In vitro synapse formation among peripheral neurons of the domestic chicken embryo, *Gallus gallus*

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INTRODUCTION:

The nervous system is composed of neurons and glial cells, the system functions via communication of these cells (Kandel, Schwatz, Jessell, Siegelbaum, & Hudspeth, 2013). Cellular communication in the nervous system is termed synaptic transmission and occurs at synapses. Neurons develop synapses as their axons choose post-synaptic partners and their axon terminals differentiate into pre-synaptic terminals while the target neuron differentiates a post-synaptic region (Kandel et al, 2013). Neuronal communication is vital for functions such as perception, voluntary movement, and learning – a loss of neural communication can result in various neurodegenerative diseases, such as Alzheimer's or Parkinson's disease (Mirnics, Middleton, Lewis, & Levitt, 2001). With the implications of synapses in mind, this experiment focuses on in vitro synapse formation.

This experiment tests the hypothesis that peripheral neurons of the domestic chicken, *Gallus gallus*, form synapses in vitro as measured by comparing the ratio of NSF fluorescence to alpha tubulin fluorescence at puncta regions to axon regions. Analysis of synaptic formation is conducted in neurons fixed by two different procedures, a methanol fixation and a formaldehyde/glutaraldehyde fixation. Sympathetic neurons of the domestic chicken are used in the testing of this hypothesis because the chicken's nervous system is remarkably similar to the human nervous system development. Since the development of chicks is mapped out by days and their nervous system development occurs fairly quickly, it is easy to obtain several embryos at the same developmental stage for dissection, making them a preferred model organism (JoVE Science Education Database, 2014). While it is well known that synapses form among the sympathetic neurons in adult chickens, little is known about their development in vitro and the conditions necessary for in vitro synaptic development. The question of whether or not synapses are able to form in vitro is significant because if synapses do form in vitro, in vitro neuronal pathways could be used to further study many brain disorders, especially those previously mentioned that involve dysfunctional neuronal communication. Aspects of diseases like Alzheimer's, Parkinson's, and amnesia could be further studied which could lead to new treatment and prevention options for patients (Mirnics et al, 2001; Sheng, Sabatini, & Südhof, 2012).

In order to determine if synapses form among sympathetic neurons from chick embryos immunofluorescence will be used. Neurons of the chick embryos will be labeled with Hoechst stain to identify DNA and DM1A anti-alpha-tubulin antibody to label tubulin. By identifying the location of DNA and tubulin, one can determine where the cell bodies are and where the axons of different neurons project. In order to test for the presence of synapses the primary antibody NSF (D31C7) rabbit antibody and the secondary antibody Tetramethyl Rhodamine goat anti-rabbit antibody will be used. NSF, N-ethylmaleimide sensitive fusion protein, is an ATPase involved in synaptic transmission. Vesicle fusing occurs at the synaptic terminal, vesicles must fuse with the plasma membrane of the pre-synaptic cell in order to be able to release neurotransmitters (Xia, Zhou, Lin, & Liu, 2000). The formation of a SNARE complex is what drives synaptic vesicle fusion. SNAREs, soluble N-ethylmaleimide-sensitive factor attachment receptors, must be disassembled after fusion for vesicle recycling. SNAP, soluble NSF-attachment protein, an adaptor protein allows NSF to bind to SNARE complexes. NSF and SNAP use the energy derived from ATP hydrolysis, by NSF, to dissociate SNARE complexes (Kandel et al, 2013). NSF also plays a role in regulating AMPA glutamate receptors on dendritic spines – therefore NSF plays a role in both the presynaptic neuron and the postsynaptic neuron (Nishimune, Isaac, Molnar, Noel, Nash, Tagaya, Collingridge, et al, 1998). Using the NSF antibody with the secondary antibody then, should signify the presence of NSF at axon terminals to provide evidence for in vitro synapse formation. Preparation for data analysis was completed in collaboration with Isabel Goncalves.

MATERIALS AND METHODS:

Dissection and Cell Culture

Dorsal root ganglia and sympathetic nerve chains were dissected from chick embryos following the "Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection" protocol (Morris, 2014d). Dulbecco's Modified Eagle's Medium (DMEM) was used in place of the Hanks Balanced Salt Solution (HBSS) that the protocol calls for.

