Local regulation and function of importin- β 1 in hippocampal neurons during transcription-dependent plasticity

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**LOCAL REGULATION AND FUNCTION OF IMPORTIN- β 1
IN HIPPOCAMPAL NEURONS DURING TRANSCRIPTION-DEPENDENT
PLASTICITY**

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**Interdisciplinary Graduate School
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**Interdisciplinary Graduate School
NTU Institute for Health Technologies @ Systems Biology and Medicine**

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Lee Yan Jun

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Ch'ng Toh Hean

Authorship Attribution Statement

This thesis contains material from one paper deposited in the BioRxiv database where I was the first author.

All materials, except Chapter 1.3, Adducin-1 data in **Fig. 3-11**, and Chapter 5, are deposited in Lee et al. (2020).

The contributions of the co-authors are as follows:

Dr. Sheeja Navakkode conducted and analysed the slice electrophysiology data. I have directed and conceptualised the experiments, including outlining the protocols necessary to obtain the results.

Tan Chee Fan prepared the samples for tandem mass spectrometry sequencing from the purified synaptosomes co-IP assays performed by me.

A/Prof Newman Sze Siu Kwan analysed the raw tandem mass spectrometry sequencing data and provided the emPAI values for the proteins identified. The subsequent analyses in shortlisting the candidate proteins were conducted by me.

Prof. Sreedharan Sajikumar and Newman Sze Siu Kwan edited the manuscript drafts and provided supervision.

14 Jan 2021

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Lee Yan Jun

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Summary

Activity-dependent transcription is critical for the encoding of long-term memories. Regulated nuclear entry of soluble proteins is one method to relay synaptic signals to the nucleus to couple neuronal excitation with transcription. To date, the role of importin- β 1 in nuclear shuttling of proteins during activity-dependent transcription has always been inferred but not directly investigated. In this study, activity-dependent nuclear accumulation of importin- β 1 from the soma and the synapto-dendritic compartments has been demonstrated. Importantly, inhibition of importin- β 1 mediated nuclear import during synaptic stimulation impairs long-term plasticity. I also show evidence that importin- β 1 mRNA-ribosome complex is distributed throughout the synapto-dendritic compartment and synaptic stimulation induces importin- β 1 local protein synthesis. Finally, several candidate proteins that associate with importin- β 1 at the synapse have been identified from which N-Myc Downstream Regulated 1 (NDRG1) is characterized as an importin- β 1 interactor that undergoes activity-dependent translocation into the nucleus. Collectively, these results highlight the crucial role of importin- β 1 in nuclear import of proteins during long-term plasticity.

Abbreviations

4AP	4-Aminopyridine
aCSF	Artificial cerebrospinal fluid
ActD	Actinomycin D
Add1	Adducin-1
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP-1	Activator protein 1
APV	(2R)-amino-5-phosphonovaleric acid
Arc	Activity-regulated cytoskeleton-associated protein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
Bic	Bicuculline
CamK	Calmodulin-dependent protein kinase
CHX	Cycloheximide
cNLS	Classical nuclear localization signal
CREB	cAMP-response element binding protein
CRTC1	CREB Regulated Transcription Coactivator 1
CTNNB1	B-catenin

DIV	Days <i>in-vitro</i>
DHPG	(RS)-3,5-Dihydroxyphenylglycine
EGR4	Early growth response protein 4
emPAI	Exponentially modified Protein Abundance Index
EPSC	Excitatory postsynaptic current
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma aminobutyric acid
GFP	Green fluorescent protein
Gly	Glycine
GO	Gene Ontology
HPRT1	Hypoxanthine phosphoribosyltransferase
IB	Immunoblotting
IBB	Imp- β 1 binding
ICC	Immunocytochemistry
IgG	Immunoglobulin G
Imp	Importin

INI43	Inhibitor of Nuclear Import-43
IP	Immunoprecipitation
IP3	Inositol 1, 4, 5-triphosphate
IPO7	Importin-7
KCl	Potassium Chloride
LMB	Leptomycin B
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MAP	Microtubule-associated proteins
mGluRs	Metabotropic Glutamate Receptors
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NDRG1	N-Myc Downstream Regulated 1
NEM	N-ethylmaleimide
NF- κ B	Nuclear factor kappa B
NFAT	Nuclear factor of activated T-cells
NLS	Nuclear localization signal
NP40	Nonidet P-40

NPAS4	Neuronal PAS domain protein 4
NMDAR	N-Methyl-d-aspartic acid receptor
NPC	Nuclear pore complex
ORF	Open reading frame
PBS	Phosphate buffered saline solution
PDPK1	3-Phosphoinositide Dependent Protein Kinase 1
pERK	Phosphorylated Extracellular Signal- Regulated Kinase
PFA	Paraformaldehyde
PKA	Protein Kinase A
PKC	Protein Kinase C
PolyA	Polyadenylation
PSD	Post-synaptic density
Puro-PLA	Puromycin-Proximity Ligation Assay
qPCR	Quantitative Polymerase Chain Reaction
SLFS	Strong low frequency stimulation
STET	Strong tetanization
STM	Short-term memory
SYPH	Synaptophysin

TX-100	Triton-X 100
TTX	Tetrodotoxin
UTR	Untranslated region
VGCC	Voltage-gated calcium channel

Chapter 1. Introduction

1.1 Long-term memory

Memory, the capacity of encoding, storing, and retrieving information, is formed through three stages; the sensory memory, the short-term memory (STM), and the long-term memory (LTM) (Camina & Güell, 2017). The newly learned information is first stored as sensory memory. This form of memory allows huge amount of information gathered from sight (known as iconic memory), audio (echoic memory), or touch (haptic memory) to be retained briefly, approximately 500 to 800 milliseconds in the case of iconic memory in young adults (Lu, 2012). These sensory registers are then converted into STM, which is labile and last for minutes to hours. According to the widely accepted Baddeley's and Hitch's structural model, the STM functions as a working memory, where there is not only a temporary retention but also processing of information with limited capacity (Baddeley, 2010). In this model, working memory consists of four subcomponents, namely the central executive responsible for controlling attention, the visuospatial sketchpad for storing and processing visual and spatial information, the phonological loop for language acquisition, and the episodic buffer for integrating information acquired from other subcomponents across time or space into a temporal representation (Baddeley, 2010).

The STM is then consolidated into LTM, which in contrast to both the sensory and working memory, has an unlimited capacity to store and retrieve information for a long period of time, ranging from several days to weeks, or even for a lifetime. LTM is stable, insensitive to disruption, and is broadly categorized into two forms, the explicit and the implicit memory. The explicit memory contains information that can be recalled consciously including personal experiences, known as episodic memory, and factual knowledge, known as semantic memory. Conversely, the implicit memory

contains information that can be recalled unconsciously, and is subdivided into four types; (1) the procedural memory that is the recalling of motor and executive skills, (2) the associative memory that is the recalling of information through association with other information, (3) the non-associative memory that is a learned behavior due to repeated exposure to an isolated stimulus and (4) priming, where the response to a specific stimulus is affected by a different stimulus presented prior to the response (Camina & Güell, 2017).

Regardless of the types of LTM, the encoding of all forms of LTM, unlike STM, requires synaptic plasticity that is long-lasting (Abraham, Jones, & Glanzman, 2019; Bailey, Kandel, & Harris, 2015; Langille & Brown, 2018). These changes can occur in both the pre- and the post-synaptic compartments. For instance, in the pre-synaptic compartments, there could be an alteration in the probability of neurotransmitter release, the quantal size and the pool of synaptic vesicles in the active zone. On the other hand, in the post-synaptic compartments, there could be an alteration in the phosphorylation states and the number of AMPAR, the surface area of the PSD, as well as the volume of the dendritic spines and thus, the area of the axon-spine interface. Alternatively, the number of synapses could be altered (Langille & Brown, 2018). Consequently, these lasting structural changes could increase the synaptic strength (potentiation) that persists beyond the early into the late phase, known as the late long-term potentiation (late-LTP). Notably, late-LTP is recognized as the cellular correlate for LTM (Langille & Brown, 2018; Lynch, 2004; Neves, Cooke, & Bliss, 2008). On the contrary, these enduring structural changes could also reduce the synaptic strength and results in long-term depression (LTD). Although LTD is not as well-studied as LTP, LTD has gained traction in the recent years as another cellular mechanism underlying certain forms of LTM (Connor & Wang, 2016).

1.2 Activity-dependent transcription in long-term memory formation

To induce enduring synaptic plasticity necessary for LTM, activity-dependent transcriptional modulation in the nucleus is essential. Numerous experiments in both vertebrates (rodents and monkeys) and invertebrates (flies and snails) have established the importance of transcription for the encoding of LTM. Blocking transcription in brain slices or cultured neurons using transcriptional inhibitors such as Actinomycin D (ActD) within a critical time window during activity reduces long-term synaptic potentiation and the efficacy of synaptic transmission (Frey, Frey, Schollmeier, & Krug, 1996a; P. Nguyen, Abel, & Kandel, 1994; J. Simon Wiegert, Hofmann, Bading, & Bengtson, 2009). Moreover, many *in vivo* experiments manipulating transcriptional modulators such as using α -amanitin that blocks RNA polymerase II have shown to negatively impact on the performance of memory-related tasks in animal models (C. M. Alberini, 2009; Sekeres, Neve, Frankland, & Josselyn, 2010; Suzuki et al., 2011). Notably, the inhibition of transcription only impairs LTM while leaving STM unimpaired (Cristina M. Alberini & Kandel, 2014; Eagle, Gajewski, & Robison, 2016; Yap & Greenberg, 2018). Collectively, these experiments establish the importance of coupling neuronal activation with transcriptional modulations for the induction of lasting synaptic changes critical for LTM formation. Indeed, the dysregulation of activity-dependent transcription is implicated in numerous neuropsychiatric and neurocognitive disorders, ranging from dementia and Alzheimer's disease to autism and depression (C. M. Alberini, 2009; Yap & Greenberg, 2018).

1.3 Nuclear signaling in neurons

The requirement for transcriptional modulation during the establishment of lasting synaptic plasticity indicates the need for signals to be relayed to the nucleus to alter transcription. Moreover, it is recently reported that the different neuronal activity patterns, including the duration of activity, can induce the expression of different activity-regulated genes that occurs in multiple temporal waves (Brigidi et al., 2019; Igaz, Vianna, Medina, & Izquierdo, 2002; Saha et al., 2011; G.-Y. Wu, Deisseroth, & Tsien, 2001). Critically, the induction of these distinct temporal waves requires nuclear signaling (Tyssowski et al., 2018). Hence, to understand how activity-dependent transcription alters the neuronal properties underlying LTM formation, it is not only critical to identify how different patterns of activity affect transcription, but also equally crucial to understand how these electrical or chemical signals are relayed to the nucleus. Through identifying and characterizing the different mechanisms of signal transduction to the nucleus, it will shed light on the temporal events that regulate the different waves of activity-driven transcription.

Neurons possess a unique morphology where the distance between the distal synaptic contacts and the nucleus are often large due to the length of the neuronal processes, which can exceed the soma by several orders of magnitude. This poses a challenge to transmit signal to the nucleus arising from different subcellular compartments. Hence, neurons have evolved multiple cellular mechanisms to overcome the physical distance between the subcellular compartments (**Fig. 1-1**).

Figure 1-1. Multiple nuclear signaling pathways in neurons

(a and b) illustrate the calcium-mediated signaling. (a) is the more rapid component where the calcium action potential propagates to the soma to trigger the opening of somatic ion channels and the subsequent influx of calcium ions directly into the soma. (b) is the slower component where the calcium, released from the ER, generates calcium waves that propagate to the soma. (c) illustrates the signaling mediated by endosomes from the axonal terminal. (d and e) illustrate the signaling mediated by the nuclear import of soluble proteins. (d) Small soluble proteins enter the nucleus by diffusion. (e) Soluble proteins enter the nucleus by active transport, commonly associated with nuclear adaptor proteins, along the cytoskeletal filaments. This study focused on this pathway highlighted in the red box. Figure adapted from (Ch'ng & Martin, 2011).

1.3.1 Electrochemical signaling

One well-studied mechanism is the rapid calcium-mediated signaling in the somato-dendritic compartment. This process, which can happen in the millisecond time scale, consists of two discrete temporal components (Augustine, Santamaria, & Tanaka, 2003). The faster component consists of the somatic calcium influx induced by synaptic activity. Upon cumulative synaptic excitation which leads to the activation of voltage-gated calcium channels (VGCCs) and other neurotransmitter receptors, calcium action potentials are generated and propagated to the soma to trigger the opening of ion channels in the somato-dendritic compartment. Consequently, the direct calcium influx at the soma signals directly to the nucleus to alter transcription and modify synaptic efficacy.

On the other hand, the slower component involves the calcium release from the intracellular stores. Local calcium influx at the synapses following depolarization at the post-synaptic membranes, besides inducing somatic calcium influx, can also trigger the release of intracellular calcium stores from the smooth Endoplasmic Reticulum (ER). The ER is detected in axons as well as in the dendrites, and the ER membrane is contiguous and can extend from the distal dendrites all the way to the nucleus. The local release of calcium coupled with the activation of inositol 1, 4, 5-triphosphate (IP3) and/or ryanodine receptors on the smooth ER surface can generate a series of calcium waves that propagate towards the nucleus along the dendrites (Ch'ng & Martin, 2011; Friel & Tsuen, 1992). The increased calcium level in the soma, regardless of the mechanisms, can then initiate downstream calcium sensitive signaling cascades, which ultimately impact the expression of activity-regulated genes.

1.3.2 Nuclear import of soluble proteins

Apart from the rapid electrochemical signaling, the movement of proteins into the nucleus through the nuclear pore complex (NPC) provides another means for the activity-dependent signals from different subcellular compartments to converge at the nucleus (A. F. Lim, Lim, & Ch'ng, 2017). Although this process is slower than the intracellular calcium signaling, it confers several advantages over the electrochemical pathways. First, depending on the type of stimulus and the proteins that enter the nucleus, this form of nuclear signaling can induce specific stimulus-driven responses through the activation of different subset of activity-regulated genes. Second, it can mediate a synapse-specific signaling to the nucleus. Third, as the transport of proteins can last for minutes to hours, this form of nuclear signaling can trigger responses long after the stimulus have been removed (W. A. Herbst & K. C. Martin, 2017; Kaushik, Grochowska, Butnaru, & Kreutz, 2014).

