Evidence that the abundance of synapses is negatively correlated with their distance from ganglion

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INTRODUCTION

Why can people form memories, acquire languages and appreciate the beautiful colors of the world? The answer is very complicated, but synaptogenesis of neurons plays a critical and fundamental role in all these daily activities. Synaptogenesis refers to the formation of synapses between neurons in the nervous system (Huttenlocher & Dabholkar, 1997). Previous study shows that spatial long-term memory is related to certain synaptogenesis (Ramírez-Amaya, Balderas, Sandoval, Escobar & Bermúdez-Rattoni, 2001). More specifically, with the synapses formed between neurons of visual area and hippocampus, it forms memory of visual objects (Torasdotter, Metsis, Henriksson, Winblad, & Mohammed, 1998). Considering the significant role of synaptogenesis, it is very important to understand its mechanism and the factors influencing it.

Recent studies consistently point out the significant role of Schwann cells in neuronal synaptogenesis. Feng and Ko suggest (2009) that Schwann cells can promote synaptogenesis by transforming growth factor-beta 1 in neuromuscular junction. Moreover, researchers also found that Schwann cells can promote neurite outgrowth of dorsal root ganglion neurons by secreting nerve growth factor (Hu, Zhou, Li, Wang & Lü, 2011). As Schanwan cells migrate from the dorsal root ganglia (Bentley & Lee, 2000), it is reasonable to expect that the farther be from the ganglion, the longer it takes for Schwann cells to travel. So, fewer Schwann cells may be found. The decrease of the amount of Schwann cells may possibly result in fewer nerve growth factors, without which synapses could hardly form. While this is a rational inference, there is no previous research directly studying on the relationship between the amount of synapses and their distance from ganglion. Therefore, I hypothesize in this study that the abundance of synapses will be greater as closer to the ganglion than father away.

In order to test this hypothesis, I conducted a study on the pattern of synaptogenesis of chick (*Gallus gallus*) embryonic nerve cells from dorsal root ganglion. I chose embryonic chick because of its rapid development, abundant availability and accessibility for visualization and experimental manipulation (Vergara & Canto-Soler, 2012). In this study, I treated chick sympathetic neurons with nerve growth factors. Then, I employed immunofluorescent method to measure the abundance of synapses and their distance toward the ganglion. The abundance of synapses is defined as the amount of synapse per axon fasciculation. The synapse's distance to its correspondent ganglion is standardized by the ratio of the direct distance between synapse and the center of ganglion and the radius of the ganglion. It was observed that there is a negative correlation between the abundance of synapses and their distance from ganglion. This result is congruent with the hypothesis and the inference from previous research. The finding provides additional solid evidence to support Schwann cell's important role in synaptogenesis.

MATERIALS AND METHODS

Materials

FITC-DM1A anti-alpha tubulin, Cat #F2168, was obtained from Sigma Chemical Co. Syntaxin 6(C34B2) Rabbit mAb #2869, Cat #8630, was obtained from Cell Signaling Co. Tetramethyl Rhodamine anti-rabbit IgG, Cay #T-2788 or 2789, was obtained from Molecular Probes Invitrogen.

Cell Culture

Dorsal root ganglion and sympathetic nerve chains of ten day *Gallus gallus* were dissected, dissociated and plated on the four coverslips treated by poly-Lysine, Lamini and growth medium (Morris, 2013a). Sea urchin nerve cells were also put on two coverslips treated in the same way. The coverslips were then put into the C-medium, consisting of Leibovitz L-15 medium, 0.5% methylcellulose, 10% fetal calf serum, 0.6% glucose, 2 mM L-glutamine, 100ug/ml streptomycin, 100U/ml penicillin, and 10-50 ng/ml NGF, to incubate at 37°C for 24 hours (Morris, 2013a).

Microtubule and Synapse staining

Two sea urchin coverslips were used as positive control. Two out of four ganglion coverslips were used as negative control. And the remaining two coverslips were employed as experiment (Morris, 2013b).

One negative control, one positive control and one experiment coverslip were fixed with formaldehyde and gluteraldehyde of 2% formaldehyde, 0.1% gluteraldehyde, 0.12M sucrose, 0.5% TX-100 and 2mM EGTA. The remaining three coverslips were fixed with methanol of 5mM EGTA in 50ml ice-cold methanol. Afterwards, the coverslips were washed with PBS-T (PBS with 0.05% TX-100) followed by a 7-day incubation at 4-6 °C in block buffer (3% BSA and 1:10 Sodium azide).

Each coverslip of positive control and experiment was treated with 200 µL of 1:100 FITC-DM1A to stain alpha tubulin, a subunit of microtubule of neuronal axons, in green. Each coverslip of positive control and experiment was treated with 200 µL of 1:100 Syntax to label synapses as primary antibody. Then, each of the six coverslips was treated with 200 µL of 1:100 Tetramethyl Rhodamine to stain syntax in red. Finally, each coverslip of the six coverslips was treated with 200 µL of 1:100 Hoechst to stain the DNA of nerve cells.