Positive Control Preparation

The positive control in this experiment consisted of Sea Urchin Embryos, *Lytechinus pictus.* The embryos were mounted onto polylysine treated coverslips via a Pasteur pipette. PBS-T was added to the coverslips to rehydrate the embryos and then they were left in block. Isabel and I each prepared our own, identical positive controls. The embryos were labeled with 0.01mg/ml Hoechst to stain for DNA, DM1A [1:50], which is used to identify alpha tubulin, NSF D31C7 primary antibody [1:100], and rhodamine goat anti-rabbit [1:100] secondary antibody. Labeling for all coverslips was completed by following the protocol starting at step D in "Immunofluor staining of SU embryos – MeOH fixation" (Morris, 2013). The protocol was adapted to reflect the antibodies and concentrations stated above. Each antibody had an incubation period of one hour at room temperature before being rinsed off. Hoechst 33342 was obtained from Moiec Probes, DM1A FITC antibody was obtained from Sigma, NSF (D31C7) rabbit antibody was received as part of an antibody sampler kid of synaptic antibodies from Cell Signaling, and Tetramethyl Rhodamine goat anti-rabbit antibody was obtained from Molec Probes.

Experimental Conditions and Negative Control Condition Preparation

There were two negative controls in this experiment – two coverslips each containing chick neurons – one was fixed via methanol fixation and the other was fixed via formaldehyde/glutaraldehyde fixation. The negative controls were labeled with the secondary antibody rhodamine goat anti-rabbit antibody and treated with block solution throughout the rest of the labeling procedure.

The methanol fixation procedure followed step I of the "Immunofluor staining of SU embryos – MeOH fixation" with some modifications to account for the use of chick neurons instead of SU embryos (Morris, 2013). Step 2 was eliminated and since the cells were on coverslips at this point the re-suspension parts can be ignored. Replace SU embryo with neurons. The Formaldehyde/glutaraldehyde fixation procedure is stated in "Immunofluorescence of Chick Neuronal Primary Cultures" (Morris, 2014c).

Two experimental conditions were prepared for this experiment. The first is a slide consisting of chick neurons that were fixed via formaldehyde/glutaraldehyde fixation and labeled with 0.01mg/ml Hoechst, [1:50] DM1A antibody, [1:50] NSF antibody, and [1:100] Rhodamine goat anti-rabbit antibody. Each antibody was present for an incubation period of one hour at room temperature before being washed off. The Hoechst stain was applied during the last wash, it was left on the coverslip for 5 minutes before being washed off. The second experimental slide was fixed via methanol fixation and labeled with 0.01mg/ml Hoechst, [1:50] DM1A antibody, [1:100] NSF antibody, and [1:100] rhodamine goat anti-rabbit antibody. Each antibody was applied for a period of one hour at room temperature before being washed off. The Hoechst stain was applied during the last wash, it was left on the coverslip for 5 minutes before being washed off.

Mounting Coverslips onto Slides

Slides were labeled with a slide number, their condition, Isabel and Kelsey's initials and the date before coverslips were mounted to them. A drop of block buffer was added to the slide and the coverslip, with its non-cell side wiped down to remove excess block solution, was placed cell side down on top of the block buffer drop. In the case of one of the positive controls coverslips chips were first added to the slide to prevent the embryos from being squished. Once the coverslip was placed on the slide the excess block buffer was wicked away and nail polish was used to seal the coverslip to the slide.

Microscopy and Imaging

Each prepared slide was placed on the stage of a Nikon Eclipse E200 microscope. The microscope was adjusted for Koehler Illumination and imagines were collected using the "Imaging Fluorescence on Gemini and Pisces computers in ICUC" protocol via a Gemini Macintosh computer with an Insight Camera (Morris, 2014b).

Analysis

Image Overlay: 3 Color Overlays were created for each imaged positive control and experimental condition slide. These imagines were also analyzed in Adobe Photoshop to confirm that the locations of DNA, alpha tubulin, and NSF made sense relative to each other. To create these overlays one copied image, renamed with "overlay", was first opened in Adobe Photoshop. Layers and windows were opened and the unwanted channels were deleted so that only the channels dedicated to chosen signals, blue, red, or green, contain the signal. The other images to overlay were also opened in Adobe Photoshop. The desired channel of each image was copied into the corresponding channel of the overlay file so that all three channels were present. The overlay image was observed by selecting "RGB Channel" which selects the red, green, and blue channels all at once.