There is now substantial evidence on the requirement of activity-dependent nuclear entry of plasticity-related proteins, such as CRTC1, CamKII and calmodulin, for the expression of activity-regulated genes and thus, transcription-dependent plasticity in neurons (W. A. Herbst & K. C. Martin, 2017; A. F. Lim et al., 2017; H. Ma et al., 2014). Of note, many of these proteins are localized in diverse subcellular compartments including synapses, dendrites, and axons (Wendy A. Herbst & Kelsey C. Martin, 2017; Lionaki, Gkikas, & Tavernarakis, 2016), and several of these proteins can directly modulate transcription in response to neuronal activity (Li et al., 2016; A. F. Lim et al., 2017). While some studies have suggested the nuclear calcium signaling alone is adequate to drive long-term plasticity (Hardingham, Arnold, & Bading, 2001), experiments blocking or manipulating the NPC to halt the movement of proteins into the nucleus have shown to impair long-term but not short-term plasticity (Thompson et

al., 2004). Furthermore, blocking specifically the nuclear shuttling of plasticity-related proteins such as CRT1 have resulted in learning and memory deficits (Uchida et al., 2017). Collectively, these data confirm the importance of activity-dependent nuclear shuttling of soluble proteins to maintain long-term plasticity.

There are various mechanisms for the nuclear entry of proteins (Cautain, Hill, de Pedro, & Link, 2015; Kimura & Imamoto, 2014; Kose, Furuta, & Imamoto, 2012; H. Ma et al., 2014). Apart from the diffusion of small proteins or direct binding to the NPC, by far the most common method for nuclear entry is through the association of the nuclear localization signal (NLS) of the cargo proteins with the nuclear adaptor proteins. To date, several classes of nuclear adaptor proteins have been discovered, of which the most ubiquitous and well-characterized is the importins (Cautain et al., 2015; Wagstaff & Jans, 2009).

Importin-mediated nuclear import processes are broadly categorized into the classical and the non-classical nuclear import pathways. In the classical nuclear import pathway, five distinct Imp- α isoforms (α 1-4, α 6 in mice) heterodimerize with Imp- β 1 via its Imp- β binding (IBB) domain. Imp- α / β 1 heterodimerization is crucial for enhancing the binding affinity between Imp- α and the cNLS-bearing cargo proteins. The resultant ternary complex then docks on the NPC, followed by the translocation into the nucleus (**Fig. 1-2**). Recently, a new member known as Imp- α 8 was identified and shown to interact with Imp- β 1 to carry cNLS bearing cargo into the nucleus (Kimoto et al., 2015).

Figure 1-2. Nucleocytoplasmic shuttling mediated by classical Imp- α / β 1 complex

(i) Imp- β 1, Imp- α and cNLS bearing cargo (blue circle) form a ternary complex in the cytoplasm. (ii) Imp- β 1 docks the ternary complex at the NPC by interacting with FG-nucleoporins of NPC. (iii) The ternary complex translocates into the nucleus. (iv) In the nucleus, the binding of Ran-GTP to Imp- β 1 dissociates the ternary complex, releasing the cargo in the nucleus. (v) Imp- α is exported out of the nucleus to the cytoplasm by associating with the exportin CAS-Ran-GTP complex. (vi) In the cytoplasm, the Ran-GAP hydrolyses Ran-GTP to Ran-GDP and triggers the dissociation of the exportin complex. The Ran-GDP can then enter the nucleus to be converted into Ran-GTP by Ran-GEF while the released Imp- α enters another transport cycle. Imp- β 1 also undergoes recycling between cytoplasm and nucleus but its export mechanism is not well-characterized (not shown in figure). Figure adapted from (Goldfarb, Corbett, Mason, Harreman, & Adam, 2004)

In contrast, the non-classical nuclear import mechanisms engage the Imp- β or β -like importins. As many as 22 Imp- β members in the superfamily have been identified in humans' proteins, of which at least 11 are known to interact with cargo proteins that bear an NLS for nuclear import (Baade & Kehlenbach, 2019; Bourgeois et al., 2020; Chook & Süel, 2011; A.-C. Ström & K. Weis, 2001). Importantly, these Imp- β members function independently from Imp- α and enter the nucleus as homodimers or as heterodimers with other Imp- β members (Baade & Kehlenbach, 2019; Chook & Süel, 2011).

<i>Mus musculus</i>	Imp- α 1	Imp- α 2	Imp- α 3	Imp- α 4	Imp- α 6	-	Imp- α 8
<i>Homo sapiens</i>	Imp- α 5	Imp- α 1	Imp- α 4	Imp- α 3	Imp- α 7	Imp- α 6	Imp- α 8
Imp-β superfamily	Importins : Imp- β 1, - β 2, -4, -5, -7, -8, -9, -11, -13. Exportins						

Table 1- 1. Importins superfamily in *Mus musculus* and *Homo sapiens* (Chook & Süel, 2011; Oka & Yoneda, 2018).

Other non-conventional nuclear adaptors that are not importins have also been discovered. One example is the Hikeshi protein that binds to the FG-nucleoporins and shuttles ATP-bound heat-shock protein 70 (Hsp70s) into the nucleus in response to heat shock stress (Kose et al., 2012). In the nucleus, the hydrolysis of ATP bound to Hsp70s dissociates the complex, leaving Hsp70s in the nucleus (Kose et al., 2012). This illustrates the sufficiency of Hikeshi protein as an importin-independent nuclear adaptor.

1.4 Importin- β 1 structure and functions in activity-dependent plasticity

Imp- β 1 is a modular protein containing 19 HEAT repeats. Each repeat consists of two α -helices connected by a short loop, forming a hairpin structure. In general, the 19 HEAT repeats fold into a superhelix structure that can adopt different conformation to allow both the cargoes and the adaptor proteins such as Imp- α and IPO7 to bind in different, but overlapping, interacting surfaces (Christie et al., 2016; A. Harel & D. J. Forbes, 2004). Due to this structural flexibility, Imp- β 1 can partake in multiple nuclear import pathways.

Figure 1-3. Imp- β 1 structure

A model of human Imp- β 1 adapted from (Amnon Harel & Douglass J. Forbes, 2004). Imp- β 1 contains 19 Heat repeats (in rectangle), where different Imp- β 1 partners interact with varying Heat repeats. For instance, Importin-7 (IPO7) is reported to bind to Heat repeats 6 to 9 while Imp- α binds at Heat repeats 7 to 19 (Christie et al., 2016; Wohlwend, Strasser, Dickmanns, Doenecke, & Ficner, 2007). Figure is adapted from (A. Harel & D. J. Forbes, 2004).

In the aspect of activity-induced nuclear signaling in neurons, there are accumulating evidence hinting at the involvement of Imp- β 1 mediated nuclear import as a mechanism to relay information to the nucleus. First, Imp- β 1, as well as Imp- α , are found localizing not only at the soma, but also at the synapses and the dendrites (Pielot et al., 2012; Pirooznia et al., 2012). Of note, both Imp- β 1 and - α accumulate in the neuronal nuclei following LTP (Thompson et al., 2004). Second, NFAT, which is a plasticity-associated protein that undergoes activity-dependent nuclear accumulation in neuron, is shown to interact with Imp- β 1 for its nuclear entry (Ishiguro, Ando, Maeda, Watanabe, & Goto, 2011; Wild et al., 2019). Other synapse-to-nucleus signaling proteins such as Jacob (Dieterich et al., 2008), CREB2 (K. O. Lai, Y. Zhao, T. H. Ch'ng, & K. C. Martin, 2008), Nf- κ β (Mikenberg, Widera, Kaus, Kaltschmidt, & Kaltschmidt, 2007) and RNF10 (Dinamarca et al., 2016) are reported to engage Imp- α for its nuclear entry following activity. Since Imp- β 1 is an obligate partner for Imp- α in the classical nuclear import pathway, these suggest Imp- β 1 involvement in the nuclear shuttling of these activity-dependent signaling proteins. Meanwhile, phosphorylated Extracellular Signal-Regulated Kinase (pERK), another synapse-to-nucleus signaling protein, is reported to bind to Importin-7 (IPO7), coupling with Imp- β 1, for its nuclear entry (Chuderland, Konson, & Seger, 2008; Li et al., 2016; Lorenzen et al., 2001).

Collectively, these data emphasize the central role of Imp- β 1 in activity-dependent nuclear signaling in neurons as it bridges the classical and the non-classical nuclear import pathways by associating with both the Imp- α and IPO7 to mediate the activity-dependent nuclear import of soluble proteins (Cherezova, Burnside, & Rose, 2011; Chook & Süel, 2011; Mehmood et al., 2011).

1.5 Research objectives

Despite the published reports on Imp- β 1, the question as to whether Imp- β 1 is truly required for transcriptional regulation underlying long-term plasticity remains. While it appears that Imp- β 1's role is indispensable for long-term plasticity, several questions have been raised to challenge this assumption. First, there is a built-in redundancy in the importin-mediated nuclear import machinery. Many nuclear proteins can associate with different importins and a loss of Imp- β 1 function could be compensated by other Imp- β members (Baade & Kehlenbach, 2019; Chook & Süel, 2011). Second, there are non-canonical nuclear import mechanisms, including other importin isoforms, that function independently from Imp- β 1 (Cautain et al., 2015; Kotera et al., 2005; M. Lever, A. Karpova, & M. Kreutz, 2015; Huan Ma et al., 2014; Oka & Yoneda, 2018). In fact, for every plasticity-associated protein that engages Imp- β 1, there could be a comparable number of proteins in the activity-driven signaling cascade, such as PKA, Akt, and MEK, that do not have a clear NLS or reported to associate with Imp- β 1. Third, the possibility that the nuclear calcium signaling alone is sufficient to trigger the first wave of transcription has been proposed (Hardingham et al., 2001). Given the spatial origin of the input signal and kinetics of nuclear import, it is possible that Imp- β 1 might be crucial for triggering or sustaining distinct transcriptional waves after the initial expression of activity-regulated genes.

This study sought to elaborate on the local function and regulation of Imp- β 1 during activity-dependent nuclear signaling in hippocampal neurons. We first verified that Imp- β 1 is crucial for activity-driven nuclear signaling and transcription-dependent plasticity. Imp- β 1 responds robustly to different neuronal stimulation paradigms including synaptic activation by accumulating in the nucleus. The blockade of Imp- β 1 function with a cell-permeable small molecule inhibitor, Inhibitor of Nuclear Import-

43 (INI43), impairs nuclear events underlying transcription-dependent plasticity, namely (1) CREB phosphorylation at serine 133 (S133), (2) the expression of select activity-regulated genes, and (3) the nuclear localization of transcriptional modulators, JUNB and NFATc3. More importantly, the inhibition of Imp- β 1 function during activity impairs both the late-LTP and the LTD in hippocampal slices, directly establishing the role of Imp- β 1 in long-term plasticity.

I next investigated Imp- β 1 local regulation in the synapto-dendritic compartment following activity as I observed the nuclear shuttling of dendritic Imp- β 1 upon synaptic activation using live imaging. To that end, I purified synaptosomes from adult male mice forebrain and detected the presence of Imp- β 1 mRNA isoforms at the synaptosomes. Critically, I observed a higher Imp- β 1 protein level in the stimulated versus basal synaptosomes, where the increment is sensitive to cycloheximide (CHX). This indicates activity-induced local Imp- β 1 translation in the synaptosomes. Correspondingly, I reported a higher dendritic and post-synaptic Imp- β 1 proteins following stimulation in cultured hippocampal neurons that abolishes with CHX but not N-ethylmaleimide (NEM) treatment. This further supports activity-induced local Imp- β 1 translation in the synapto-dendritic compartments and eliminates the possibility that the increment is due to movement of newly synthesized Imp- β 1 from the soma. Consistent with these results, I also observed an increased Imp- β 1 translational machineries localized at the dendrites of the stimulated cultured neurons using Puromycin-Proximity Ligation Assay (Puro-PLA). Taken together, these results revealed activity-dependent local Imp- β 1 translation at the synapto-dendritic compartments.

Given that there are up to 10 % of the synaptic proteome reported to harbor bona fide NLS yet, only a subset of these proteins are known to undergo activity-

dependent nuclear shuttling, we hypothesize that there are additional synapse-to-nucleus signaling proteins that remain to be identified (Jordan et al., 2004; B. A. Jordan, B. D. Fernholz, L. Khatri, & E. B. Ziff, 2007; Jordan & Kreutz, 2009; Karpova, Bar, & Kreutz, 2012; M. B. Lever, A. Karpova, & M. R. Kreutz, 2015; Pielot et al., 2012). Identifying a comprehensive list of these synapto-nuclear signaling proteins are vital as they have been implicated in a wide spectrum of brain disorders including both the neuropsychiatric and the neurodegenerative disorders (Parra-Damas & Saura, 2019). Therefore, we casted a wide net to identify potential synapse-to-nucleus signaling proteins by conducting co-immunoprecipitation (co-IP) using Imp- β 1 as the bait, since it involves in both the classical and the non-classical nuclear import pathways. With subsequent stringent filtering steps on the tandem mass spectrometry sequencing data and extensive literature search on nuclear presence and roles, I have narrowed down the candidate list from which N-Myc Downstream Regulated 1 (NDRG1) is characterized. I validated NDRG1 interaction with Imp- β 1 in the synaptosomes via reverse co-IP and further showed NDRG1 nuclear enrichment after synaptic activity, with a corresponding reduction in dendrites and synapses using cultured hippocampal neurons. Notably, its activity-induced nuclear enrichment is mediated by Imp- β 1.

Collectively, our results confirm the importance of Imp- β 1 and more broadly, adaptor protein-mediated nuclear import of soluble proteins during transcription-dependent plasticity.

Chapter 2. Experimental Procedures

2.1 Animals

All experiments were performed using protocols approved by Nanyang Technological University Institutional Animal Care and Use Committee (IACUC #E0019 and #A18091). C57BL/6 mice and Sprague-Dawley rats were used. Mouse and rat hippocampal neurons were isolated from P0 to P1 pups and maintained for 14 to 17 days in-vitro (DIV) prior to any experiments. Synaptosomes and acute slices were isolated from the forebrain of P56 to P60 male mice.