An observation chamber for each coverslip was made using ProGold Anti-Fade Agent as the medium and Revlon Cherries in the Snow as the sealant and stored at 4-6°C in the dark for 24 hours (Morris, 2012)

Measurement of Abundance of Synapses and Distance from Ganglion

Individual synapse was selected to perform analysis. A synapse was defined as only stained in red and located right next to at least one axon. Also the synapse's diameter can be no larger than that of its corresponding axon.

All fluorescent images of the microtubules and synapses were gathered on a Mac OS X Version 10.5.8 using a Nikon Eclipse E200 Epi-fluorescence microscope with a Nikon Plan Fluor 10x or Plan Apo 40x (with Nikon Type A immersion oil) objective, diagnostic instruments

0.76x HRD076-NIK c-mount, and SPOT RT3 camera (25.4 2 Mp Slider), using SPOT version 4.6.1.41.

The fluorescent images of microtubules and synapses were overlaid using Adobe Photoshop CS2 Version 9.0.2. Overlay images were used to identify the location of the individual synapses. Because many synapses locates inside of ganglion and the axons are hard to trace, Freehand Lines tool was used to determine the straight distance between the center of ganglion and the synapses. The length was measured in pixels in ImageJ 1.40g. Then length in pixels was later converted to micrometers. Considering the difference in the radius of ganglion, the ratio of synapse's distance to the center of ganglion and the radius of the ganglion was calculated to standardize the distance of synapses from its correspondent ganglion.

The florescent images of synapses and microtubules were analyzed with ImageJ respectively to select possible synapses and measure the average brightness of the synapses. Then, the brightness of the background of florescent images of synapses was measured and subtracted from the average brightness of synapses and axons. Afterwards, the ratio of newly obtained brightness of synapses and axons was calculated to represent the abundance of synapses. The abundance of synapses and the distance from ganglion was then drawn with MS Excel in a scatter plot with linear regression equation to find out their relationship.

RESULT

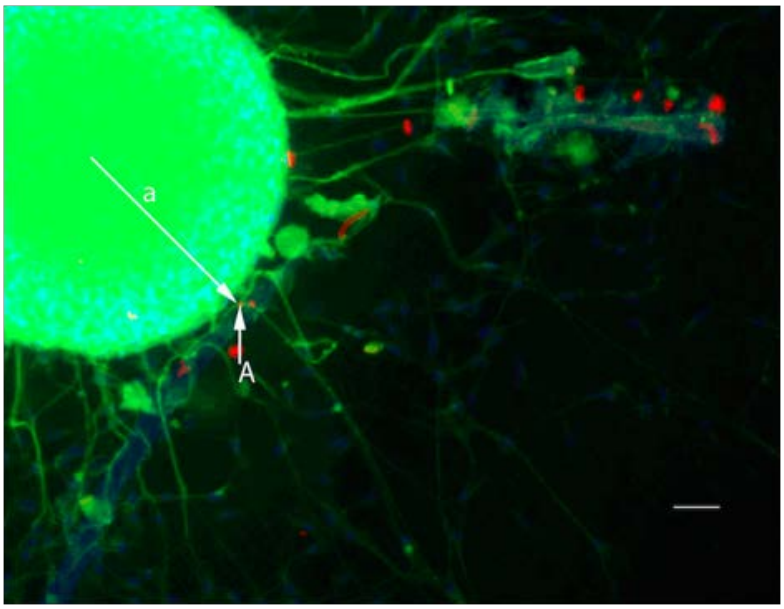

Figure 1. Dorsal root ganglion neurons on the experiment coverslip fixed with formaldehyde and gluteraldehyde and labeled first with Syntaxin illuminated by Tetramethyl Rhodamine (red), FITC-DM1A (green) and Hoechst (blue) taken with a Plan Apo 10X objective with Nikon Type A immersion oil. Overlaid by ImageJ. Label A indicates the synapse measured. Line a demonstrates the distance between synapse A and the center of the ganglion. Bar, 10 micrometer.

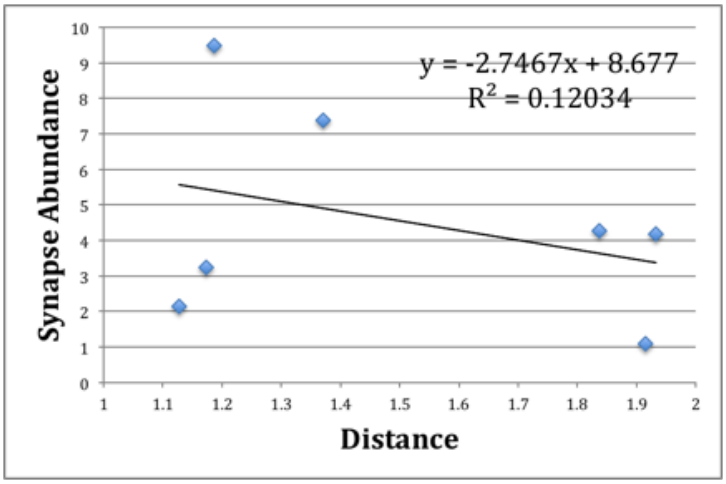

Figure 2. Synapse abundance was plotted according to their distance to ganglion. It shows that the synapse abundance is negatively correlated with their distance to ganglion. Synapse abundance was calculated by the ratio of the brightness of synapses and the corresponding axons. The distance to ganglion was obtained by the ratio of the synapses' direct distance to the center of its corresponding ganglion and the radius of correspondent ganglion. Seven synapses from six ganglions were analyzed. The linear regression line was drawn. The linear regression equation and the regression coefficient were presented.