Scale bars were added to the images using the "Image Analysis for Independent Research 2014" protocol (Morris, 2014a). Each scale bar represents 10 micrometers.

Puncta in the fluorescence of NSF were identified while viewing experimental condition images in ImageJ. Puncta, for the purposes of this experiment, can be defined as bright spots within the NSF fluorescence. The bright spots have a diameter similar to that of the axons and are much smaller than the cell bodies. Puncta can be found anywhere along axons, whether it be where two axons cross or at the ends where axons meet. Six puntca and five puncta were randomly selected in the methanol fixed condition and the formaldehyde/glutaraldehyde fixed condition respectively. Brightness values of the puncta location, thought to represent a synapse, and the axon locations were determined in both the NSF fluorescence and alpha tubulin fluorescence images of each condition using the "Image Analysis for Independent Research 2014" protocol (Morris, 2014a). Brightness values were recorded in a Microsoft Excel file and brightness values were averaged for all puncta regions and axon regions in both fluorescent images for each fixation condition. The ratio of brightness of NSF fluorescence to alpha tubulin fluorescence at puncta locations versus axon locations was calculated for each fixation condition using the average brightness values.

Microsoft Excel was used to create a bar graph representation of the brightness ratios in the methanol fixed condition compared to the brightness ratios of the formaldehyde/glutaraldehyde fixed condition in order to draw conclusions about synapse formation in each condition.

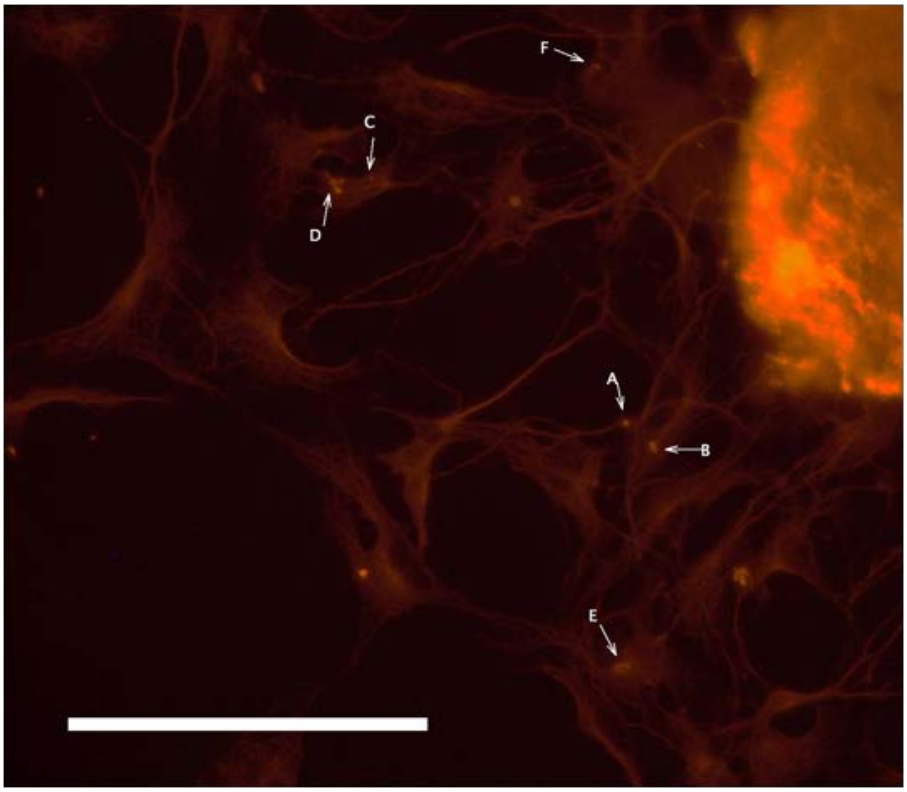
RESULTS:

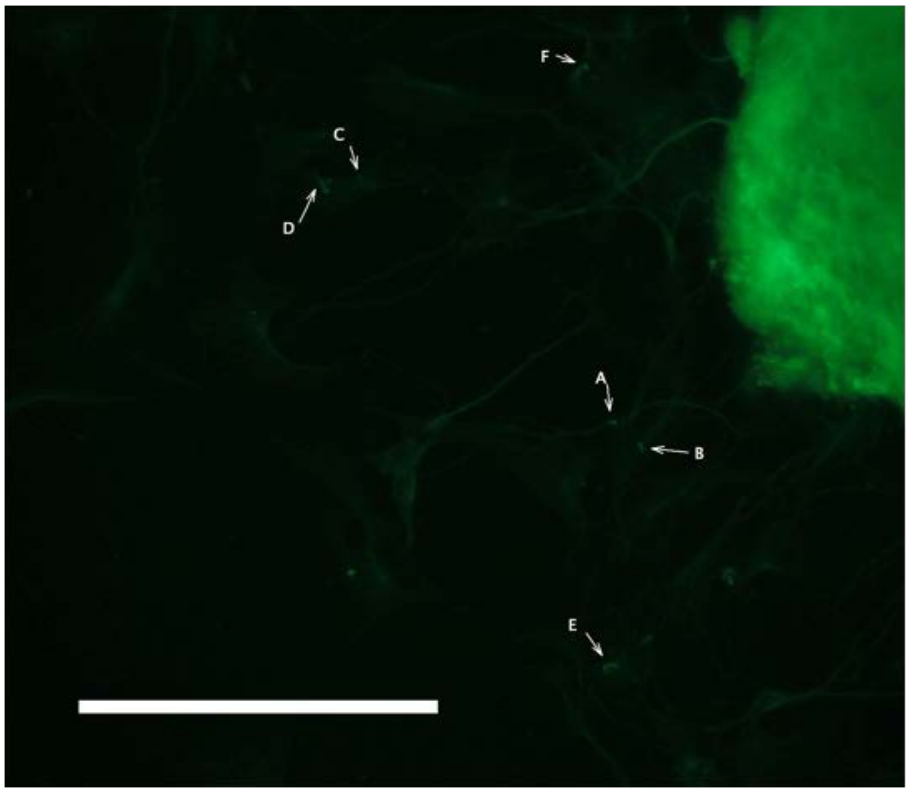
Analysis was conducted on fluorescent light images in Adobe Photoshop and ImageJ. Isabel Goncalves and I obtained all original images, used in figures 1 through 3, together and then analyzed them separately. As depicted in Figure 1, negative controls where secondary antibodies were added but not primary antibodies, show minimal autofluorescence of the rhodamine goat anti-rabbit antibody in both the methanol fixed and formaldehyde/glutaraldehyde fixed condition. All other antibodies and stains used were primary, without their presence the other fluorescent channels did not show any illumination. Only one of the positive control slides had Sea Urchin, *Lytechinus pictus*, present. Observation of the fluorescent images of an embryo showed that Hoechst stain, DM1A antibody, NSF antibody, and rhodamine goat anti-rabbit antibody were working to identify what they are supposed to identify.

1A.  1B. 

Figure 1. Images of the negative controls at 40X under fluorescent light exposure. The neurons in part A. were fixed by methanol fixation while the neurons in part B. were fixed by formaldehyde/glutaraldehyde fixation. Rhodamine goat anti-rabbit antibody was added to each of these slides without the addition of NSF (D31C7) rabbit antibody to check for autofluorescence of rhodamine. Part A. has no fluorescence visible, meaning rhodamine goat anti-rabbit antibody does not auto fluoresce, confirming that anywhere it lit up in the experimental conditions it was bound to the NSF antibody. Part B. has some slight autofluorescence but not enough to discount the brighter fluorescent results obtained in the experimental conditions – rhodamine fluorescence in the experimental conditions was primarily a result of the presence of the NSF antibody.