2.2 Antibodies

Antibodies	Source	Dilution
Alexa Fluro conjugated secondary Ab	Life Technologies	1:1000 (ICC)
Chicken-anti-MAP2	Phosphosolutions	1:10,000 (ICC)
HRP-linked anti-mouse and anti-rabbit secondary Ab	Cell Signaling	1:1000 (IB)
Mouse specific light chain (HRP conjugated) secondary Ab	EMD Millipore	1:1000 (IB)
Mouse-anti-Add1	Santa Cruz	1:200 (ICC)
Mouse-anti-GAPDH	ProteinTech	1:5000 (IB)
Mouse-anti-HA	Sigma	1:1000 (IB)
Mouse-anti-importin- β 1 (3E9)	Thermo Fisher	1:1000 (ICC); 3 μ g (IP); 1:1000 of PLA probe conjugated 3E9 (420 μ g/ mL)
Mouse-anti-Imp- α 1	Santa Cruz	1:200 (IB)
Mouse-anti-IPO7	Santa Cruz	1:200 (IB)
Mouse-anti-JUNB	Santa Cruz	1:100 (ICC)
Mouse-anti-PSD95	Invitrogen	1:5000 (IB); 1:1000 (ICC)
Mouse-anti-puromycin	Kerafast	1:1000 of PLA probe conjugated antibody (420 μ g/ mL)

Mouse-anti-synaptophysin (Clone SY38)	EMD Millipore	1:1000 (IB)
Mouse IgG	Sigma	3 μ g (IP)
Rabbit-anti-Imp- α 2, 3, 4, 6	Gift from Martin K.C	1:10,000 (IB)
Rabbit-anti-cleaved caspase 3	Cell Signaling	1:400 (ICC)
Rabbit-anti-CRTC1	Bethyl labs	1:3000 (ICC)
Rabbit-anti-GFP	Thermo Fisher	1:1000 (ICC)
Rabbit-anti-H3	Cell Signaling	1:1000 (IB)
Rabbit-anti-NDRG1	Gift from Kokame, K	1:100 (ICC); 3 μ g (IP)
Rabbit-anti-pCREB (S133)	Cell Signaling	1:500 (ICC)
Rabbit-anti-synaptophysin	EMD Millipore	1:500 (ICC)
VeriBlot for IP Detection Reagent (A131366)	Abcam	1:1000 (IB)

2.3 Plasmids and viruses

FLAG-tagged Imp- α (1-3,6), photoactivatable GFP-tagged Imp- α 4 plasmids and GFP-tagged NF- κ B plasmids. β -catenin, NDRG1 and PDPK1 plasmids were obtained from Mammalian Gene Collection (NIH) and used to generate GFP- β -catenin, NDRG1-GFP and GFP-PDPK1 plasmids, respectively. HA-tagged human Imp- β 1 plasmid was a kind gift from Chook, Y. M (UT Southwestern) and used to generate Imp- β 1-Dendra2 (L22-Camk2 α -hImp- β 1-Dendra2 from L22-Camk2 α -Dendra2; GenScript BioTech, Hong Kong). sGFP-hNFATc3 plasmid was a kind gift from Mark Dell'Acqua (U. of Colorado, Denver). pFCK (1.3) GW was a kind gift from Pavel Osten (Addgene plasmid #27230; <http://n2t.net/addgene:27230>).

2.4 Mammalian and hippocampal neuronal cultures

Hippocampal neurons were isolated as previously described in (Ch'ng et al., 2012) and plated on poly-DL-lysine (0.5 mg/ml; Sigma) coated coverslips (Carolina Biologicals, Burlington, NC), maintained in Neurobasal media (Invitrogen, Carlsbad,

CA), supplemented with B27 (Invitrogen), GlutaMAX (Invitrogen), fetal bovine serum (FBS) and gentamycin (Life Technologies) one day post-culture for mouse cultures. For rat cultures, Neurobasal media was supplemented with B27, GlutaMAX, monosodium glutamate (Sigma) and β -mercaptoethanol (Sigma) during plating. After one day post-culture, both rat and mice cultures were maintained in Neurobasal media supplemented with B27 and GlutaMAX for the remaining days of the culture (Ch'ng et al., 2012). For most experiments, unless otherwise indicated, neurons were incubated with various pharmacological agents in the conditioned neuronal media, kept in humidified incubator (37°C, 5% CO₂) for the appropriate amount of time before cells were processed for immunoblotting (IB) or immunocytochemistry (ICC).

HEK 293T cells were grown in Dulbecco's Modified Eagle Media (DMEM; Nacalai) supplemented with 10% FBS (Hyclone) and 1% Penicillin/Streptomycin mixed solution (Nacalai), kept in a humidified incubator (37 °C, 5 % CO₂) for the appropriate amount of time before experiments.

2.5 Pharmacological treatment

The following pharmacological agents were used: APV (40 μ M; Tocris), Bicuculline (Bic, 40 or 50 μ M; Sigma), Brain-derived neurotrophic factor (BDNF, 50 ng/mL), Cycloheximide (CHX, 50 μ M; Sigma), Glutamate (40 μ M; Sigma), Glycine (20 or 100 μ M; Sigma), H₂O₂ (500 μ M, Sigma), INI43 (5 μ M; Sigma), Kynurenic acid (Kyn, 1 mM, Sigma), Leptomycin B (LMB, 20 nM; Sigma), MG132 (5 μ M), N-ethylmaleimide (NEM, 50 μ M; Sigma), N-Methyl-d-aspartic acid (NMDA, 20 μ M), Potassium Chloride (KCl, 50 mM; Merck), (RS)-3,5-Dihydroxyphenylglycine (DHPG, 100 μ M; Tocris), Tetrodotoxin (TTX, 1 μ M; Tocris), and 4-Aminopyridine (4AP, 200 μ M). Inhibitors were added to neuronal cultures prior to and during stimulation as described in each experiment. For neuronal depolarization experiments, culture

medium was replaced either with basal artificial cerebrospinal fluid (aCSF; 126 mM NaCl; 24 mM NaHCO₃; 1 mM NaH₂PO₄; 2.5 mM KCl; 2 mM CaCl₂; 2 mM MgCl₂; 10 mM D-Glucose; 0.4 mM ascorbic acid) or high K⁺, no Mg²⁺ aCSF (78.5 mM NaCl; 24 mM NaHCO₃; 1 mM NaH₂PO₄; 50 mM KCl; 10 mM CaCl₂; 0 mM MgCl₂; 10 mM D-Glucose; 0.4 mM ascorbic acid). For sGFP-NFATc3 stimulation, culture medium was replaced with basal Tyrode's solution (140 mM NaCl, 10 mM HEPES, 5 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose) or high K⁺, no Mg²⁺ Tyrode's solution (95 mM NaCl, 10 mM HEPES, 50 mM KCl, 4 mM CaCl₂, 10 mM glucose) for stimulation. Recovery was conducted in basal Tyrode's solution. All experiments using NEM was conducted in basal Tyrode's solution.

2.6 Forebrain Acute Slices

Coronal acute slices (250 µm) were obtained from adult male mice forebrain, sectioned using vibratome in cold cutting solution, gassed with 5 % CO₂ and 95 % O₂. The slices were then recovered for three hours at interface in aCSF (126 mM NaCl, 24 mM NaHCO₃, 1 mM NaH₂PO₄, 10 mM D-Glucose, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 0.4 mM ascorbic acid) at 32 °C, at 5 % CO₂ and 95 % O₂. Chemical stimulation were conducted in aCSF with no Mg²⁺ (78.5 mM NaCl; 24 mM NaHCO₃; 1 mM NaH₂PO₄; 50 mM KCl; 10 mM CaCl₂; 0 mM MgCl₂; 10 mM D-Glucose; 0.4 mM ascorbic acid; 40 µM forskolin) for 15 min, followed by high KCl (50 mM) for 10 min (Ch'ng et al., 2012). All slices were then used for immunocytochemistry (ICC) experiments.

2.7 Cellular Transfection and Transduction

Calcium phosphate transfection of cultured hippocampal neurons (DIV13-16) were conducted as previously published (Dudek, Ghosh, & Greenberg, 1998). In brief, cultured neurons in 24-wells plate were incubated for 20 to 60 min with the

DNA/calcium phosphate precipitate in neat Neurobasal media before returning to conditioned media. Unless otherwise stated, transfected neurons were allowed 16-24 h of expression prior to experiments.

Transfection of plasmids in HEK 293T cells was carried out with DNAfectin™ (Invitrogen) following manufacturer's protocols. Length of expression of these constructs varied depending on the experiments and are individually described.

For lentiviral transduction of cultured hippocampal neurons, the viral inoculum was diluted in 250 µl of conditioned medium and incubated with the neurons (DIV 11) overnight, before swapping into the remaining conditioned medium without viral inoculum. In general, lentiviral integration and expression were allowed for a minimum of 3 days post-transduction before conducting any further experiments.

2.8 Immunocytochemistry (ICC)

Cultured hippocampal neurons were fixed in 4 % paraformaldehyde (PFA) diluted in Tyrode's solution, permeabilized in PBS containing 0.1 % Triton-X100 (TX-100) and blocked in 10 % goat serum diluted in PBS. Acute forebrain slices were fixed in 4 % PFA diluted in aCSF, permeabilized in PBS containing 0.4 % TX-100, and blocked with 10 % goat serum diluted in 0.2 % TX-100. All primary antibody incubations were performed overnight in 4 °C and secondary antibodies staining were done 1 h at room temperature. For all preparations, samples were washed 3X with PBS between every change in post-fixation treatment solutions. After antibody incubations, samples were mounted on slides using Aqua-Poly/Mount (PolySciences).

2.9 Confocal Imaging and Analyses

Unless otherwise stated, all confocal images, either static or live cell imaging, were performed on a Zeiss LSM800 (Carl Zeiss, Inc.) inverted scanning confocal

microscope using either 40 X or 63 X, 1.4 NA oil objectives. For live cell imaging of lysosomal movement, cultured mouse hippocampal neurons grown on glass coverslips were incubated in N-ethylmaleimide (NEM, 50 μ M, 10 min) containing Tyrode's solution, followed by the addition of LysoTracker Deep Red (1 μ M, 10 min, Thermofisher) before washing out, and transferred to a live-cell imaging buffer (140 mM NaCl, 10 mM HEPES, 5 mM KCl, 3 mM CaCl₂, 0.1 mM MgCl₂, 10 mM glucose, 10 nM ascorbic acid, pH 7.35). Cells were imaged on a heated stage assembly and images were acquired at 1 s per frame for 60 s. The displacement length and cumulative displacement of the lysosomes were calculated using Imaris "track displacement length" plugin.

For live cell imaging of Imp- β 1 movement, rat hippocampal neurons were transfected with Imp- β 1-Dendra2 constructs (DIV 13-16). After 16 h of expression, neurons were transferred to a buffer containing low (for basal) or no (for stimulation) Mg²⁺ Tyrode's solution (140 mM NaCl; 10 mM HEPES pH 7.3; 5 mM KCl; 3 mM CaCl₂; 0.1 mM MgCl₂; 10 mM glucose; 10 nM Trolox pH 7.35) in the presence or absence of bicuculline (Bic; 40 μ M). The entire assembly was placed on a heated stage for imaging. Neurons with comparable expression levels were selected for imaging. Three regions of interest (15 μ m x 5 μ m) on dendrites within 60 μ m from the soma were converted by brief exposure to UV laser three times (405 nm, 6.0 %, scan speed: 3). Immediately after photoconversion, photoconverted red-shifted Dendra2 signal in the soma was tracked for 15 min (1 frame per min: t = 1 to 15). The photoconverted (red) signal from Dendra2 and Imp- β 1-Dendra2 in the nucleus (based on pre-capture, low intensity snapshot of Hoechst) were quantified and normalized (t =1) across all time points in each condition.

For all confocal micrographs, quantifications were done using ImageJ unless otherwise stated. For nuclear-to-cytoplasmic ratio, cytoplasmic intensity was calculated by subtracting the nuclear mask (based on Hoechst) from the whole cell body mask (based on MAP2). All dendritic measurements were obtained from dendrites taken 70 μm from the base of the cell body, unless otherwise stated. For the dendritic Puro-PLA, signals were processed with a constant threshold applied within the same experimental set using threshold function in ImageJ before quantifying for its integrated intensity.

Specific for Puro-PLA synaptic experiments, confocal images of dendrites taken 50 μm from the base of the cell body were captured in a fixed 50 μm x 15 μm field using 63X, 1.4 NA oil DIC Plan Apochromat AiryScan objective lens on an LSM800 scanning confocal microscope, attached to a 32-element GaAsP-PMT array detector running the AiryScan module. Images were then deconvoluted using AiryScan deconvolution algorithm post-acquisition and the colocalization analyses were performed using IMARIS 6.3.1, with its surface-surface colocalization plugin. Prior to volumetric analysis, a constant threshold to remove background was used within each experimental set for each fluorescence channel: MAP2 (10 % lower intensity threshold); PSD95 and synaptophysin (15 – 20 % lower intensity threshold and split object); Imp- β 1-Puro PLA (30 % lower intensity threshold, smoothing and split object). Within each dendritic field, a 3-D volumetric surface was independently generated for all neuronal markers and PLA signals. The marker surfaces were then mapped onto the PLA surfaces to generate a 3-D mask that shows overlapping regions (**Fig. 3-5D**).

For analyses of Imp- β 1 in spines (**Fig. 3-6B**), the spines were identified by thresholding the intensity and circularity of the GFP puncta that are found within 1 μm away from the dendrites.

2.10 Quantitative PCR (qPCR)

Total RNA was extracted with TRIzol (Invitrogen) and column purified with either PicoPure RNA isolation kit (Cultured neurons; Thermofisher) or RNeasy Mini Kit (synaptosome and cytosolic fractions from homogenized mouse forebrain; Qiagen). For RT-PCR, cDNA was generated using RevertAid First strand cDNA synthesis kit (Thermofisher) with either poly-dT or random hexamer priming. SYBR Green Master Mix (Invitrogen) with specific primer pairs (**Table 2-1**) were used for all qPCR assays and analyzed via QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Technical and experimental triplicates were performed for all samples. For cultured neurons analyses, ΔC_t values were calculated by normalizing the average C_t values for each gene against the neuron specific, activity independent, hypoxanthine phosphoribosyltransferase gene (HPRT1). $\Delta\Delta C_t$ values were then calculated by normalizing the ΔC_t of the treated samples against basal controls. For synaptosomal and cytosolic Imp- β 1 isoforms analyses, the mean C_t values obtained from primers targeted against long (L1 and L2) and full-length (F1 and F2) isoforms were normalized against the mean C_t values obtained from the common primer (S1).

Genes	Primers
<i>arc</i>	Forward: GGT GAG CTG AAG CCA CAA AT Reverse: GGT GTC ATT CTC CTG GCT CT
<i>junB</i>	Forward: TCA CGA CGA CTC TTA CGC AG Reverse: CCT TGA GAC CCC GAT AGG GA
<i>egr4</i>	Forward: TAT CCT GGA GGC GAC TTC TTG Reverse: AGA TGC CAG ACA TGA GGT TGA
<i>cfos</i>	Forward: CAC ACA GGA CTT TTG CGC

	Reverse: GAC ACG GTC TTC ACC ATT CC
<i>npas4</i>	Forward: AGG GTT TGC TGA TGA GTT GC Reverse: CCC CTC CAC TTC CAT CTT C
<i>hprt1</i>	Forward: TGT TGT TGG ATA TGC CCT TG Reverse: GGC CAC AGG ACT AGA ACA CC
Imp β 1-S1	Forward: ATG ATG ACC TGA GAC CCC CA Reverse: AGC CTC ACT CAC TCA TCC TCT
Imp- β 1-L1	Forward: GAG GAG AAA GAT GGA GCC CG Reverse: GAG AAT GTG GGT ACC GCC TC
Imp- β 1-L2	Forward: GGC GGT ACC CAC ATT CTC AA Reverse: TTG TGC TGC ATC AGT AGC CA
Imp- β 1-F1	Forward: GGG CAT CGG ATA GAC TTG GG Reverse: TCA CAG TTG CCT CTG ACA GC
Imp- β 1-F2	Forward: GGT GTC AGA TGG TGA CCC TG Reverse: GGG TGC CTT GTT CCC TTG TA

Table 2- 1. qPCR primer pairs.

