Ectodomain Shedding in Neurodevelopment

***Rationale and Hypothesis***

Synaptic plasticity is a fundamental process of nervous system development. Proper synaptic plasticity is necessary for functional connectivity of the nervous system. Aberrant synapse formation leads to numerous neurodevelopmental and neurodegenerative disorders, such as Down syndrome and Alzheimer’s disease, respectively. It is known that various cell adhesion molecules, including neuroligin, neurexin, ephrin, and Eph receptor mediate synaptic plasticity throughout neurodevelopment. One group of cell adhesion molecules described in literature involved in synaptic plasticity is the immunoglobulin superfamily of cell adhesion molecules1. Within said superfamily, proteins of the IgLON subfamily have been demonstrated as important regulators of cell adhesion, subdomain target recognition, and neurite outgrowth1. The IgLON subfamily consists of Neurotrimin (NTM), Opioid-binding cell adhesion molecule (OBCAM), Limbic system associated membrane protein (LSAMP), and Neuronal growth regulator 1 (NEGR1)2. The implication of IgLON proteins in synaptogenesis has been suggested in literature. Through immunohistochemistry and electron microscopy, OBCAM and NEGR1 have been shown to localize with vesicle-associated membrane protein 2, a synaptic marker3. In addition, IgLON proteins also play a role in neurological disorders that may have a basis in synaptic dysfunction. LSAMP and NEGR1 have been reported to be involved in major depressive disorder and autism spectrum disorder, respectively4, 5. Thus, it would be of interest to systematically investigate the role of IgLON members in synaptogenesis and synaptic plasticity.

It was also demonstrated by our lab that IgLON proteins undergo cell surface proteolysis through a post-translational mechanism known as ectodomain shedding2. Members of the metzincin family of metalloproteinases, including matrix metalloproteinases (MMPs) and adamalysins (ADAMs), mediate this shedding process2. Components shed by MMPs and ADAMs are released as biologically active or dominant negative fragments. Consequently, ectodomain shedding affects myriad neuronal processes, such as cell survival, axon outgrowth and guidance, and synaptogenesis. Out lab has shown in rat dorsal root ganglion cells that ectodomain shedding of LSAMP by ADAM promotes neurite outgrowth6. Furthermore, our research demonstrated that the observed neurite outgrowth was due to the shedding relieving the outgrowth inhibitory signal from LSAMP at the cell surface6. However, it is unknown whether the shedding of individual IgLON proteins affect synapse formation. Considering the effect of IgLON ectodomain shedding on promoting neurite outgrowth, it is then reasonable to hypothesize here that IgLON ectodomain shedding plays a positive role in facilitating other neurodevelopmental processes, such as synapse formation and plasticity.

***Objectives***

The short-term goal is to determine the role of metalloproteinase mediated shedding of IgLON proteins in synaptogenesis. Subsequently, the long-term objective of research herein is to elucidate the molecular mechanisms by which shedding of IgLON proteins regulate the formation and plasticity of synapses.

***Methods and Analyses***

A rat model will be adopted to study IgLON members, including NTM, OBCAM, LSAMP, and NEGR1. First, the expression of IgLON proteins at excitatory and inhibitory synapses will be investigated by immunocytochemistry. Cortical cultures will be stained using antibodies against specific IgLON proteins and antibodies against either excitatory or inhibitory synapses. The cultures will be imaged using confocal microscopy. These experiments will define the repertoire of IgLON members at excitatory and inhibitory synapses.

Gain and loss of function approaches will be applied to determine the role of IgLONs in synapse formation. The direct effect of IgLONs on promoting synapse formation will be evaluated by co-culturing cortical neurons with fibroblasts expressing specific IgLONs. Next, the effect of IgLON overexpression on synapse formation between cortical neurons will be examined in cortical cultures transduced with individual IgLONs. Then, the necessity of IgLONs for excitatory and inhibitory synapses will be examined using shRNAmirs to knockdown individual IgLONs in cortical neurons. In vivo role of IgLONs in synaptogenesis will be investigated using in utero electroporation of shRNAmirs for specific IgLONs.

Cortical neurons transduced with either wild-type or artificially cleavable IgLON constructs for individual IgLON members will be used to study the role of IgLON shedding in synapse formation. These constructs will be resistant to the shRNAmirs. Hence, the wild-type construct should rescue neurons from shRNAmir knockdown while the artificially cleavable constructs should lead to changes in synapse formation compared to the wild-type. In parallel, metalloproteinase inhibitors will be used to investigate the importance of IgLON as a substrate for metalloproteinase-dependent effects on synaptogenesis. If effects are metalloproteinase-dependent, then treatment with inhibitors should suppress effects due to shRNAmir knockdown.