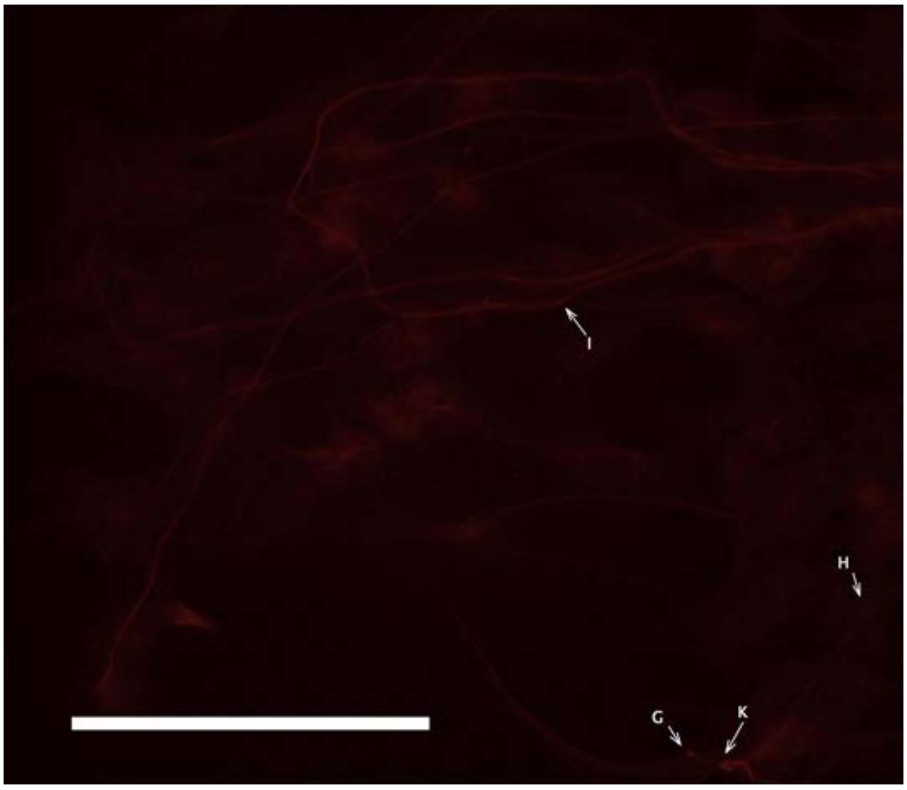
Figure 2 and figure 3 show regions of neurons in the two experimental conditions, methanol fixation and formaldehyde/gluteraldehyde fixation respectfully, and point out the puncta randomly chosen for brightness analysis of all the puncta observed in the images displaying NSF fluorescence.

2A. 