2.11 Electrophysiology[#]

Acute hippocampal slices were prepared from 5 to 7-week-old wild type BL-6 mice for electrophysiological recordings. Briefly, after anesthetization using CO₂, the mice were decapitated, and the brains were quickly removed and transferred into cold, oxygenated aCSF. The aCSF contained the following (in millimolar): 124 NaCl, 3.7 KCl, 1.0 MgSO₄·7H₂O, 2.5 CaCl₂·2H₂O, 1.2 KH₂PO₄, 24.6 NaHCO₃, and 10 D-glucose, equilibrated with 95 % O₂ and 5 % CO₂ (carbogen; total consumption 16 L h⁻¹). From each hippocampus, 8 – 10 transverse hippocampal slices (400 μ m thick)

were prepared using a manual tissue slicer. The slices were incubated in an interface brain slice chamber (Scientific Systems Design, Mississauga, Ontario, Canada) at 32 °C for three to four hours at an aCSF flow rate of 1 mL min⁻¹. For INI43 treated slices, aCSF containing 5 µM INI43 was bathed in the slices 30 min prior and 30 min post-stimulation or -depression.

For stimulation, a monopolar stainless-steel electrode (5 MΩ; A-M Systems, Sequim, WA, USA) was positioned within the stratum radiatum of the CA1 region. For the recordings of field EPSP (measured as its slope function), a monopolar stainless-steel electrode (5 MΩ; A-M Systems, Sequim, WA, USA) was placed in the CA1 apical dendritic layer. Signals were amplified by a differential amplifier (Model 1700; AM Systems) and digitized using a CED 1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and monitored online.

Input–output relation (afferent stimulation vs. fEPSP slope) was determined for each slice and the stimulus intensity that evokes 40 % of the maximum fEPSP slope is set as test stimulus intensity. For baseline recording and testing at each time point, four 0.2-Hz biphasic constant-current pulses (0.1 ms per polarity) were used. Late-LTP was induced using three stimulus trains of 100 pulses ('strong' tetanus [STET], 100 Hz; duration, 0.2 ms per polarity; intertrain interval, 10 min). Late-LTD was induced using a strong low-frequency stimulation (SLFS) protocol of 900 bursts [one burst consisted of three stimuli at 20 Hz, and the interburst interval was 1 s (i.e., $f = 1$ Hz; stimulus duration, 0.2 ms/half wave; total number of stimuli, 2700)].

In collaboration with Professor Saji Kumar Sreedharan. Dr. Sheeja Navakkode Gangadharan performed and analyzed the data for all electrophysiology experiments.

2.12 Puromycin Proximity Ligation Assay (Puro-PLA)

Puro-PLA on cultured hippocampal neurons was conducted according to published protocol (tom Dieck et al., 2015). Briefly, after stimulation, all samples were first treated with cycloheximide (CHX; 355 μ M, 30 min) to terminate nascent chain elongation and to prevent the release of nascent peptide from the ribosomal complex (David et al., 2012; tom Dieck et al., 2015). This is followed by puromycin (3 μ M, 15 min) treatment in conditioned medium in a humidified incubator (37 °C, 5 % CO₂). Incubation was stopped by immediate fixation with 3.2 % PFA diluted in Tyrode's solution. After fixation, the neurons were washed, permeabilized and treated for PLA according to protocol described in Duolink[®] PLA Fluorescence kit (Sigma). Both the antibodies targeted against Imp- β 1 and puromycin were conjugated directly to the PLA probes and identified as 'Plus probe' and 'Minus probe' respectively using Duolink[™] In Situ Probemaker PLUS and MINUS kits (Sigma). Neurons were then fixed again with 3.2 % PFA before processing for immunocytochemistry (ICC).

2.13 Synaptosomes extraction and stimulation

Synaptosomes were isolated from four male adult mice in each extraction as described in Jeffrey *et al.* (2009) with minor modifications including: 18 up-and-down strokes for the first homogenization; 6 up-and-down strokes for the second homogenization; 700 x g for the first round of centrifugation; crude pellet was homogenized in 4.5 mL of Solution B, layered on the discontinuous sucrose gradient and subsequently ultracentrifuged (82,500 x g for 2 h 15 min). Synaptosomes were harvested at the sucrose interface between 1.0 M and 1.2 M and 2.5 μ M of CaCl₂ was added to the purified synaptosomes before dividing it into basal and stimulated groups. Stimulation of synaptosomes involved a 5 min incubation with 100 μ M glycine followed by another 5 min incubation with 50 mM KCl. Cycloheximide (CHX, 50 μ M)

was added to the specified group 30 min before stimulation. The synaptosomes were kept in cold except during the stimulation, which was conducted in 37 °C water incubator. These synaptosomes were then used for qPCR analyses or immunoprecipitation experiments.

Figure. 2-1. Synaptosome extraction. Figure adapted from (Au - Bermejo, Au - Milenkovic, Au - Salahpour, & Au - Ramsey, 2014).

2.14 Immunoprecipitation (IP)

Cold lysis buffer (0.025% digitonin, 150 mM NaCl, 10 mM Tris-HCl-pH 7.4), supplemented with protease inhibitor cocktail (Roche), was added to synaptosomes in a 1:1 ratio and rotated end-to-end in cold room for 30 min prior to IP assays. For HEK 293T cells, the lysates were prepared by scraping the (transfected) cells in PBS, followed by centrifugation at 5000 rpm for 3 min in cold. Subsequently, cold lysis buffer (1% NP-40 (for Imp- β 1) or 0.1% TX-100 (for GFP-candidate proteins), 50 mM Tris-HCl pH 8.0 (for Imp- β 1) or pH 7.6 (for GFP-candidate proteins), 150 mM NaCl supplemented with protease inhibitor cocktail) was added to the cell pellet and incubated for 15 min on ice.

After lysis, 3 µg of the appropriate antibodies were added to each sample and incubated overnight, followed by 4 h incubation with 300 µg /mL of Protein G magnetic beads (Thermofisher), all rotated end-to-end in 4 °C. The beads were placed on a magnetic plate for 15 min on end in ice and washed 3X with cold lysis buffer before resuspending the samples in 2X sample buffer and boiled at 105°C for 10 min. The samples were used for immunoblotting (IB) or tandem mass spectrometry (MS/MS) sequencing.

2.15 Immunoblotting (IB)

For HEK 293T cell lysates that were not prepared for co-IP experiments, HEK 293T cells were harvested in RIPA buffer (150 mM NaCl, 1 % TX-100, 0.5% sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 50 mM Tris pH 8.0). Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermofisher) according to the manufacturer's protocol. Both the lysed HEK 293T cells and synaptosomes were then diluted with 1X sample buffer, boiled at 105 °C for 10 min.

All proteins were resolved in 10 to 12 % SDS-PAGE and transferred to Immobilon transfer membrane (EMD Millipore). Membranes were blocked in ChemiBlocker (EMD Millipore) for at least 1 h at room temperature before incubating in primary antibodies overnight at 4 °C, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. All membranes were washed 3X with 0.1 % Tween 20 in PBS, with 20 min per wash, after primary and secondary antibodies incubation. SuperSignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was then added to the blots and imaged immediately with Bio-Rad imaging system with analyses and quantifications of bands performed using Image LabTM.

2.16 Co-immunoprecipitation and LC-MS/MS analysis[#]

The synaptosomal co-IP lysates boiled in 2X sample buffer were separated on a 12 % SDS-PAGE and subjected to in-gel digestion using trypsin. The tryptic peptides were separated and analyzed using a Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive instrument (Thermo Fisher Scientific, San Jose, CA) as previously described (Gallart-Palau et al., 2019). Raw data files were converted to mascot generic file format using Proteome Discoverer 1.4 (Thermo Fisher Scientific, San Jose, CA). The MS/MS spectra were subjected to Mascot database search against UniProt mouse protein database. The exponentially modified protein abundance index (emPAI) of identified individual proteins were used for label-free protein quantitation as previously described (Gallart-Palau et al., 2019). Imp- β 1 specific binding proteins determined by subtracting the Imp- β 1 co-IP proteins with the background proteins in the Immunoglobulin G (IgG) control sample were shortlisted for further analyses.

[#] In collaboration with Associate Professor Sze Siu Kwan. Graduate student Tan Chee Fan performed the in-gel digestion and trypsinization and Assoc Prof. Sze analyzed the raw mass spectrometry results and generated the emPAI values.

2.17 Statistical analyses

All statistical analyses and graphs were done using GraphPad Prism 7.0. For two comparison groups, parametric t-test or Mann-Whitney non-parametric t-test were performed unless otherwise stated. For more than two comparison groups, one-Way ANOVA coupled with Tukey's post-hoc analysis (parametric) or Dunn's post-hoc analysis (non-parametric) was conducted unless otherwise specified. To test for normality, Shapiro-Wilk normality test was conducted for small sample size ($n < 30$) while D'Agostino & Pearson normality test was conducted for large sample size prior to One-Way ANOVA test. A probability value of $\alpha = 0.05$ was used to determine

statistical significance. Only under specific and rare instances, an outlier test using ROUT method, $Q = 1\%$ has been performed, specifically for the NDRG1 dendritic analyses (**Fig. 3-12D**) and Imp- β 1 localization analyses in KCl stimulation experiments in cultured hippocampal neurons (**Fig. 3-1A**).

Chapter 3. Results

3.1 Importin- β 1 is critical for transcription dependent plasticity

3.1.1 Neuronal stimulation triggers nuclear entry of importin- β 1 in hippocampal neurons

We performed a series of experiments to demonstrate the importance of Imp- β 1 during transcription-dependent plasticity. Previous reports have shown that the classical nuclear import pathway is sensitive to neuronal activity. In particular, Imp- α and - β 1 are shown to enter the nucleus during glutamatergic stimulation (Thompson et al., 2004). I replicated and extended these experiments by focusing specifically on the subcellular localization of Imp- β 1 in the neurons, labeled with the neuron specific antibody targeting against microtubule-associated protein 2 (MAP2). I tested several antibodies for Imp- β 1 and selected (3E9) monoclonal antibody which showed specificity for Imp- β 1 and robustly detects the protein in multiple subcellular compartments including the soma, dendrites, and the synapses (**Fig. S1**). As expected, 3E9 detects high concentration of Imp- β 1 around the nuclear membrane, presumably reflecting the docked Imp- β 1 at the NPC as reported by several publications (Gorlich, Vogel, Mills, Hartmann, & Laskey, 1995; A. C. Ström & K. Weis, 2001).

I then asked if different stimuli drive the nuclear entry of Imp- β 1 in neurons. I stimulated matured mouse hippocampal neurons in culture using a brief pulse of glutamate (40 μ M, 5 min) followed by immediate fixation. I observed rapid accumulation of Imp- β 1 in the nuclei of pyramidal neurons and this nuclear translocation is blocked in the presence of APV, an NMDA receptor (NMDAR) antagonist (**Fig. 3-1A**). Similarly, the stimulation of neurons with a brief pulse of KCl (50 mM; 5 min), coupled with glycine to preferentially enhance the activation of

NMDARs (Corera, Doucet, & Fon, 2009), also resulted in a robust nuclear accumulation of Imp- β 1, which is reversed in the presence of APV (**Fig. 3-1A**). Interestingly, the presence of tetrodotoxin (TTX; 1 μ M, 1 h), which blocks action potential firing in cultured neurons, did not further lower the Imp- β 1 proteins in the nucleus. This may reflect the baseline nucleocytoplasmic shuttling of Imp- β 1 that is independent of neuronal activity.

In addition to bath applications of glutamate and KCl, I also stimulated neurons with bicuculline (40 μ M, 30 min), a GABA_A receptor antagonist, to enhance overall network activity by blocking inhibitory inputs and promoting action potential bursting in excitatory neurons (Eisenman, Emnett, Mohan, Zorumski, & Mennerick, 2015). Unlike KCl and glutamate stimulation, bicuculline stimulation resulted in a moderate nuclear accumulation of Imp- β 1 (**Fig. 3-2A**). We reasoned that since bicuculline is a synaptic stimulus, the activity induced will be less intense. And since importins undergo rapid nucleocytoplasmic shuttling, it is therefore, not surprising that the bicuculline stimulation resulted in a modest enrichment of Imp- β 1 in the nucleus.

I further investigated if stimuli triggering LTD can also drive Imp- β 1 into the nucleus. Both DHPG (100 μ M, 20 min) and NMDA (20 μ M, 5 min) with glycine (20 μ M, 5 min) are known chemical stimuli to initiate LTD in cultures by targeting the metabotropic glutamate receptors (mGluRs) and NMDARs respectively (K. O. Lai et al., 2008; Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008). Using both stimulation paradigms, I observed a robust nuclear accumulation of Imp- β 1 in NMDA-dependent LTD with a milder, yet significant, nuclear accumulation of Imp- β 1 in DHPG-dependent LTD (**Fig. 3-1B**).

To complement the studies in cultured hippocampal neurons, I induced chemical stimulation in acute forebrain slices by incubating these slices in magnesium-free aCSF supplemented with forskolin (40 μ M, 15 min) and KCl (50 mM, 10 min) (Ch'ng et al., 2012; Chotiner, Khorasani, Nairn, O'Dell, & Watson, 2003; Thompson et al., 2004). The increase in the activity-dependent nuclear localization of CRTCL following chemical stimulation validated the slices were sufficiently stimulated (Ch'ng et al., 2015). Consistent with the observations made in the hippocampal cultures, Imp- β 1 accumulates in the nucleus in response to chemical stimulation in acute slices (**Fig. 3-1C**).

Taken together, these experiments indicate Imp- β 1 nuclear entry can be driven by various neuronal activity and it requires the stimulation of NMDARs. In addition, I also provide evidence showing Imp- β 1 nuclear shuttling during LTD.

