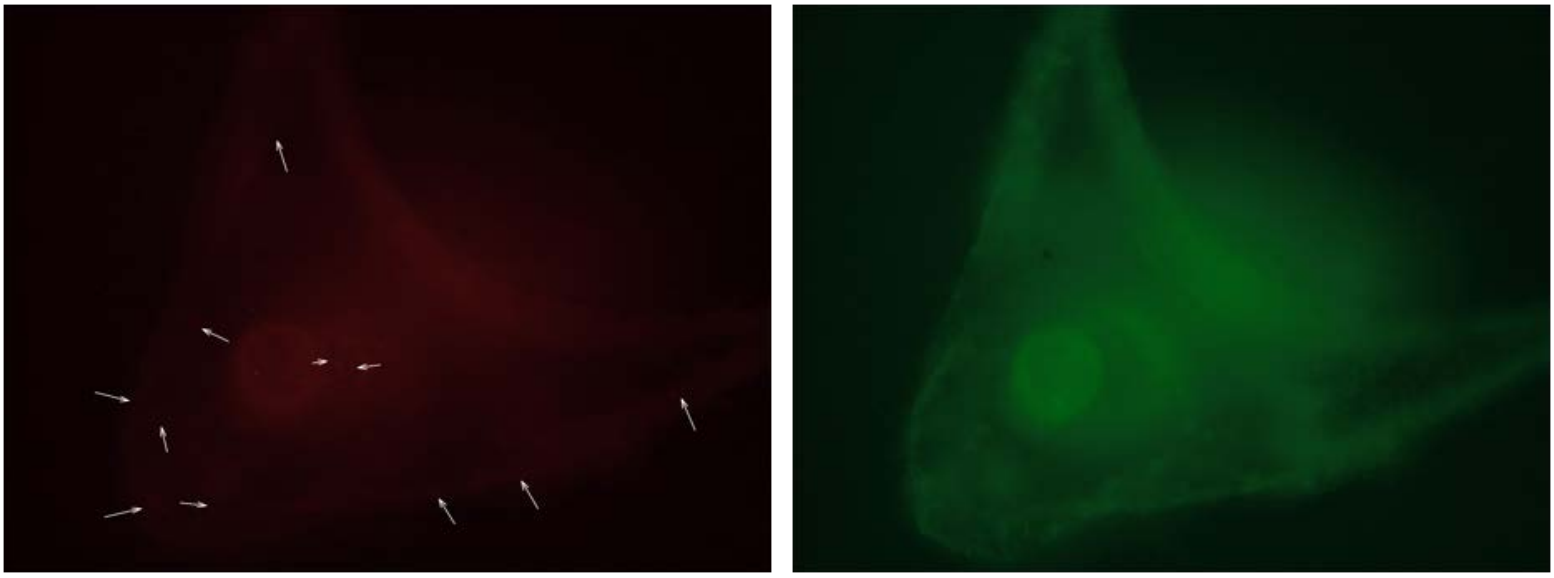

Figure 1: Image from the positive control, sea urchin embryo taken at a 40x magnification. Sample was treated with a 1:100 concentration of Syntaxin 6 solution. This image shows some differential staining between the RGAR and the DM1A.