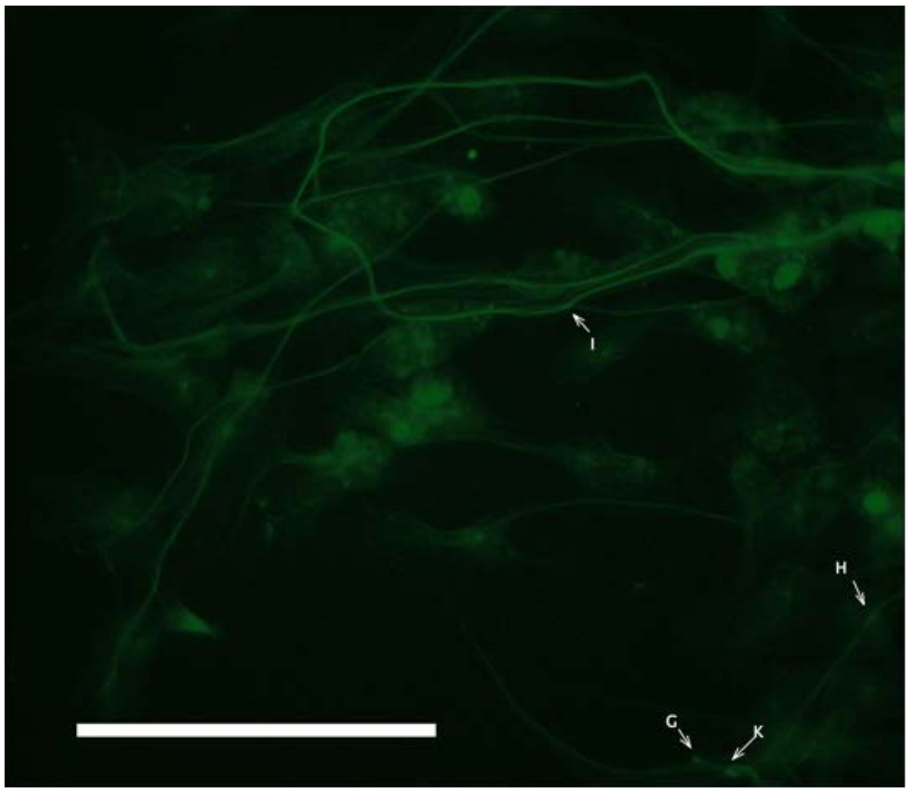


2B.

Figure 2. Images of the MeOH fixed experimental condition with exposure to fluorescent light at 40X. Part A. shows fluorescence of rhodamine goat anti-rabbit antibody, which represents NSF (D31C7) antibody fluorescence of NSF present in the neurons. Part B. shows fluorescence of DM1A antibody, which represents the alpha tubulin present in the neurons. Arrows labeled A through F in part A. point to the puncta where brightness of fluorescence of NSF was measured using ImageJ. The arrows labeled A through F in part B. point to the corresponding spots where brightness of fluorescence for alpha tubulin was measured using ImageJ. The scale bar is 10 micrometers.



3A.



3B.

Figure 3. Images of the formaldehyde/glutaraldehyde fixed experimental condition with exposure to fluorescent light at 40X. Part A. shows the fluorescence of rhodamine goat anti-rabbit, which represents the presence of NSF. Part B. shows the fluorescence of DM1A, which represents the presence of alpha tubulin. Arrows labeled G through K point to the puncta found in part A. that were randomly chosen for brightness analysis using ImageJ. The arrows labeled G through K in part B indicate the same regions that were analyzed in part A. The scale bar is 10 micrometers.

Higher ratios of NSF fluorescence to alpha tubulin fluorescence were measured at puncta regions compared to axonal regions in both fixation conditions (Figure 4). Overall, brightness values of NSF fluorescence and alpha tubulin fluorescence were higher in the methanol fixation condition than in the formaldehyde/glutaraldehyde fixation condition. The difference between the brightness ratios in the formaldehyde/glutaraldehyde fixation condition was 0.235 and the difference between the brightness ratios in the methanol fixation condition was 0.575.

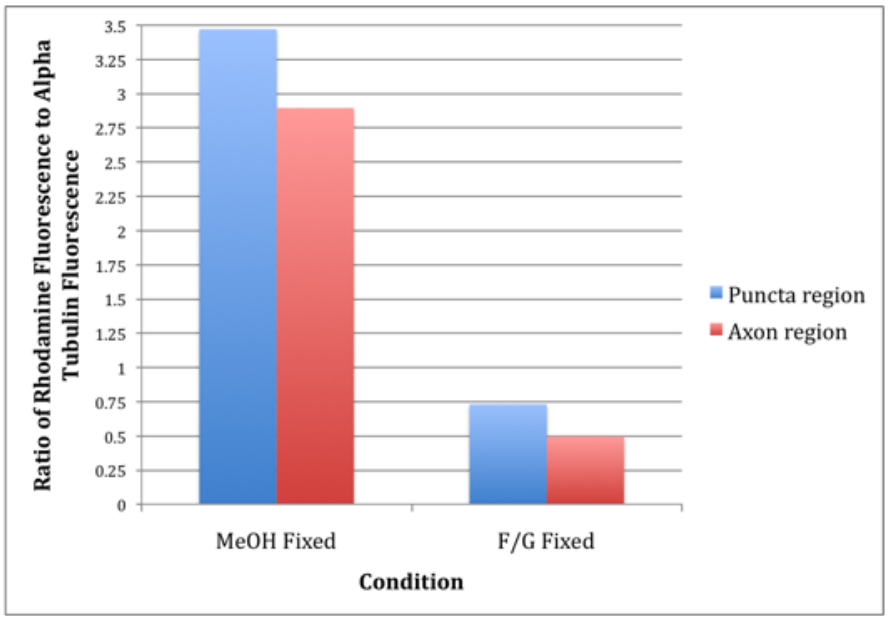


Figure 4. Average ratio of NSF fluorescence to alpha tubulin fluorescence at puncta regions compared to axon regions in both the formaldehyde/glutaraldehyde (F/G) fixed condition and the methanol (MeOH) fixed condition (n=5 in the F/G fixed condition and n=6 in the MeOH fixed condition). In both cases the average ratio of brightness is greater in puncta regions than in axonal regions.