Figure 3-1. Neuronal stimulation triggers nuclear entry of Imp- β 1 in hippocampal neurons

(A) Cultured mouse hippocampal neurons were treated with either tetrodotoxin (TTX; 1 μ M, 1 h), glutamate (Glut; 40 μ M, 5 min) or KCl (50 mM, 5 min) with glycine (100 μ M, 5 min) in the presence or absence of APV (40 μ M, 1 h) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), Imp- β 1 (red) and Hoechst nuclear dye (blue in merged image). The Imp- β 1 nuclear-to-cytoplasmic ratio was quantified and the group data from independent experiments were plotted. One way-ANOVA with Dunn's post-hoc analyses were conducted (Glutamate: N=3, KCl: N=2; **** p < 0.0001). Scale bar, 10 μ m. **(B)** Cultured hippocampal neurons were bath applied with DHPG (100 μ M, 20 min) or NMDA (20 μ M, 5 min) with glycine (20 μ M, 5 min) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), Imp- β 1 (red) and Hoechst nuclear dye (blue in merged image). The Imp- β 1 nuclear-to-cytoplasmic ratio was quantified and the group data from independent experiments were plotted. Unpaired T-test analyses were conducted between stimulated and basal conditions (N=2; ** p < 0.01, **** p < 0.0001). Scale bar, 10 μ m. **(C)** Representative confocal micrographs of neurons in dentate gyrus of the basal and stimulated acute forebrain slices. Stimulated acute forebrain slices were treated with forskolin (40 μ M, 15 min) and KCl (50 mM, 10 min) in magnesium free aCSF before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), CRTCl (red), Imp- β 1 (green) and Hoechst nuclear dye (blue). The CRTCl and Imp- β 1 nuclear-to-cytoplasmic ratio were quantified and the group data from independent experiments were plotted. Unpaired T-test analyses were conducted between stimulated and basal condition (N=3; *** p < 0.001, **** p < 0.0001).

3.1.2 Inhibiting importin- β 1 function negatively impacted on the nuclear events underlying transcription dependent plasticity

Imp- β 1 plays a critical role in the nuclear shuttling of many proteins. Thus, it is not surprising that a homozygous germline loss of Imp- β 1 in mice is embryonically lethal (Miura et al., 2006). To circumvent the deleterious effects of knocking out or knocking down Imp- β 1 protein in neurons, we employed a small molecule inhibitor, Inhibitor of Nuclear Import-43 (INI43), to study Imp- β 1 functions specifically during activity. INI43 was identified from an *in-silico* screen, using a structure-based approach, for compounds that bind to overlapping Imp- α 2 and Ran binding sites on Imp- β 1 with high affinity (van der Watt et al., 2016). Subsequent characterization on INI43 function show that it blocks the nuclear entry of endogenous Imp- β 1 and several of Imp- β 1 cargo proteins such as Nuclear factor of activated T-cells (NFAT), Nuclear factor kappa B (NF- κ B) and Activator protein 1 (AP-1) complex (Forwood, Lam, & Jans, 2001; Stelma & Leaner, 2017; van der Watt et al., 2016). It is noteworthy that NF- κ B is reported to engage Imp- α 3 and - α 4 (Fagerlund, Kinnunen, Kohler, Julkunen, & Melen, 2005) whereas NFAT nuclear entry is reported to engage Imp- β 1 only, independent of Imp- α , for its nuclear entry (Ishiguro et al., 2011). These indicate that INI43 is not limited to blocking only Imp- α 2 cargoes. Instead, INI43 directly inhibits Imp- β 1 nuclear entry and thus, blocking the nuclear shuttling of all Imp- β 1 cargoes. Notably, several studies reported that the use of either siRNA or INI43 to block Imp- β 1 function led to the same outcomes (Chi, van der Watt, Wei, Birrer, & Leaner, 2021; Stelma & Leaner, 2017). Moreover, the overexpression of Imp- β 1 can rescue the effect of INI43 (van der Watt et al., 2016). Together, these provide evidence on the specificity of INI43 targeting Imp- β 1.

I optimized INI43 treatment parameters (5 μ M, 1 h) in cultured hippocampal neurons and show that it attenuates activity-dependent nuclear accumulation of Imp- β 1 following bicuculline and glutamatergic stimulation in pyramidal neurons (**Fig. 3-2A**). In addition, I also asked if INI43 can inhibit the nuclear entry of NLS-bearing cargo proteins by selecting NFATc3, a transcription factor that associates with Imp- β 1 and responds to activity by undergoing nuclear translocation in neurons (Ishiguro et al., 2011; Murphy, Crosby, Dittmer, Sather, & Dell'Acqua, 2019; Torgerson, Colosia, Donahue, Lin, & Hawiger, 1998; Wild et al., 2019). Consistent with the published reports, depolarization drives transiently expressed sGFP-NFATc3 into the nucleus and this translocation is partially inhibited by INI43 (**Fig. 3-2B**). Of note, I also validated the specificity of INI43 by showing the activity-induced CRTCl nuclear accumulation in neurons is unaffected by INI43 treatment since CRTCl nuclear entry is independent of Imp- β 1 (Ch'ng et al., 2015) (**Fig. 3-2C**).

After validating INI43 efficiency and specificity, I next investigated if the blockade of Imp- β 1 function in neurons using INI43 alters the molecular pathways associated with transcription dependent plasticity. I first looked at the phosphorylation of cAMP-response element binding protein (CREB) at the residue serine 133 (pCREB S133) (Ch'ng et al., 2012; Shaywitz & Greenberg, 1999). The phosphorylation of CREB S133 depends largely on the activity of different kinases including calmodulin-dependent protein kinase I and II (CamkI, CamkII) as well as protein kinase A and C (PKA, PKC) (Johannessen & Moens, 2007). As expected, bicuculline (50 μ M, 30 min) stimulation of the cultured hippocampal neurons, with the addition of 4AP (200 μ M, 30 min) to increase the bursting frequency of bicuculline-induced action potential (Hardingham et al., 2001), enhances the phosphorylation of CREB S133. In contrast, when Imp- β 1 function is blocked using INI43, the phosphorylation of CREB S133 is

greatly diminished (**Fig. 3-2D**). Similarly, I asked if the nuclear enrichment of JunB, a component of AP-1 complex, after stimulation is altered in the presence of INI43 (C. M. Alberini, 2009). The data indicate that INI43 reduces the nuclear accumulation of JunB upon bicuculline + 4AP stimulation, suggesting the activity-induced nuclear localization of JunB depends on Imp- β 1 (**Fig. 3-2D**).

Since the transcription of many activity-regulated genes rely on the activation of CREB (Saha et al., 2011), we anticipate the absence of stimulus-driven Imp- β 1 mediated nuclear import, which we have shown to inhibit the phosphorylation of CREB S133, would negatively impact on the expression of activity-regulated genes. The quantitative PCR (qPCR) analyses of the treated cultured hippocampal neurons show the expression of the activity-regulated genes, namely the *arc*, *junB*, *egr4*, *cfos* and *npas4*, increases following bicuculline + 4AP stimulation, and this increment is completely abolished in the presence of INI43 (**Fig. 3-2E**). Importantly, the transcript level of HPRT1, an activity-independent housekeeping gene, is comparable across all treatment conditions (**Fig. 3-2E**) (Santos & Duarte, 2008). In contrast, the HPRT1 transcript level was much lower in the H₂O₂ induced apoptotic neurons compared to the basal neurons, with most of the independent runs failing to detect the transcripts within 40 amplification cycles (**Fig. 3-2F**). This at least indicates that the cells are viable throughout the INI43 treatment and the degradation of housekeeping genes associated with apoptosis is not triggered during the treatment period (X. Liu et al., 2018). Moreover, the immunoreactivity of cleaved caspase 3, a hallmark for apoptosis, is comparable between basal and INI43 treated neurons, and both are significantly lower than the H₂O₂ induced apoptotic neurons (**Fig. 3-2F**; (J. H. Park et al., 2016)). This further illustrates that the transient exposure of INI43 does not trigger apoptosis in neurons and that the loss of nuclear signaling following INI43 treatment is not due

to the activation of cell death pathway. Collectively, the data indicate Imp- β 1 mediated nuclear import mechanism is required for initiating the nuclear events critical for the establishment of long-term plasticity.

Figure 3-2. Inhibiting Imp- β 1 function negatively impacted on the nuclear events underlying transcription-dependent plasticity

(A) Cultured hippocampal neurons were treated with bicuculline (Bic; 40 μ M, 30 min) or glutamate (Glut; 40 μ M, 5 min) in the presence or absence of INI43 (5 μ M, 1 h) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), Imp- β 1 (red) and Hoechst nuclear dye (blue in merged image). The Imp- β 1 nuclear-to-cytoplasmic ratio was quantified and the group data from independent experiments were plotted. One way-ANOVA with Tukey's (Bic) or Dunn's (Glut) post-hoc analyses were conducted (N=2; * p < 0.05, *** p < 0.001, **** p < 0.0001). Scale bar, 10 μ m. **(B)** Representative confocal micrographs of TTX-silenced and KCl-depolarized cultured hippocampal neurons (DIV14) transfected with sGFP-NFATc3 plasmids on DIV13, with or without INI43 treatment, immunolabeled with antibodies targeted against MAP2 (cyan), GFP (red) and Hoechst nuclear dye (blue in merged image). All neurons were pre-treated with TTX (1 μ M, 30 min) to reduce baseline activity. INI43 (5 μ M, 30 min) treatment was performed (where indicated) in the presence of TTX. Neurons were then depolarized with KCl (50 mM, 3 min) in the presence of INI43 (where indicated). All the neurons were then recovered in Tyrode's solution containing TTX (1 μ M, 10 min), with INI43 (5 μ M) where applicable. The nuclear-to-cytoplasmic ratio of sGFP-NFATc3 staining in neurons were quantified and group data is represented as bar plots. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses (N=2; * p < 0.05, ** p < 0.05, **** p < 0.0001). Scale bar, 5 μ m. **(C)** Representative confocal micrographs of basal and stimulated cultured hippocampal neurons, in the presence or absence of INI43, immunolabeled with antibodies targeted against MAP2 (cyan), CRTc1 (red) and Hoechst nuclear dye (blue in merged image). Cultured neurons were treated with bicuculline (Bic; 50 μ M, 30 min) + 4AP (200 μ M, 30 min), with or without INI43 (5 μ M, 1 h). For experiments with INI43, neurons were pre-treated with the inhibitor prior to and during stimulation. The

nuclear-to-cytoplasmic ratio of CRTCl in neurons were quantified and group data is represented as bar plots. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses (N=2; **** $p < 0.0001$). Scale bar, 10 μ m. **(D)** Representative confocal micrographs of basal and stimulated cultured hippocampal neurons, in the presence or absence of INI43, immunolabeled with antibodies targeted against MAP2 (cyan), pCREB (S133) or JunB (red) and Hoechst nuclear dye (blue in merged image). Cultured neurons were treated with various pharmacological agents including tetrodotoxin (TTX; 1 μ M, 1 h), bicuculline (Bic; 50 μ M, 30 min) + 4AP (200 μ M, 30 min) and INI43 (5 μ M, 1 h). For experiments with INI43, neurons were pre-treated with the inhibitor prior to and during stimulation. The mean nuclear intensity of pCREB (S133) staining and the nuclear-to-cytoplasmic ratio of JunB in neurons were quantified and the group data is represented as bar plots. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses (pCREB: N=3, JunB N=2; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Scale bar, 10 μ m. **(E)** Quantitative PCR (qPCR) analyses on the expression of activity-regulated genes in basal and stimulated cultured hippocampal neurons, with or without INI43 treatment. Cultured neurons were treated with various pharmacological agents including bicuculline (Bic; 50 μ M, 30 min) + 4AP (200 μ M, 30 min) and INI43 (5 μ M, 1 h). For experiments with INI43, neurons were pre-treated with the inhibitor prior to and during stimulation. Bar graphs depict the $2^{-\Delta\Delta C_t}$ normalized to basal condition. Statistical analyses performed on group data use one way-ANOVA with Tukey's post-hoc analyses (N=3; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). The mean C_t values of the housekeeping gene, HPRT1, for each treatment are plotted. **(F)** The mean HPRT1 C_t values for basal and H₂O₂ induced apoptotic cultured hippocampal neurons (100 μ M, 24 h) for 5 independent experiments were listed in table.

“Undetermined” indicate the number of amplified transcripts did not reach the threshold with 40 cycles of amplification. Representative confocal micrographs of basal neurons, with or without INI43 treatment, as well as H₂O₂ treated neurons immunolabeled with antibodies targeted against MAP2 (cyan), cleaved caspase 3 (red) and Hoechst nuclear dye (blue in merged image). Cultured neurons were treated with various pharmacological agents including INI43 (5 μ M, 1 h) and H₂O₂ (500 μ M, 2 h). The mean cleaved caspase 3 staining in the soma were quantified and normalized against the basal neurons. The group data is represented as bar plots. Statistical analyses performed with one way-ANOVA with Dunn’s post-hoc analyses (N=2; *** p < 0.001, **** p < 0.0001). Scale bar, 10 μ m.

3.1.3 Inhibition of importin- β 1 function impairs late-LTP and LTD

Late phase LTP (late-LTP) in the CA3 to CA1 hippocampal circuit is transcription dependent (P. V. Nguyen & Kandel, 1997). Experiments have shown the addition of ActD and other transcriptional inhibitors impair late-LTP (Frey, Frey, Schollmeier, & Krug, 1996b; P. Nguyen et al., 1994). To evaluate if Imp- β 1 mediated nuclear entry is also necessary for transcription dependent late-LTP, a two-pathway model (**Fig. 3-3A**) is employed using *in vitro* electrophysiology. Bath application of INI43 for 60 min after recording a stable baseline of 30 min in wild-type mouse hippocampal slices did not alter the baseline recordings (**Fig. 3-3B**). The slope of the field excitatory postsynaptic potential (fEPSP) evoked every 5 min after drug application remained unchanged for a total of 180 min. No significant changes were observed in fEPSP values throughout the recording when compared with its own baseline ($p > 0.05$, Wilcoxon test). The results confirm that the basal synaptic transmission is not affected when Imp- β 1 mediated nuclear transport is blocked by INI43.

As a control experiment, the data showed the application of a strong tetanus (STET) that consisted of 100 Hz, 100 pulses at 0.2 ms resulted in late-LTP that was stable for a recorded period of 180 min (**Fig. 3-3C**). A statistically significant potentiation was observed immediately after tetanus at 1 min ($p = 0.0156$, Wilcoxon test; $p = 0.0006$, U-test) and it persisted until 180 min ($p = 0.0156$, Wilcoxon test; $p = 0.0006$, U-test). The synaptic potentiation was input specific as no significant shift in fEPSPs in the unpaired control input S2 was observed (open circles). Next, to test the role of Imp- β 1 in transcription dependent plasticity, late-LTP was induced using STET in the presence of INI43 (**Fig. 3-3D**). After recording a stable baseline for 30 min, INI43 was bath applied to the hippocampal slices for 60 min and late-LTP was induced

30 min after the beginning of the drug application in S1. Induction of late-LTP during the inhibition of Imp- β 1 mediated nuclear import by INI43 prevented the late-LTP without affecting the persistence of early-LTP. A significant potentiation was observed immediately after tetanus at 1 min ($p = 0.0156$, Wilcoxon test; $p = 0.0006$, U-test), and it remained significant until 120 min ($p = 0.0156$, Wilcoxon test; $p = 0.0262$, U-test), and then it decayed back to baseline at 180 min ($p = 0.973$, Wilcoxon test) (**Fig. 3-3D**; filled circles). No significant changes were observed in the control input S2 and it remained stable throughout the period of recording (open circles). When compared between the two groups of LTP with and without INI43 (**Fig. 3-3C & D**), no significant difference was observed from 1 min until 60 min (at 1 min, $p = 0.2086$, U-test; at 60 min, $p = 0.7104$, U-test) but from 120 min onwards, it showed significant difference and remained significant until 180 min (at 120 min, $p = 0.0012$, U-test; at 180 min $p = 0.0023$, U-test).

