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The Research Grants Council of Hong Kong
NSFC/RGC Joint Research Scheme
Joint Completion Report

(Please attach a copy of the completion report submitted to the NSFC by the Mainland researcher)

Part A: The Project and Investigator(s)

1. Project Title

Functional and in vivo study of the Neuroligin and Itch Interaction

2. Investigator(s) and Academic Department/Units Involved

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Prof. Jun Xia	Prof. Jianhong Luo
Post	Professor	Professor
Unit / Department / Institution	LIFS/HKUST	Medicine/Zhejiang University
Contact Information	jxia@ust.hk	luojianhong@zju.edu.cn
Co-investigator(s) *(with title and institution)*		Junyu Xu (Associate Professor)

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted*)
Project Start date	2016-1-1		
Project Completion date	2019-12-31		
Duration *(in month)*	48		
Deadline for Submission of Completion Report	2020-12-31		

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. *Determine the site of Itch-neuroligin-2 interaction and investigate how this interaction regulates neuroligins' function in synaptogenesis using biochemical and cell biology approaches.*

2. *Generate brain-specific Itch knockout mice and conduct biochemical and cell biology analysis on their synapse formation.*

3. *Conduct electrophysiology study to investigate the function of Itch in synapse formation and function.*

4. *Perform behavioral analysis of the Itch knockout mice to examine whether they have behavioral defects and what those defects are.*

5.2 Revised Objectives

Date of approval from the RGC: _____

Reasons for the change: _____
_____

1.
2.
3. ....

6. Research Outcome

Major findings and research outcome
(maximum 1 page; please make reference to Part C where necessary)

We identified ITCH as a neuroligin2-binding protein. ITCH is a E3 ubiquitin ligase with three distinct domains: N-terminus C2 domain, middle tandem WW domains (four WW domains), and C-terminus HECT domain. We found that the WW domains but not the other domains interact with neuroligin2. It has been reported that WW domain binds to PY motif. One PY motif was found on the intracellular sequence of neuroligin2 and sequence alignment revealed that other neuroligin

proteins, including neuroligin1 and neuroligin3, also possess the same PY motif. Immunoprecipitation experiment showed that all the neuroligin proteins bound to ITCH and mutation of the PY motif abolished ITCH-neuroligin2 interactions.

ITCH is one family member of NEDD4 family of E3 ubiqutin ligase, which all contains WW domains. This prompted us to test interactions of neuroligin2 to all NEDD4 family proteins. The results showed that only three members of NEDD4 family interacted to neruoligin2, including ITCH, WWP1, and WWP2. Interestingly, we found that only ITCH but not the other NEDD4 family members is the functional E3 ligase of neuroligin2, as our antibody-feeding endocytosis assay and surface biotinylation experiments revealed that only ITCH could induce endocytosis and surface reduction of neurolgin2 and the other NEDD4 family members failed to do so. This prompted us to investigate why ITCH specifically induces endocytosis of neuroligin2 given ITCH and the other NEDD4 family members have the same domain arrangement. First, point mutations of ITCH C830A and neuroligin2 K749R, which inactivates ITCH and abolished ubiquitination site on neuroligin2 respectively, prevented the endocytosis of neuroligin2. These results indicate ubiquitination is required for neuroligin2 endocytosis, which is consistent with ubiquitination's role in endocytosis. Unexpectedly, mutations of ITCH and neuroligin2, which disrupt WW-domain-mediated interaction between ITCH and neuorligin2, inhibited endocytosis of neuroligin2. Moreover, disruption of ITCH-neurolgin2 interaction did not weaken ubiquitination of neuroligin2, suggesting that the ITCH-neuroligin2 interaction independently regulates endocytosis of neuroligin2. In summary, we found that both ubiquitination and interaction are required for ITCH-induced endocytosis of neuroligin2.

Cultured rat hippocampal neurons were used to investigate function of ITCH to neuroligin2-positive-synapse. Neuroligin2 is a cell adhesion protein exclusively localizes on GABAergic inhibitory post-synaptic sites. Quantitative immunofluorescent showed that expression of ITCH in hippocampal neurons decreased size, density and intensity of neuroligin2-positive inhibitory neuron, while inhibitory pre-synapses were not affected. On the other hand, excitatory synapses were unaffected by ITCH expression. ITCH shRNA was generated to knock down ITCH in cultured hippocampal neurons. In contrast to ITCH expression, knock-down of ITCH increased the size and intensity of neuroligin2-positive inhibitory post-synapse. In collaboration with Prof. Luo's group, we also measured mIPSC (miniature inhibitory postsynaptic current) in cultured neuron with expression or knock-down of ITCH. Consistent with staining results, frequency and amplitude of mIPSC of cultured neuron was decrease upon expression of ITCH, while knock-down of ITCH increased the frequency and amplitude.

We measure mIPSC in cultured neurons after itch overexpression or RNAi-mediated itch knockdown to investigate whether altered levels of ITCH and morphological changes of synaptic was associated with altered neural transmission. We found that the mIPSC frequency and amplitude was decreased in neurons overexpressing ITCH, which can be reversed by expressing the mutant itch-C830a. Meanwhile, the mIPSC and amplitude was increased when we knockdown ITCH. Combined with previous results, this result indicates that Itch can negatively regulate GABAergic transmission via mediating NL2 membrane expression.