DISCUSSION AND CONCLUSIONS:

The hypothesis that synapses form among peripheral neurons in vitro is supported by the data. Puncta were observed in the images showing NSF fluorescence. These puncta were hypothesized to represent synapses as they signal a greater abundance of NSF present in the designated regions. As depicted in figure 4, the brightness ratios of these puncta regions calculated in NSF fluorescence versus in alpha tubulin fluorescence compared to the brightness ratio of axonal regions in NSF fluorescence versus in alpha tubulin fluorescence were noticeably higher to suggest that there is more NSF present at the puncta than at the non-puncta axonal regions of the neurons. Since NSF is required for membrane fusion in the process of neurotransmitter release from synaptic vesicles, and this membrane fusion occurs at synapses, areas of high NSF abundance, puncta, are likely to be synapses (Whiteheard, Rossnagel, Buhrow, Brunner, Jaenicke, & Rothman, 1994). Though the fluorescent labeling of rhodamine labeled with a higher concentration of NSF antibody, [1:50] versus [1:100], in the formaldehyde/glutaraldehyde fixed condition appeared fuzzier (figure 3) and was measured to be less bright than the brightness of rhodamine on the slide labeled with the less concentrated NSF antibody and fixed in methanol (figure 4), the presence of brighter puncta spots where axons overlapped or met were observed and suggested to represent synapse locations. While the brightness levels were higher with methanol fixation, the same conclusions were drawn from each fixation condition – there is evidence that peripheral chick neurons are able to form synapses in vitro.

In future experiments it would be beneficial to test the effects of more varied antibody concentrations with each fixation method in order to obtain the clearest distinction of puncta within the images. In order to do this the same concentration of all antibodies should be used across both fixation conditions to compare the effects of each fixation method and then varied concentrations of antibodies should be used on two separate sets of slides, one set fixed with methanol and the other fixed with formaldehyde/glutaraldehyde fixation. Keeping the fixation constant while varying concentrations would allow analysis of the direct effects of the concentrations. In this experiment both the fixation method and the concentration of the NSF antibody changed between the experimental conditions, making it difficult to assess the causes of the fuzziness and decreased brightness observed in fluorescent imagine. Keeping the model organism consistent throughout all conditions would also yield stronger evidence. If the positive controls were prepared using chick embryos, instead of sea urchin embryos, it would confirm that the antibodies are specific and actually stain what they are supposed to stain in the chick cells – it is inaccurate to take evidence that the antibodies work in sea urchin embryos and apply it to the analysis of chick embryo cells.

If enough data were collected that all confirmed the presence of puncta in NSF fluorescence when also labeled with NSF antibody it would confirm that NSF proteins are abundantly present in specific regions of the cell. Since these regions appear to be located where axons cross or line up, one could conclude that peripheral neurons form synapses in vitro and that NSF proteins are more concentrated at synapses than anywhere else in the neuron. If slides prepared with methanol fixation were consistently more clear and the brightness values were higher one could also conclude that NSF fluorescence of chick embryo cells should be analyzed using methanol fixation with [1:100] NSF antibody labeling. All of this together means that axons are able to grow, choose a post-synaptic partner, and differentiate a pre-synaptic terminal region where synaptic communication occurs, as evidenced by the abundance of NSF protein working with the synaptic vesicle fusion to release neurotransmitters in vitro (Xia, Zhou, Lin, & Liu, 2000). With evidence that synapses form among peripheral neurons in vitro, further studies could look at the neurotransmitters being released and the patterns of synaptic pathways forming. By studying neurotransmitters and neuron communication in vitro scientists could work toward developing pharmacological treatment for many of the neurodegenerative disorders that do not yet have cures (Cheung, Fu, & Ip, 2006; Sheng et al, 2012).

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