LTD is another form of long-term plasticity and several forms of LTD have been reported to be transcription dependent (Kauderer & Kandel, 2000; Kemp, Tischmeyer, & Manahan-Vaughan, 2013; O'Riordan et al., 2006). In addition, since importins are released upon the activation of NMDARs and given that LTD also involves the activation of NMDARs, we hypothesized that the Imp- β 1 mediated transcriptional mechanisms may play a role in LTD. To test this hypothesis, a two-pathway model (**Fig. 3-3A**) is again employed using *in vitro* electrophysiology, but in this case, late-LTD was induced using a strong low frequency stimulation (SLFS) consisted of 900 pulses (Sajikumar & Frey, 2003; Shetty et al., 2015). A robust synaptic depression was recorded for up to 180 min post-SLFS (**Fig. 3-3E**). Application of SLFS led to a significant decrease in fEPSP values from 1 min ($p = 0.0313$, Wilcoxon test; $p = 0.0022$, U-test) and it remained stable until the end of the recording at 180 min ($p =$

0.0313, Wilcoxon test; $p = 0.0022$, U-test). Subsequently, to study the role of Imp- β 1 in LTD, INI43 is applied during the induction of late-LTD. The same experiment as in **Fig. 3-3E** is repeated but now, in the presence of INI43, 30 min before and after SLFS (**Fig. 3-3F**). Interestingly, unlike LTP, a significant impairment in LTD is observed at an earlier time point. Field EPSP values were significantly different at 1 min ($p = 0.0391$, Wilcoxon test; $p = 0.0112$, U-test) and then from 5 min onwards, it was not significant ($p = 0.7422$, Wilcoxon test; $p > 0.9999$, U-test) until the recorded period of 180 min ($p > 0.9999$, Wilcoxon test; $p = 0.7416$, U-test). The control input S2 did not show significant changes in both sets of experiments for the entire recording period (open circles). When compared between the two groups of LTD with and without INI43 (**Fig. 3-3E & F**), we observed a significant difference from 1 min ($p = 0.008$, U-test) until 180 min ($p = 0.0027$, U-test).

Altogether, the hippocampal slice recordings provide the first direct evidence illustrating the importance of Imp- β 1 in transcription dependent plasticity by showing the inhibition of Imp- β 1 mediated nuclear entry negatively affected long-term plasticity. Specifically, we reported an impaired late-LTP following the blockade of Imp- β 1's function while leaving the early-LTP unaffected in tetanized hippocampal slices. In addition, the data suggest a role of Imp- β 1 in LTD. The potential reasons for the observed early LTD impairment are revisited in discussion.

Figure 3-3. Inhibition of Imp- β 1 function impairs late-LTP and LTD

(A) Schematic representation of a transverse hippocampal slices showing the electrodes positioning in the CA1 region for field EPSP (fEPSP) recording. Two stimulating electrodes S1 and S2 were placed in the stratum radiatum to stimulate two independent Schaffer Collateral synaptic inputs to the same neuronal population. Recording electrode (Rec) was placed in the CA1 apical dendrites between the two stimulating electrodes. Calibration scale bars for all analog traces: vertical: 3 mV horizontal: 5 ms.

(B) Basal synaptic transmission is not affected by the application of INI43. Application of INI43 for 60 min after recording a stable baseline for 30 min did not alter the baseline values and it remained stable for 180 min.

(C) Late-LTP was induced by STET (arrows) in S1 (filled circles) after recording a stable baseline for 30 min. Open circles represent control input S2, which remained stable throughout the 180 min period of recording. The test stimulation was given every 5 min.

(D) Experimental design was the same as in (C), but with the exception that INI43 was applied 30 min before and 30 min after the induction of late-LTP, which resulted only in an early-LTP.

(E) Late-LTD was induced by SLFS (arrow) after recording a stable baseline for 30 min which resulted in a depression that was stable for a recording period of 180 min.

(F) Experimental design was the same as in (E), but with the exception that INI43 was applied 30 min before and 30 min after the induction of late-LTD. This resulted in the total abolishment of LTD. Control inputs S2 were stable in both experiments throughout the period of recording.

Dr. Sheeja Navakkode performed and analyzed the data for all electrophysiology experiments.

3.2 Importin- β 1 is localized at the synapto-dendritic compartment and undergoes activity-dependent nuclear translocation

Having established the importance of Imp- β 1-mediated nuclear import during transcription dependent plasticity, I next explored the role and regulation of Imp- β 1 in the synapto-dendritic compartment. Imp- β 1 is ubiquitously detected cell-wide and to verify endogenous localization of Imp- β 1 in the synapto-dendritic compartment, I virally expressed GFP under the CamKII α promoter to label spines in excitatory neurons (Thompson et al., 2004). Imp- β 1 fluorescent puncta are found throughout the synapto-dendritic compartment, including a large majority in the GFP-positive spines (**Fig. 3-4A**).

Since Imp- β 1 is a nuclear adaptor protein, I performed time-lapse imaging to examine if the synapto-dendritic localized Imp- β 1 undergoes activity-dependent movement into the nucleus. To that end, I transiently expressed Imp- β 1 tagged to a photoconvertible fluorescent protein, Dendra2 (Imp- β 1-Dendra2), or Dendra2 alone constructs in the excitatory neurons in rat hippocampal cultures using a CamKII α promoter for a short period (14-16 h) to limit overexpression. To label a subpopulation of Imp- β 1-Dendra2 or Dendra2 alone protein exclusively in the synapto-dendritic compartments, UV laser was used to photoconvert the Dendra2 signal from green to red in three distinct synapto-dendritic compartments labeled R1 to R3. Subsequently, to detect the nuclear shuttling of these labeled synapto-dendritic localized Imp- β 1 in response to activity, I measured the intensity of the photoconverted Imp- β 1-Dendra2 or Dendra2 red-shifted signals in the nucleus in the presence or absence of bicuculline stimulation.

Importantly, each photoconverted region showed an increase in the red-shifted (568nm) Dendra2 signal immediately upon photoconversion without a corresponding

increase at the soma (**Fig. 3-4B**). Time-lapse images obtained over 15 min, at 1 min per frame, indicate a gradual but significant nuclear accumulation (starting at 5 min post-conversion) of the red-shifted Imp- β 1-Dendra2 signal only in the bicuculline stimulated neurons (**Fig. 3-4C**). Unlike other synapse-to-nucleus signaling proteins that show increasing nuclear accumulation over time, the increase in the nuclear, red-shifted Imp- β 1-Dendra2 reaches a plateau and achieves equilibrium, which is consistent with the idea that Imp- β 1 undergoes rapid nuclear-cytoplasmic shuttling. Notably, I did not observe a significant, red-shifted Imp- β 1-Dendra2 nuclear accumulation in neurons under basal conditions. Similarly, there is no significant accumulation of red-shifted Dendra2 alone in both the basal and the bicuculline stimulated neurons (**Fig. 3-4C**). The appearance of red-shifted Dendra2 control signal in the nucleus shortly after photoconversion is likely to be due to diffusion and is independent of bicuculline stimulation. Taken together, the live cell imaging experiments show the activity-dependent translocation of synapto-dendritic Imp- β 1 to the nucleus.

(A) Cultured mouse hippocampal neurons expressing virally delivered GFP driven by CamKII α promoter. Neurons were fixed and immunolabeled with antibodies targeted against MAP2 (blue), GFP (green) and Imp- β 1 (red). White dotted boxes (A & B) are magnified regions with arrows indicating Imp- β 1 positive spines. Scale bar 10 μ m (1 μ m in magnified images). **(B)** Cultured rat hippocampal neurons expressing Imp- β 1-Dendra2 or Dendra2 alone constructs were photoconverted at three regions indicated by the white boxes (R1-R3) using UV (405 nm) laser. Representative confocal micrographs of R1-R3 and soma pre- and post-photoconversion are shown with the corresponding before-after photoconversion graphs of the red-shifted Dendra2 (568 nm) intensity, shown as heat map for better contrast. Statistical analyses performed on group data use Mann-Whitney non-parametric t-test (n=16; * p < 0.05, ** p < 0.01). **(C)** After photoconversion, the nuclear accumulation of the photoconverted red-shifted signal (568 nm) was tracked for 15 min (at 1 frame per min). Time lapse images of the somatic photoconverted Imp- β 1-Dendra2 and Dendra2 alone signals (every 5th frame) are shown for basal and bicuculline (Bic; 40 μ M) stimulated neurons. Quantification and plotting of line graphs show normalized photoconverted signal in the nucleus. Statistical analyses performed on group data use Mann-Whitney non-parametric T-test for comparing between treatment groups at the same time point and one way-ANOVA with Dunn's post-hoc analyses to compare between the different time points against t=1 within the same treatment group. (Basal: Dendra2, n=7; Bic: Dendra2, n=5; Basal: Imp- β 1-Dendra2, n=10; Bic: Imp- β 1-Dendra2, n=11, * p < 0.05, Bic-Imp- β 1-Dendra2, t=1 as compared to t=3 to 13; * p < 0.05; Bic-Imp- β 1-Dendra2 as compared to Basal-Imp- β 1-Dendra2, from t=5 to 15 * p < 0.05). Bar graph depicts the change in red-shifted Dendra2 (568 nm) nuclear intensity between the first- and the final-time points (t=1 and t=15). One way-ANOVA with Dunn's post-hoc analyses were performed

between the different treatments against the change in nuclear intensity of red-shifted Imp- β 1-Dendra2 in basal neurons (* $p < 0.05$).

3.3 Importin- β 1 is regulated through local translation in the synapto-dendritic compartment in response to synaptic stimulation

3.3.1 Importin- β 1 is locally translated in the synapto-dendritic compartment basally

Neurons are highly compartmentalized cells with some synaptic junctions situated far from the soma. Since Imp- β 1 moves from the synapto-dendritic compartment to the nucleus upon stimulation, it is likely that the Imp- β 1 is locally regulated at or near the synapses. Local translation in the synapto-dendritic compartment is well-accepted as a rapid and efficient way to respond to synaptic needs during stimulation (Biever, Donlin-Asp, & Schuman, 2019; Tushev et al., 2018). Local synthesis of new proteins is critical for regulating many cellular mechanisms associated with synaptic plasticity including spine growth, cytoskeletal reorganization, and ribosomal translation (De Rubeis et al., 2013; Miller et al., 2002; Newpher, Harris, Pringle, Hamilton, & Soderling, 2018; Poon, Choi, Jamieson, Geschwind, & Martin, 2006). Unlike Imp- α , where it has been shown to tether to the cytoplasmic tail of the NR1-1a subunit of NMDARs and is only released during glutamatergic stimulation (Jeffrey, Ch'ng, O'Dell, & Martin, 2009), nothing is known about the regulation of Imp- β 1 at the synapses.

Imp- β 1 transcripts has been reported in the mid-axons, dendrites and in the presynaptic compartment (Hafner, Donlin-Asp, Leitch, Herzog, & Schuman, 2019; R. B. Perry et al., 2012; Poon et al., 2006). Specifically, two Imp- β 1 isoforms have been identified in rat neurons, with the longer isoform preferentially targeted to the axons (R. B.-T. Perry et al., 2012). Notably, local translation of the long Imp- β 1 isoform occurs in response to axonal injury (R. B.-T. Perry et al., 2012). This hints at the possibility that Imp- β 1 could be regulated via local translation in the synapto-dendritic

compartment following activity (Biever et al., 2019; Holt, Martin, & Schuman, 2019; Tushev et al., 2018).

The presence of multiple Imp- β 1 isoforms mainly arises from the differential usage of alternate polyadenylation (PolyA) signals at the 3' untranslated region (UTR). Using algorithms to detect the differential usage of PolyA signals (Proudfoot, 2011), I identified alternate PolyA signals in the mouse Imp- β 1 mRNA (NM_008379.3) that would correspond to a full-length Imp- β 1 mRNA (5.9 kb) as well as the long and short isoforms described by Perry *et al.* (2012) (**Fig. S2**). Next, using primers targeting different regions of the Imp- β 1 3' UTR, specifically to identify the presence of full-length (F1 and F2), long (L1 and L2) and short (S1) isoforms, I performed qPCR to not only detect, but also to determine the relative abundance of each isoform in the purified synaptosomes from the mouse forebrain (**Fig. 3-5A, Table 2-1**). The data indicate that first, all three Imp- β 1 mRNA isoforms are detected in the synaptosomes (**Fig. 3-5A**). Second, the proportion of the combined long and full-length isoforms represented approximately 18.6 % of the total Imp- β 1 mRNA species at the synaptosomes, of which 5.1 % of the transcripts were attributed to the full-length Imp- β 1 species alone (**Fig. 3-5A**). To further distinguish if a specific Imp- β 1 isoform is enriched in the synaptosomes, I extracted and profiled the Imp- β 1 transcripts from the cytosolic fraction of the mouse forebrain. I observed a similar distribution as seen in the synaptosome preparation for all three Imp- β 1 isoforms, suggesting no enrichment of any specific type of Imp- β 1 mRNA isoform at the synapse (**Fig. 3-5A**). Importantly, using either random hexamers or oligo-dT priming to synthesize cDNA from the total synaptosomal RNAs generated the same results and all primer pairs selected for this study are highly specific for each isoform with no background contamination (**Fig. 3-5A, S3**).

To directly demonstrate if Imp- β 1 local translation occurs at the synapto-dendritic compartment, I next performed puromycin labeled proximity-ligation assay (Puro-PLA) in cultured mouse hippocampal neurons. Puro-PLA enables the visualization of newly synthesized Imp- β 1 proteins by detecting the puromycylated nascent protein chains at the site of synthesis. The subsequent spatial coincidence between the two oligonucleotide probes, one conjugated to an antibody targeted against puromycin (known as minus probe) and the other conjugated to an antibody targeting Imp- β 1 (known as plus probe), will produce a fluorescent signal that identifies newly translated Imp- β 1 proteins. In addition, I also pre-incubated the cultured neurons with cycloheximide (CHX) prior to puromycin labeling. The CHX treatment serves to stall the ribosome and immobilizes the nascent chain, thereby allowing the detection of the entire ribopuromycylated structure at the site of synthesis (**Fig. 3-5B**) (David et al., 2012; tom Dieck et al., 2015).

To examine the specificity of the labeling and to establish baseline signals, I optimized the assay by incubating the cultured neurons with only a single probe, either the minus or the plus probe, as well as in the absence of puromycin to demonstrate low background signals. In contrast to the controls, using the complete protocol for Puro-PLA generated a robust signal for Imp- β 1 translational machineries not only in the neuronal soma, but also in the MAP2 positive dendrites (**Fig. 3-5C**). Since Imp- β 1 is not neuron-specific, the Imp- β 1-Puro-PLA structures that did not colocalize with MAP2 staining is likely to be the Imp- β 1 translational complexes present in non-neuronal cells.