***Feasibility***

The methods and antibodies that will be used throughout this research have all been validated. Using neuroligin transfected COS-7 cells as positive control has been established for co-culture experiments. Production of lentivirus with either knockdown or overexpression vector has been set up. Generation of artificially cleavable immunoglobulins has been done previously in our lab using thrombin cleavage site. The aforementioned in vivo procedure has been practiced previously in collaboration with Dr. Keith Murai. One major challenge of said in vivo experiment is the analysis of synaptogenesis. In literature, dendritic spine density is often used as a quantification of synaptogenesis. However, the accurate and precise identification of individual spines is difficult as different software and packages lead to dissimilar measurement of spines.

In the previous collaboration with Dr. Keith Murai, the objective was to examine the role of matrix metalloproteinase-dependent Nogo-66 receptor ectodomain shedding in synapse formation. One experiment that determined the dependency of the effect on membrane-type-3 matrix metalloproteinase (MT3-MMP) involved examining dendritic spine density in the cerebral cortex of control animals compared to MT3-MMP-knockdown animals. The control animals were in utero electroporated with a control vector, whereas the MT3-MMP-knockdown animals were in utero electroporated with a MT3-MMP shRNAmir vector that knocks down MT3-MMP expression. Animals were perfused and brains were cryosectioned. Images were taken as stick figure files using spinning disk microscopy.

The proper analysis of dendritic spines required a program to visualize the images. Therefore, Imaris (a microscopy image analysis software developed by Bitplane) and Fiji (a image processing package based on ImageJ) were assessed for this purpose. Figure 1 and 2 below show image of control and shMT3 neuron, respectively, opened in Fiji. Figure 3 and 4 below show image of the identical control and shMT3 neuron, respectively, opened in Imaris. In figures 3 and 4, the protrusions labeled in blue, which were manually selected, represent spines from the dendritic branch of interest. The neuron in Fiji is a 2D image constructed by projection of the original stick figure file onto a single plane. In contrast, the neuron in Imaris is a 3D image reconstruction of the original stick figure file.

Figure 5 and 6 show quantification of dendritic spine density of control compared to shMT3 neurons using Fiji and Imaris, respectively. Interestingly, quantification using Imaris, but not Fiji, led to significant result that corroborated the hypothesis and other findings of the project. Since spines were manually selected for both the analysis performed using Fiji and Imaris, it is reasonable to speculate that the difference between the two quantifications was due to how the images were constructed and interpreted. Unlike the 3D images from Imaris, the 2D images from Fiji exclude the spines that overlap or protrude in and out of the 2D plane when the stick figure files are projected onto a single plane. This was demonstrated as the spine density of both control and shMT3 neurons decreased when images were examined using Fiji compared to Imaris. Said effect was more severe in control compared to shMT3 neurons because the total number of spines is greater in control neurons. There is the option of analyzing each stick figure file using Fiji plane by plane. However, this method considerably reduces the contrast between signal and noise, and therefore, was not implemented here. It is also important to note that the length of the dendrite of interest is slightly decreased when a file is projected onto a 2D plane. This would theoretically increase the spine density computed using Fiji, as spine density is a function of number of spines divided by length of dendrite. However, perhaps this effect was negligible here. Due to the abovementioned challenges regarding dendritic spine measurement using Fiji, the proposal here is to first use Imaris to generate a 3D reconstruction of the dendrites and spines and then compute the spine density as a quantification of synaptogenesis.

***Contributions***

A number of neurodevelopmental and neurodegenerative disorders are associated with aberrant synaptogenesis. Therefore, elucidating molecular mechanisms underlying synapse formation and plasticity leads to potential therapeutic interventions for these disorders. The translational aspect of this research is further strengthened due to the fact that the IgLONs studied here have been identified in both rodents and humans.

***References***

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***Figure 1 – Fiji image of control neuron***



***Figure 2 – Fiji image of shMT3 neuron***



***Figure 3 – Imaris image of control neuron***



***Figure 4 – Imaris image of shMT3 neuron***



***Figure 5 – Quantification of spine density of control versus shMT3 using Fiji***

***Figure 6 – Quantification of spine density of control versus shMT3 using Imaris***

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