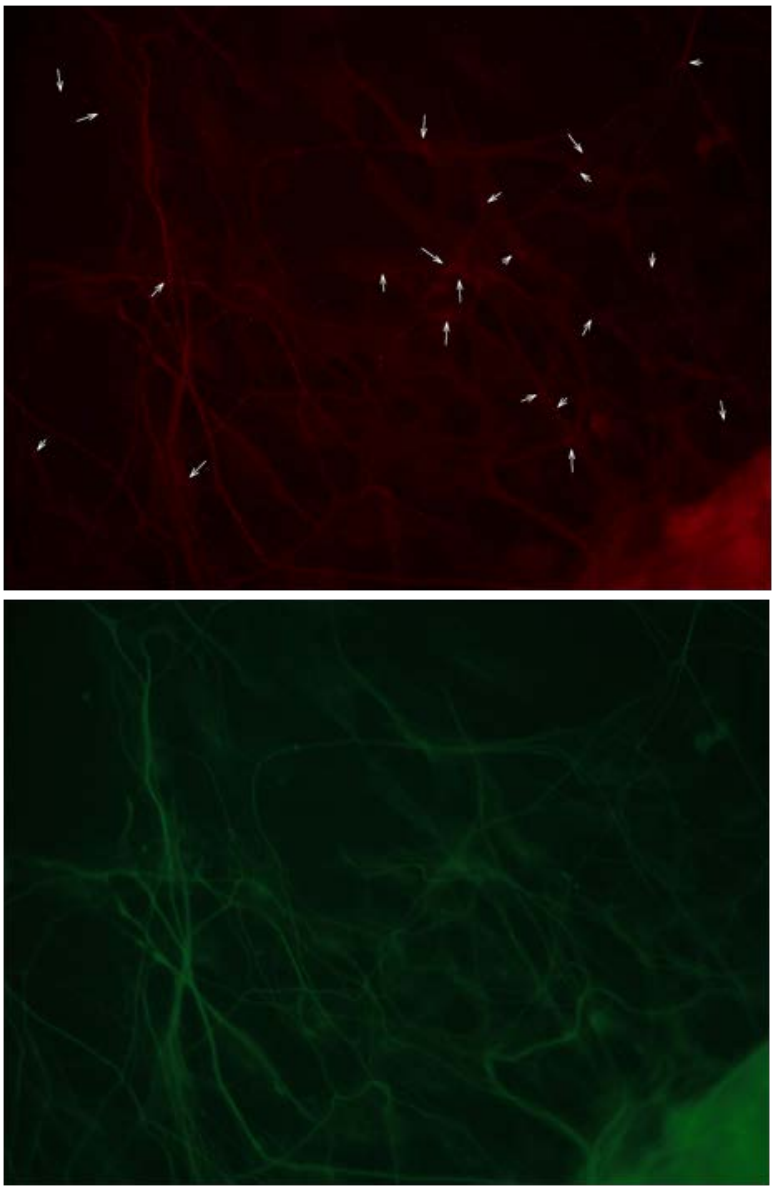

Figure 2: Image taken at a 40x magnification from coverslip treated with a 1:100 concentration of the Syntaxin 6 antibody. Arrows indicate puncta that are present in the RGAR stain that are not present in the DM1A stain.

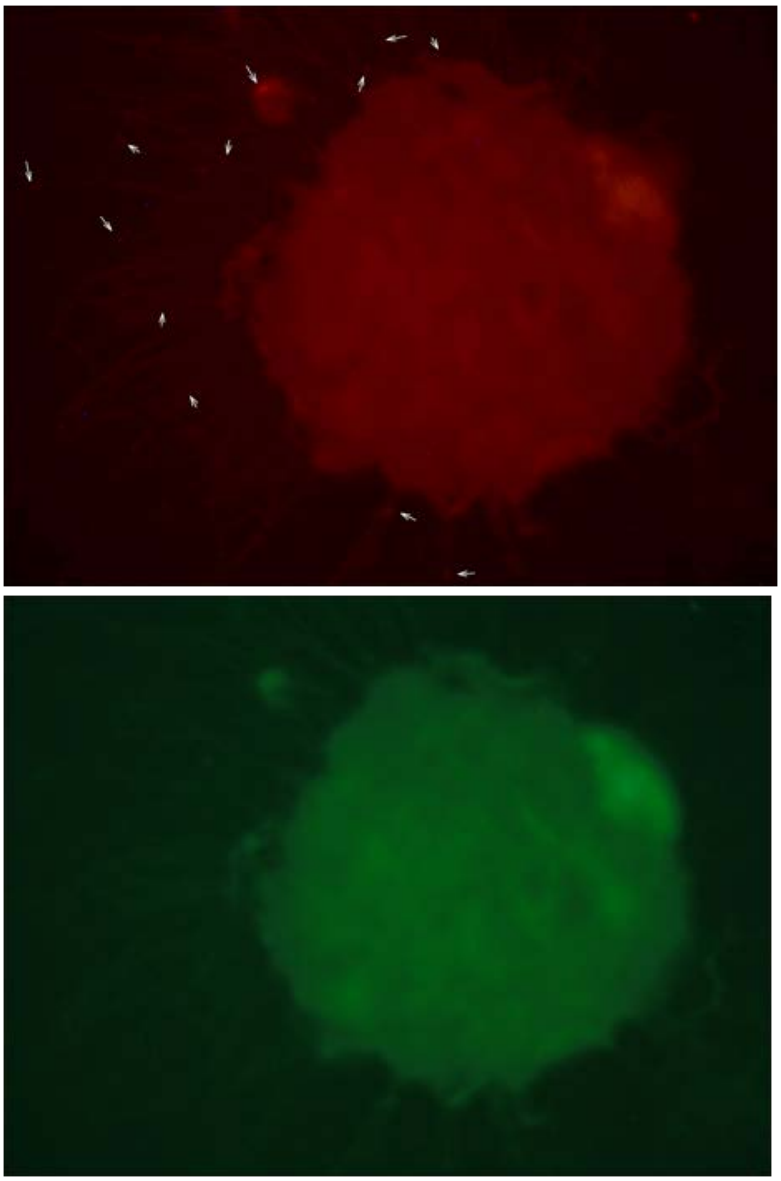

Figure 5: Image taken at a 40x magnification from coverslip treated with a 1:50 concentration of the Syntaxin 6 antibody. Arrows indicate puncta that are present in the RGAR stain and not in the DM1A stain.