As Imp- β 1 local translation can be detected along the proximal and the distal segments of the dendrites, we wanted to further examine the distribution of these ribopuromycylated structures in the synapses. To get a quantitative measure of the

Puro-PLA signals for Imp- β 1 at the synapses, I triple labeled all samples with antibodies targeted against the pre-synaptic marker synaptophysin (SYPH), post-synaptic marker (PSD95), along with MAP2 to identify dendrites. Since synaptic junctions are three-dimensional structures, a two-dimensional rendition cannot accurately resolve the colocalization between the ribopuromycylated signals and the neuronal markers staining. As such, I performed a high resolution AiryScan confocal imaging of the dendrites from the pyramidal neurons (70 μ m from soma), followed by image deconvolution and volumetric reconstruction of the synapses with IMARIS. I rendered three-dimensional surfaces based on the Imp- β 1 Puro-PLA signals and examined the extent of colocalization between Imp- β 1 Puro-PLA signals and all the synaptic and dendritic markers (**Fig. 3-5D**).

Most of the Imp- β 1 Puro-PLA structures can be classified into one of the four categories: First, approximately 19.0 % of the analysed Imp- β 1 Puro-PLA structures shared at least partial colocalization with MAP2 dendritic marker alone, indicating the presence of ribosome-bound Imp- β 1 translational complexes in the dendrites (**Fig. 3-5D**). I next analysed the Imp- β 1 Puro-PLA structures that colocalized with the synaptic markers and found a proportion of them exhibiting at least a partial overlap with either the post-synaptic compartment (19.7 %), the pre-synaptic compartment (13.7 %) or with both the synaptic markers (16.4 %) (**Fig. 3-5D**). Importantly, most of these volumetric structures are localized close to or overlap with MAP2 signal, indicating its proximity to dendrites. Finally, around one-third (31.7 %) of the Imp- β 1 Puro-PLA structures do not colocalize with any of the neuronal markers and hence, are likely to be present in the surrounding glial cells (**Fig. 3-5D**). Collectively, these assays reveal the presence of Imp- β 1 transcripts in the synaptosomes and its local translation in the synapto-dendritic compartment in basal hippocampal neurons.

Figure 3-5. Imp- β 1 transcripts are found in synaptosomes and are locally translated in the synapto-dendritic compartment in basal hippocampal neurons

I) Dendrite

II) Post-synaptic

III) Pre-synaptic

IV) Pre- and Post-synaptic

13.7 ± 2.77	Pre-synaptic
16.4 ± 4.44	Pre- and Post-synaptic
19.0 ± 3.58	Dendrite
19.7 ± 3.92	Post-synaptic
31.2 ± 3.17	Others

(A) Schematic of a mouse Imp- β 1 mRNA showing the last two exons followed by the full length 3' UTR. The red boxes indicate exons in the open reading frame (ORF) while the white box indicates the full length 3' UTR with the relative size of the long and short isoforms indicated as lines. The bidirectional arrows indicate the relative location of primers used to detect the full-length isoform (F1 and F2), long isoform (L1 and L2), and short isoform (S1). The qPCR products were separated on agarose gel. The qPCR results were represented in bar graph depicting the fold change ($2^{-\Delta\Delta CT}$) normalized to the common primer (S1), obtained from both the cytosolic and the purified synaptosomes fractions. The percentage of each splice isoform relative to S1 is indicated at the top of the bar graph (N = 3). Note that L1/L2 values include both the long and the full-length isoforms. **(B)** Schematic for puromycin labeled proximity ligation assay (Puro-PLA). Cycloheximide (CHX) is first added to the cultured hippocampal neurons to stall translation at the site of synthesis. Puromycin is then added to label the truncated nascent peptides. In another word, if there is newly synthesized Imp- β 1, the newly synthesized Imp- β 1 will be labeled with puromycin. Next, the oligonucleotide conjugated antibodies targeted against puromycin (termed as minus probe; red Y) and Imp- β 1 (3E9) (termed as plus probe; blue Y) were added. The antibodies targeted against puromycin (minus probe) will bind to the puromycin while the antibodies targeted against Imp- β 1 (plus probe) will bind to Imp- β 1. Consequently, the minus and the plus probes would be in proximity for the newly synthesized Imp- β 1, and this would allow the template linker oligo to ligate both the plus and minus probes into a circle, enabling circling amplification. Lastly, the binding of the fluorescent molecules on the amplified probes generates the Puro-PLA signal (green fluorescence). **(C)** Cultured mouse hippocampal neurons were pre-treated with cycloheximide (CHX; 50 μ M (for single probe controls) or 355 μ M (for puromycin

control), 30min) before the addition of puromycin (Puro; 3 μ M, 15 min) where indicated. After Puro-PLA, the neurons were fixed and immunolabeled with antibodies targeted against MAP2 (violet) and Hoechst nuclear dye (blue in merged image). The Puro-PLA signal specific for Imp- β 1 appears as green puncta (inverted to black and white for contrast). The integrated Puro-PLA intensity normalised to total MAP2 area in the field was quantified and the group data from independent experiments were plotted. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses for single probe controls (N=2; * p < 0.05, ** p < 0.01) and unpaired T-test analysis for comparison between conditions with and without puromycin (N = 3; n = 30; **** p < 0.0001). Scale bar, 2 μ m. **(D)** Cultured mouse hippocampal neurons were pre-treated with cycloheximide (CHX; 355 μ M, 30 min), before undergoing Puro-PLA (green) in the presence of puromycin (Puro; 3 μ M, 15 min), followed by fixation and immunolabeling with antibodies targeted against MAP2 (grey), PSD95 (red) and synaptophysin (Syph; violet). Neurons were imaged and deconvoluted using Zeiss AiryScan deconvolution module and signals for each channel were rendered into a three-dimensional object and analysed for colocalization with Imp- β 1-Puro-PLA signals using IMARIS (*Upper panels*). Individual Puro-PLA puncta specific for Imp- β 1 that colocalized with synapto-dendritic markers are projected along their 3 axes (xy , xz and yz) and categorized as translation occurring in dendrites (MAP2; Category I), post-synaptic (PSD95; Category II), pre-synaptic (SYPH; Category III), and pre- and post-synaptic (PSD95 and SYPH; Category IV) compartments. The relative distribution for each category is indicated in the bar chart (*lower panels*, N = 3, n = 161). Scale bar, 10 μ m (dendrite) or 2 μ m (puncta).

3.3.2 Increased importin- β 1 protein level in stimulated synaptosomes and in the dendrites and spines of stimulated hippocampal cultures that is sensitive to cycloheximide

After showing Imp- β 1 local translation in cultured neurons under basal conditions, we next asked if neuronal activity and synaptic stimuli can enhance local translation of Imp- β 1 in the synapto-dendritic compartment. To address this, I first purified synaptosomes from the forebrains of male mice by extracting and homogenizing the forebrain tissues and subjecting the homogenized lysates through gradient centrifugation (**Fig. 2-1**). The purified synaptosomes were then stimulated with KCl in the presence of glycine to trigger local translation. I chose this stimulation paradigm as it has been used to induce an increased translation of α -CaMKII and BDNF in the stimulated synaptosomes (Bagni, Mannucci, Dotti, & Amaldi, 2000; Gharami & Das, 2014). Following stimulation, I then performed affinity purification using 3E9 antibody to extract and enrich for Imp- β 1 protein. While Imp- β 1 protein is readily detected in the synaptosomes via immunoblotting, we reasoned that the affinity purification step will enhance the detection of any changes in Imp- β 1 concentration due to protein synthesis. As predicted, I consistently observed an average of 1.5-fold increment in Imp- β 1 protein level in the stimulated synaptosomes as compared to those in mock treatment (**Fig. 3-6A**). Notably, the activity-induced increment of Imp- β 1 is lost when the synaptosomes were stimulated in the presence of a translation inhibitor, CHX, indicating the increment is due to local synthesis of Imp- β 1 (**Fig. 3-6A**).

As synaptosomes contain both the pre- and the post-synaptic structures, I was not able to determine if the local translation of Imp- β 1 occurs in one or both the subcellular compartments in response to activity. Specifically, we wanted to identify if the activity-dependent increase in Imp- β 1 local translation is detected in the synapto-

dendritic compartment. Unlike bath application of KCl or glutamate, which led to a robust Imp- β 1 nuclear accumulation (**Fig. 3-1A**) with a corresponding reduction in dendritic Imp- β 1 (**Fig. S4**), bicuculline-induced action potential bursting produces a moderate Imp- β 1 nuclear accumulation (**Fig. 3-2A**). We reasoned that under these circumstances, the effect of local translation could be observed during the initial stages of bicuculline stimulation as the stimulus would not drive all the newly synthesized Imp- β 1, if any, into the nucleus. I briefly stimulated neurons with bicuculline (40 μ M, 30 min) and showed mild nuclear accumulation of Imp- β 1 that is not sensitive to CHX treatment (**Fig. 3-6B**). I then quantified Imp- β 1 intensity in the dendrites (0-70 μ m from soma) as well as in the spines labeled with GFP. I observed a significant increase in Imp- β 1 staining in both the subcellular compartments in the stimulated as compared to the basal neurons (**Fig. 3-6B**). Importantly, unlike in the nucleus, the increase in both the dendritic and post-synaptic Imp- β 1 in the stimulated neurons was abolished after CHX treatment (**Fig. 3-6B**).

Figure 3-6. Increased Imp-β1 protein level in stimulated synaptosomes and in the dendrites and spines of stimulated hippocampal cultures that is sensitive to cycloheximide

(A) Purified synaptosomes from male mice forebrain were left untreated (mock) or stimulated with KCl (50 mM, 5 min) and glycine (100 μ M, 5 min) in the presence or absence of cycloheximide (CHX; 50 μ M, 30 min prior to and during KCl and glycine treatment), followed by co-IP using Imp- β 1 antibody (3E9). Blots were probed with 3E9 and the normalized Imp- β 1 against the light chain (LCh) band intensities were quantified. Unpaired T-test analysis was conducted between the two conditions ($*p < 0.05$). (B) Representative confocal micrographs of basal and bicuculline (Bic; 40 μ M, 30 min) stimulated cultured hippocampal neurons, with or without cycloheximide (CHX; 50 μ M, 30 min) treatment. The neurons were then immunolabeled with antibodies targeted against MAP2 (cyan), Imp- β 1 (green) and Hoechst nuclear dye (blue in merged image). Imp- β 1 intensities were quantified for the nucleus (as nuclear / cytoplasmic) and dendrites (MAP2 normalized). Group data is represented as bar plots and in the table. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses ($*p < 0.05$, $**p < 0.01$). Scale bar, 10 μ m. For spines, neurons expressing GFP as described in **Fig. 3-4A** were fixed and immunolabeled with antibodies targeted against MAP2 (blue), GFP (green) and Imp- β 1 (red). The synaptic Imp- β 1 staining were quantified and the group data is represented as bar plots and indicated in the table. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses ($****p < 0.0001$). Scale bar, 2 μ m.

3.3.3 Activity-dependent increase in importin- β 1 protein level in synapto-dendritic compartment is not due to movement of newly synthesized proteins from soma

To rule out the possibility that the activity-driven increase of synapto-dendritic Imp- β 1 is due to the transport of newly synthesized proteins from the soma, I next employed N-ethylmaleimide (NEM), a small molecule inhibitor, commonly used to block ATP- driven molecular motors including kinesin, dynein and myosin (Pfister, Wagner, Bloom, & Brady, 1989). In cultured hippocampal neurons, the addition of NEM blocks both the bidirectional vesicular trafficking in axons and the motor-dependent movement of cytosolic cargo proteins (Scott, Das, Tang, & Roy, 2011).

Since NEM is a potent inhibitor, I first set out to optimize its concentration in our hippocampal cultures by tracking the lysosome movement, using lysotracker, as a proxy for active transport since lysosome trafficking engages both the dynein and the kinesin motor proteins for its bidirectional transport in neurons (Goo et al., 2017; Pu, Guardia, Keren-Kaplan, & Bonifacino, 2016). I found that a brief incubation with NEM (50 μ M; 10 min) effectively blocks the bidirectional displacement of lysosomes, with a significant reduction in the average displacement of lysosomes in the NEM-treated culture compared to those in mock treatment (**Fig. 3-7A**). Individual traces of the fastest moving lysosomes within each field show NEM treatment severely impacted its cumulative displacement (Mock: 13.3 μ m $\pm$ 5.4; NEM: 3.3 μ m $\pm$ 1.6; **Fig. 3-7A**).

At this NEM concentration, I expect most of the active transport in the neuronal processes to be halted without substantially impacting the translational machinery as NEM inhibits translation only when it is applied in millimolar range (Hatey & Gaye, 1978; J. M. Wu & Ibrahim, 1980). To stimulate local translation in NEM pre-treated

neurons, I first stimulated the cultures with bicuculline (40 μ M, 30 min) and observed that the cells were unhealthy. I reasoned that the inhibition on the motor-dependent movements in the cells for this long period of time would be detrimental to the cells. Hence, I reduced the bicuculline stimulation time and observed the health of the cells were not impacted. However, at this condition, I failed to detect a significant increase in the dendritic Imp- β 1 protein level, suggesting the stimulation paradigm is not sufficient to trigger substantial and detectable local translation in the shorter time frame of our NEM experiments. As such, I turned to stimulation paradigms that are known to induce local protein synthesis in the synapto-dendritic compartments within this short duration.

BDNF is a well-studied neurotrophic factor that regulates different forms of neuronal plasticity including the induction and maintenance of late-LTP in hippocampus (Kuipers et al., 2016; Leal, Comprido, & Duarte, 2014). Application of BDNF in hippocampal neurons can also enhance glutamatergic synaptic transmission through increasing glutamate release pre-synaptically while increasing the probability of NMDAR opening and the number of surface AMPAR post-synaptically (Cunha, Brambilla, & Thomas, 2010; Santos, Comprido, & Duarte, 2010). More recently, the addition of BDNF has also been reported to result in an accumulation of GluN2B containing NMDARs in the post-synaptic compartment and a corresponding increase in the amplitude of NMDAR-mediated miniature excitatory post-synaptic current (mEPSC) recorded in rat hippocampal culture (Afonso et al., 2019). Critically, BDNF-induced plasticity requires local protein synthesis in dendrites (Kang & Schuman, 1996; Santos et al., 2010). In the NEM-pre-treated cultured hippocampal neurons, BDNF bath application (50 ng/mL; 20 min) resulted in a significant increase in dendritic Imp- β 1 signal, which is abolished in the presence of CHX (**Fig. 3-7B**).