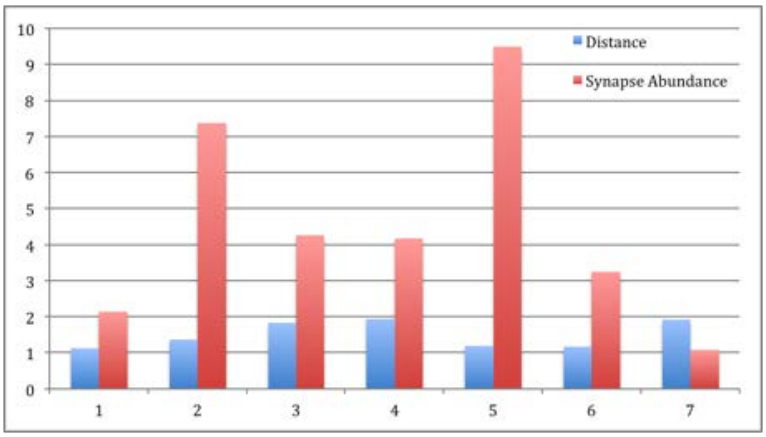

Figure 3. The distance and synapse abundance of seven synapses are presented in column chart, which shows the pattern of the synapse formation that all the synapses measured are formed within two ganglion-radius distance. The synapse abundance is determined by the brightness of synapse and the brightness of its corresponding axon. The distance to ganglion was obtained by the ratio of the synapses' direct distance to the center of its corresponding ganglion and the radius of correspondent ganglion.

Only blue fluorescence was observed and neither red nor green was observed in the negative control. It indicates that the red and green fluorescence gathered in the images is from stained synapses and microtubules. The average brightness of analyzed synapses and microtubules are significantly larger than their background. The data are not provided.

Figure 1 shows that axons are green and process from ganglion. Synapses are selected according to its definition of red stain, close to axon and small diameter. Label A points out the data point 1, which is the synapse measured in the second neuron found in the coverslip fixed with formaldehyde and gluteraldehyde. Line a shows the direct distance from the center of the ganglion to the synapse.

Figure 2 displays the distance between selected synapses and the center of their correspondent ganglion as x-axis. The synapse abundance calculated from the ratio of the brightness of synapses and the brightness of its correspondent microtubules is the y-axis. As the distance increases, the synapse abundance decreases accordingly, verified by the negative slop of the linear regression equation.

An obvious pattern of synapse formation is observed in Figure 3. All the synapses measured are found between one and two ganglion-radius. The first three synapses were fixed in formaldehyde and gluteraldehyde, while the other four synapses were fixed in methanol. The column graph indicates that the fix method does not play a significant role in the measurement of synapse abundance.

DISCUSSION

The data obtained from this study supports the hypothesis that the abundance of synapses is negatively correlated to their distance from ganglion. Demonstrating by the negative slope of linear regression equation, the farther is the synapse from the ganglion, the less is synapse abundance. These data is congruent with the previous study about Schwann cell's migration from ganglion (Bentley & Lee, 2000). Because Schwann cells are mostly gathered in ganglion, the father is from the ganglion, the fewer Schwann cells can be found (Kandel, Schwartz & Jessel, 2000). As Schwann cells provides neural growth factors to promote synaptogenesis (Feng & Ko, 2009), the decreasing amount of Schwann cells will lead to the reduction of neural growth factors, leading to fewer synapses formed. Although the regression coefficient is only 0.12034, indicating the correlation found between synapse abundance and its distance from ganglion is not significant, it is may be caused by the small data samples. A follow-up experiment with more neurons cultured and synapses measured should be performed to gain more power for this result.

Moreover, according to the discovery that all seven synapses I have measured are within two ganglion-radius range, it shows a primitive synaptogenesis pattern that for one week vitro culture, synapses tend to form within two ganglion-radius range. It indicates that most Schwann cells are found within two ganglion-radius. This result is coherent with previous studies that most Schwann cells are found in ganglion (Kandel, Schwartz & Jessel, 2000). The data also indicates that there may exist a significant decrease in the amount of Schwann cells after two ganglion-radius. However, considering the extreme small sample, more data should be collected to confirm this assumption.

During the whole experiment, I should not have stained the negative control with Hoechst, because I am also interested to see if it is Hoechst that stains DNA in blue instead of other factors.

Since all the molecular explanation behind the correlation between the synapse abundance and its distance to the ganglion is related to the abundance of Schwann cells, future studies should focus on the examination of the location, concentration and spread pattern of Schwann cells. Therefore, we could develop a more direct understanding of the mechanism of synaptogenesis.

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