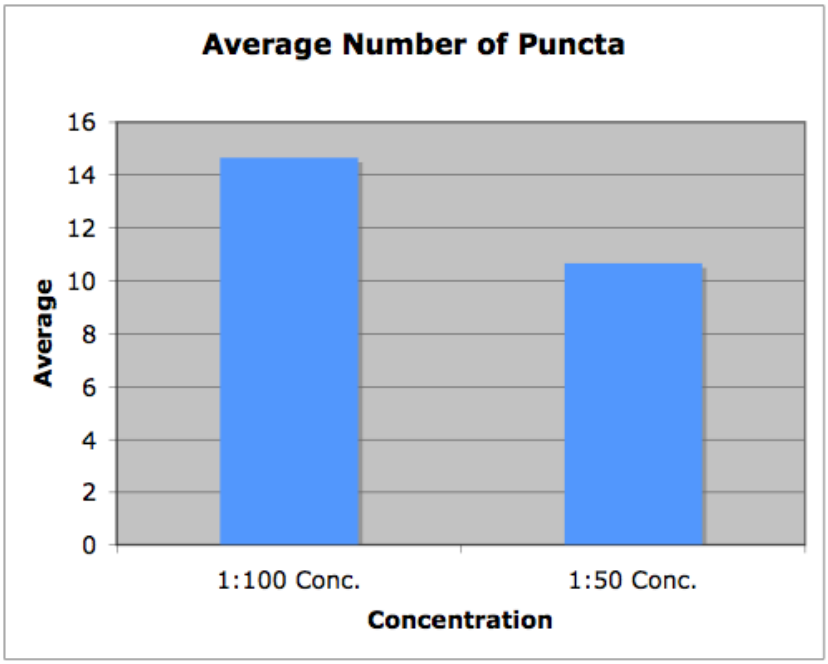

Figure 7: Graph depicts the average number of puncta in each concentration of the secondary antibody.

There were many puncta that were evident in both stains, some that were only evident in the Syntaxin 6 and some that were evident exclusively in the DM1A. The puncta were only counted in the Syntaxin 6 stain because those that appeared in both the Syntaxin 6 and the DM1A, were often much brighter in the DM1A stain.

Discussion:

This experiment tested the hypothesis that synapses form in neuronal cultures taken from domestic chicken, *Gallus gallus*, embryos. Synapses were qualified as puncta that were evident in the Syntaxin 6 antibody stain but not in the DM1A antibody stain. Based on the data analyzed, there is evidence that synapses do in fact, form in these cell cultures. When the concentrations of the secondary antibody solutions are compared, it is not clear whether there is significance in the more concentrated solution. The 1:50 concentration of the Syntaxin 6 antibody shows a lower average number (10.67) of puncta, in comparison to the 1:100 concentration of the same antibody solution, which has a higher average number (14.67) of puncta. This result does not support the prediction that the more concentrated solution would show more synapses. The images obtained from the 1:50 slide (figures 4-6) were not as clear as those obtained from the 1:100 slide (figures 1-3). This could be because there was more nonspecific binding and this made determining which areas contained puncta under red immunofluorescence versus green immunofluorescence difficult, so no definitive conclusions could be made about the concentration differences because there could very well have been many more synapses in this concentration, but due to the unclear images there is no way to be certain that this was the case.

If this experiment was repeated multiple times and the same results were seen, this would further support that the puncta observed were synapses. If the 1:50 concentration resulted in unclear pictures every time the experiment was performed, it would suggest that the higher concentration is not effective in determining whether synapses form in culture, and that using the 1:100 concentration of Syntaxin 6 solution would actually result in the clearest images.

Syntaxin 6, which was the primary antibody that was used to stain the potential synapses in the culture, is a synaptic vesicle protein ("Syntaxin 6," n.d.), which means that it is staining the vesicles present in the synapses. Studies have shown that synaptic vesicles are actually depots for neurotransmitters. The vesicles release their contents into the synaptic cleft via exocytosis (Purves, Augustine & Fitzpatrick, 2001). The puncta observed in the cells examined in this experiment are likely areas at which synaptic vesicles are heavily concentrated, which could mean that in addition to the formation of synapses, there are also certain neurotransmitters being produced in culture. Further experiments could attempt to test if this is actually the case and if so, which neurotransmitters are produced in neuronal cell cultures.

There were several sources of error in this experiment. When preparing the negative control coverslips, which were supposed to show no immunofluorescence, they were unintentionally washed in Hoechst, which labeled the DNA and caused them to fluoresce in blue under the microscope. The negative controls also showed very faint traces of fluorescence in both the red and green channels, which is likely due to an error during the application and washing process. In the future the preparation of these slides would need to be done with more care. There were also bacteria observed on the coverslips. These bacteria are from improper or unsterile lab techniques and could have been introduced into the medium at any point in preparing the coverslips. In future experiments, working to minimize these errors would be crucial in making any further conclusions. In spite of the sources of error valuable information was gained from this experiment, including the knowledge that synapses could be forming in neuronal cultures of the *Gallus gallus* embryo as well as many ways to improve lab techniques.

References:

Allyssa Lumbert, collaboration in fixation, data collection and data analysis

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I have abided by the Wheaton College honor code in this work. Rachel Kirk