To investigate if Itch regulated Neuroligin2 expression plays any role in behavioral level. We bilaterally injected ITCH-shRNA-control or ITCH-shRNA into

the BLA of wildtype mice. Three weeks later, we examined the anxiety related behaviors and fear memory of the mice. We found that knockdown of Itch in the BLA of wild type mice does not have significant effect in the anxiety behavior, but it impaired contextual memory formation.

Mainland visit paid by Jun Xia from Hong Kong team
Dates: Jun 8, 2016-Jun 12, 2016
duration and purpose of visit: 4 days, for project discussion
activities carried out: meeting to discuss data and plan for experiments.

Hong Kong visit paid by Junyu XU from mainland team:
Dates: 23rd May, 2016-24th May, 2016
duration and purpose of visit: 2 days, for project discussion
activities carried out: progress meeting was carried out between the Hong Kong research group and Zhejiang University research group. Mice transfer schedule was determined and experimental results were exchanged and discussed. Research plan modifications were made based on the results obtained and progresses made.

Potential for further development of the research and the proposed course of action *(maximum half a page)*

Our study found that Itch is a E3 ubiquitin ligase that could ubiquitinate neuroligin2 and regulate synapse formation. Further investigation on how this ubiquitination of neuroligin 2 could be removed to keep a balance of ubiquitination and de-ubiquitination will provide insight to fully understand the dynamics of synapse formation.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Human brain is made of billions of neurons. Neurons communicate with each other via specialized structures called synapse. Synapses are important for neuronal communication and information processing in the brain. Formation of synapses, or synaptogenesis, ensures the proper wiring of neuronal circuitry in the brain. Abnormal synaptogenesis is believed to be the underlying cause of brain disorders such as autism and mental retardation. The process of synapse formation is tightly controlled, but the molecular mechanism underlying this process is not fully understood. In this study, we investigate the roles of two molecules, neuroligin and Itch, to elucidate their functions in synapse formation. We found that Itch add a small protein called ubiquitin to neuroligin. This process then removes neuroligin from the synapses. With less neuroligin on the synapse, the number of synapses is reduced. As synapses are important for our brain function, reducing Itch in the brain changes synapses number and behavior. This study will eventually help us understand the molecular mechanism of synaptogenesis and provide molecular basis to learn how abnormal synaptogenesis contribute to human diseases such as autism.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising <u>directly</u> from this research project

(Please attach a copy of each publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(**bold** the authors belonging to the project teams and denote the corresponding author with an asterisk*)*	Title and Journal/ Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Year of publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*						
J1			2020	Hong-lei Tian, Yong-lan Du, Chuen Kam, Zheng Rui, Tai-lin Liao, **Junyu Xu, Jian-hong Luo, Jun Xia***	Ubiquitination of Neuroligin-2 by ITCH Promotes its Endocytosis and Inhibits Synaptogenesis. submitted	No	No	Yes	No
2018 J2				He J, Xia M, Yeung PKK, Li J, Li, Z, Chung KK, Chung SK, **Xia J***	PICK1 inhibits the E3 ubiquitin ligase activity of Parkin and reduces its neuronal protective effect. <u>*PNAS*</u> 115(30):E7193-E7201	2020	Yes	Yes	Yes
2018 J3				Wu M, Tian HL, Liu X, Lai JHC, Du S, **Xia J***	Impairments of Inhibitory Synapse Formation and Motor Behaviors in Mice Lacking the NL2 Binding Partner LHFPL4/GARLH 4. <u>*Cell Reports*</u> 23:1691-1705.	2020	Yes	Yes	Yes
2018 J4				Wei Cao, Shen Lin, Qiang-qiang Xia, Yong-lan Du, Qian Yang, Meng-ying Zhang, Yi-qing Lu, Shu-min Duan, Guoping Feng, **Xia Jun, Junyu Xu*, and Jian-hong Luo***	Gamma Oscillation Dysfunction in mPFC Leads to Social Deficit in Neuroligin 3 R451C Knock-in Mice. <u>*Neuron*</u>, 97(6):1253-1260	2020	Yes	Yes	Yes

2016 J5				**Junyu Xu,** Na Wang, **Jian-hong Luo and Jun Xia***	Syntabulin regulates the trafficking of PICK1-containing vesicles in neurons. *Sci Rep.* 6:20924	Dec, 2017	No	Yes	

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered

(Please attach a copy of each delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)

Month/Year/ Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
11/2018 C1	Impairment of inhibitory synapse formation and motor behavior in mice lacking the NL2 binding partner LHFPL4/GARLH4	Society of Neuroscience annual conference 2018	2020	Yes	Yes	No

10. Student(s) trained

(Please attach a copy of the title page of the thesis.)

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Min Wu S1	PhD in Life Science	2014-9	2018-6

11. Other impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)

N/A

12. Statistics on Research Outputs

(Please ensure the summary statistics below are consistent with the information presented in other parts of this report.)

	Peer-reviewed journal publications	Conference papers	Scholarly books, monographs and chapters	Patents awarded	Other research outputs (Please specify)
No. of outputs arising directly from this research project [or conference]	5	1	0	0	0