To complement BDNF stimulation, I used another small molecule agonist, DHPG, that is known to bind and activate mGluR1/5 receptors to trigger mGluRs-LTD (Fitzjohn, Kingston, Lodge, & Collingridge, 1999; Huber, Kayser, & Bear, 2000). Like BDNF, DHPG-mediated mGluRs-LTD is rapid and requires local protein synthesis (Waung et al., 2008). Similar to the BDNF results, bath application of DHPG (100 μ M, 5 min) in NEM pre-treated neurons resulted in a significant increase in dendritic Imp- β 1 signal, which is eliminated in the presence of CHX (**Fig. 3-7B**). Importantly, neither NEM treatment nor BDNF or DHPG bath application altered dendritic MAP2 protein level, which is used for normalization (**Fig. 3-7B**).

Figure 3-7. Activity-dependent increase in Imp- β 1 protein level in synapto-dendritic compartment is not due to movement of newly synthesized proteins from soma

(A) Cultured hippocampal neurons were treated with or without N-ethylmaleimide (NEM; 50 μ M, 10 min) in Tyrode's solution and lysosomes from both conditions were labeled with LysoTracker (1 μ M, 10 min). The movement of the lysosomes were then imaged using live confocal microscopy (1 s per frame for 60 s) and tracked using particle displacement module in IMARIS. The lines were color coded based on individual lysosomal displacement length. The cumulative displacement of the five fastest moving lysosomes for each experiment (N = 3; n = 15) were plotted against time along with the average displacement of all the lysosomes captured in each condition. Unpaired T-test analysis was conducted between NEM-treated and mock condition (N = 3, mock: n = 2971, NEM: n = 2393; **** $p < 0.0001$). **(B)** Representative confocal micrographs of basal, BDNF- (50 ng/mL, 20 min) and DHPG- (100 μ M, 5 min with 15 min recovery) treated cultured hippocampal neurons, with or without cycloheximide (CHX; 50 μ M, 30 min) prior to and during stimulation. All neurons were pre-treated with NEM (50 μ M, 10 min) and MG132 (5 μ M, 30 min), to prevent proteasomal degradation (if any), before and during stimulation. All treatments were conducted in Tyrode's solution. The neurons were then immunolabeled with antibodies targeted against MAP2 (cyan) and Imp- β 1 (green). The normalized dendritic Imp- β 1 to MAP2 staining were quantified and the group data is represented as bar plots. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses (N=2; * $p < 0.05$, *** $p < 0.001$). Scale bar, 10 μ m. The mean MAP2 intensity of these dendrites were also plotted in bar graph and statistical analyses performed on the group data use one way-ANOVA with Dunn's post-hoc analyses (N=2).

3.3.4 Activity-induced increase in importin- β 1 translational machineries in synapto-dendritic compartment

If neuronal activity drives local translation of Imp- β 1 at synapto-dendritic compartment, we should expect an increase in the recruitment of its ribosomal translation machinery in the dendrites and the synapses. To that end, I stimulated neurons with bicuculline (40 μ M, 30 min) and immobilized the ribosomes with their nascent peptides in the presence of CHX before performing Puro-PLA. I observed an activity-dependent increase in the integrated signal of Imp- β 1 Puro-PLA both in the soma and in the dendrites (**Fig. 3-8A**). This is consistent with our hypothesis that Imp- β 1 local translation occurs in response to activity.

Next, we wanted to ask if the neuronal activity alters the subcellular distribution of these Imp- β 1 Puro-PLA structures. We anticipate that the activity-dependent increase of Imp- β 1-ribosomal complex in the synapto-dendritic compartment to be disproportionately localized near to or at the synaptic sites according to the spine-specific and clustered plasticity models, which will be revisited in discussion section (Rangaraju, Tom Dieck, & Schuman, 2017). Using the same three-dimensional classification scheme previously described in **Fig. 3-5D**, I mapped and categorized all the Imp- β 1 Puro-PLA structures in bicuculline stimulated neurons and compared that against the neurons under basal condition. Although I observed a moderate increase in the total number of Imp- β 1-ribosome structures and the number of Imp- β 1-ribosome structures sharing at least partial overlap with PSD in the bicuculline stimulated neurons versus those in the mock treatment, the results nonetheless show no statistically significant difference in these increase as well as the distribution of Imp- β 1 Puro-PLA structures in all the five categories between the basal and the stimulated neurons (**Fig. 3-8B**). While the data would suggest an equal increase in local translating

Imp- β 1 throughout the synapto-dendritic compartment, there is a trend towards a higher proportion of Imp- β 1 Puro-PLA structures co-localizing with both the pre- and post-synaptic markers (p -value = 0.15) in the bicuculline stimulated neurons versus those in the mock treatment (**Fig. 3-8B**). These suggest the possibility that the Imp- β 1 local translation is disproportionately elevated at the synaptic junctions in response to activity.

Figure 3-8. Activity-induced increase in Imp-β1 translational machineries in synapto-dendritic compartment

(A) Cultured mouse hippocampal neurons in basal and bicuculline (Bic, 40 μ M, 30 min) stimulated states were treated with cycloheximide (CHX; 355 μ M, 30 min) followed by Puro-PLA in the presence of puromycin (Puro; 3 μ M, 15 min) before being fixed and immunolabeled with antibodies targeted against MAP2 (violet) and Hoechst nuclear dye (blue in merged image). The Imp- β 1 Puro-PLA signal is in green. The integrated Puro-PLA intensity (Int. PLA) normalized to the total MAP2 area of soma or the dendrites were quantified and the group data from independent experiments were plotted. Unpaired T-test analyses were conducted between basal and stimulated groups (N=3; * $p < 0.05$). Scale bar, 2 μ m. (B) Cultured mouse hippocampal neurons in basal and bicuculline (Bic, 40 μ M, 30 min) stimulated states were treated and processed as described in (A). The normalized number of Puro-PLA puncta (green) against the total MAP intensity as well as the normalized number of PSD colocalized Puro-PLA puncta against total PSD intensity of each field of image were plotted against the basal condition, expressed in percentage. The data indicates a trend in an increased number of Imp- β 1 translation machineries and an increased number of Imp- β 1 translation machineries in the PSD in the bicuculline stimulated versus the basal neurons. Next, the Imp- β 1 Puro-PLA puncta were analyzed and categorized as in **Fig. 3-5D**. The percentage of each category in basal and stimulated states were reflected in bar graph and in the table.

3.4 Importin- β 1 associates with NDRG1 at the synapse, and NDRG1 undergoes importin- β 1 mediated nuclear accumulation following glutamatergic stimulation

3.4.1 Importin- β 1 association with nuclear adaptors at the synapse

Our data shows Imp- β 1 undergoes activity-dependent local protein synthesis in the synapto-dendritic compartment. In parallel, it has been shown that activity releases Imp- α from its NR1-1a subunit anchor in the synapses (Jeffrey et al., 2009). Moreover, activity-dependent nuclear entry of proteins from the synapses and dendrites have been previously described, with a subset of these proteins reported to associate with nuclear adaptor complexes, mainly Imps- α , for its nuclear entry (Behnisch et al., 2011; Dinamarca et al., 2016; W. A. Herbst & K. C. Martin, 2017; K. O. Lai et al., 2008; A. F. Lim et al., 2017; Mikenberg et al., 2007). Taken together, we postulate that the activity-dependent local Imp- β 1 translation provides more Imp- β 1 in the synapto-dendritic compartments to cater to the need for the activity-induced retrograde shuttling of synaptic proteins to the nucleus in response to activity (**Fig. 4-1**).

To test this model, I performed co-IP experiments on purified synaptosomes in both the basal and the stimulated states using Imp- β 1 as bait (**Fig. 3-9A**). This assay allows the identification of additional nuclear adaptor proteins and synaptic proteins that respond to neuronal activity by entering the nucleus through interacting with Imp- β 1. I purified forebrain synaptosomes from adult male mice and profiled the fractions via immunoblotting to identify fractions enriched with pre- and post-synaptic markers. As expected, the synaptosome fraction is enriched in both the pre- and post-synaptic markers, the synaptophysin and PSD95 respectively. Furthermore, I also detected the presence of Imp- β 1, all the Imp- α isoforms (α 1, α 2, α 3, α 4 and α 6) and IPO7 in the synaptosome fraction (**Fig. 3-9B and C**). Importantly, since we are looking for nuclear

shuttling proteins at the synapse, the absence of resident nuclear proteins, as indicated by the absence of Histone H3, ensures that there is no false positive when we are identifying potential synapse-to-nucleus shuttling proteins (**Fig. 3-9B**).

Next, I stimulated the purified synaptosomes with KCl in the presence of glycine, a protocol that has shown to depolarize synaptic membranes and trigger AMPA receptor insertion and local translation (Corera et al., 2009). Here, I show that the stimulation triggers the dephosphorylation of CRTC1 via calcineurin in the synaptosomes (**Fig. 3-9D**), thereby validating that the depolarization trigger calcium influx into the synaptosomes while maintaining functional signaling pathways that trigger CRTC1 activation (Ch'ng et al., 2012). To ensure that the Imp- β 1 remains associated with other nuclear adaptor proteins, I immunoblotted both the basal and the stimulated synaptosomes with antibodies targeted against other importins. All Imp- α isoforms and IPO7, except Imp- α 2, were found associated with Imp- β 1 in the synaptosomes. These data suggest the heterotrimeric interaction of Imp- α / β 1-cargo is stable during affinity purification since the IBB domain of Imp- α is inaccessible to Imp- β 1 in the absence of cargo binding (Lott & Cingolani, 2011; Oka & Yoneda, 2018). Not surprisingly, I observed a higher Imp- β 1 level in the stimulated synaptosomes and an enhanced interaction between Imp- β 1 and most of its binding partners, namely the Imp- α 1, - α 4 and - α 6, as well as IPO7. This likely reflects an increased association of these nuclear adaptor proteins with Imp- β 1 after activity-induced Imp- β 1 local translation (**Fig. 3-9E**).

Figure 3-9. Imp- β 1 association with nuclear adaptors at the synapse

	Act/Basal		Act/Basal		Act/Basal		Act/Basal
Imp-α1	1.81	Imp-α3	1.20	Imp-α4	1.60	Imp-α6	1.24
Imp-β1	1.36	Imp-β1	1.64	Imp-β1	1.12	Imp-β1	3.66

(A) The workflow of synaptosomal purification, stimulation, digitonin lysis and co-IP with Imp- β 1 antibody for tandem mass spectrometry (MS/MS) sequencing. (B) Different fractions from the synaptosomal extraction protocol were harvested, subjected to immunoblotting, probed with antibodies targeted against PSD95, synaptophysin (SYPH), Histone-H3, and Imp- β 1. Lys: forebrain homogenate; P1: nuclear fraction; P2 and P3: pellets; S1 and S2: supernatants harvested during enrichment; Syn, purified synaptosomes fraction. The fold change of the synaptic markers in the purified synaptosomes were quantified and normalized against the forebrain homogenate (Lys) and the group data represented as bar graph (N=3 for SYPH; N=5 for PSD95). (C) Purified synaptosomes were probed with antibodies targeted against Imp- α isoforms and IPO7. (D) Purified synaptosomes in basal and stimulated (glycine, 100 μ M, 5 min followed by KCl, 50 mM, 5 min) states were probed with antibodies targeted against CRTTC1 and the band intensity was quantified. The ratios of dephosphorylated to total CRTTC1 were quantified and plotted on a before-after graph (N=3). (E) Lysates obtained after Imp- β 1 co-IP of basal and stimulated (glycine, 100 μ M, 5 min followed by KCl, 50 mM, 5 min) synaptosomes were probed with antibodies targeted against Imp- β 1, Imp- α isoforms and IPO7. The fold change (activated/basal) of normalized band intensities against light chain (LCh) for Imp- α and - β 1 were quantified and stated in the table.

3.4.2 Importin- β 1 association with cargo proteins at the synapse

To complement the immunoblotting data from co-IP experiment, tandem mass spectrometry (MS/MS) sequencing was conducted on three independent Imp- β 1 co-IP samples and used exponentially modified protein abundance index (emPAI) to quantify the abundance of proteins in the samples. This is because emPAI scores overcome the limitation of overestimating the quantity of large proteins due to more tryptic peptides generated, thereby providing a more accurate quantification as compared to the conventional PAI scores (Ishihama et al., 2005). As expected, Imp- β 1 protein is one of the highest enriched proteins for each of these co-IP experiments, with more Imp- β 1 signal detected in the stimulated (average 2-fold increase) versus the basal synaptosome samples (**Fig. 3-10A**). Consistent with the immunoblot data, the MS/MS results confirmed a robust Imp- β 1 association with Imp- α s and IPO7 (**Fig. 3-10A**). Specifically, Imp- β 1 interactions with Imp- α 1, α 3, α 4, α 6 and IPO7 were readily detected in synaptosomes and all but Imp- α 3 had a stimulation score of more than 1.0, with Imp- β 1-IPO7 interaction achieving the highest increase at 3.2-fold (**Fig. 3-10A**). Importantly, the MS/MS emPAI scores for IgG co-IP controls detected negligible binding for all Imp- α isoforms and IPO7, indicating the high specificity in the identification of protein complexes. Curiously, both immunoblotting and MS/MS sequencing data confirmed the absence of Imp- α 2/ β 1 complex in both the basal and the stimulated synaptosomes (**Fig. 3-9E and 3-10A**). This is surprising and hints at the possibility of selective Imp- β 1 heterodimerization that is stimulus-dependent or subcellular compartment specific.

I next analysed the MS/MS data for Imp- β 1 co-IP to identify synaptic proteins that bound to Imp- β 1 complexes. I applied a series of stringent filters on the MS/MS sequencing data to obtain strong candidates for Imp- β 1 binding (**Fig. 3-10B**). I

considered only candidate proteins from the co-IP that registered no background binding to IgG controls (emPAI scores of -1). This criterion greatly reduces false positives even though some *bona fide* candidate proteins that bind to Imp- β 1 may be excluded. Next, I shortlisted 277 candidate proteins which showed stable (Imp- β 1 emPAI in stimulated sample > 0.1) and enhanced binding to Imp- β 1 in stimulated synaptosomes (Imp- β 1 emPAI stimulated > basal).

I then analysed the shortlisted candidate proteins using two parallel approaches. In the first approach, I compared the 277 candidate proteins against published databases for subcellular localization. For synaptic proteins, I cross-referenced the candidates against three databases: SynGO (Koopmans et al., 2019), SynaptomeDB (Pirooznia et al., 2012) and a published synaptosome proteomic database (Filiou *et al.* 2010). Synaptic proteins from all three databases share a significant overlap with 71 % (199 / 277) of our candidate proteins appearing at least once out of the three databases (**Fig. 3-10B**). I then compared the 277 candidates against the mouse nuclear proteome listed in Mouse Genome Informatics (MGI) database and identified approximately 38 % (105/277) of the candidate proteins annotated as nuclear proteins (**Fig. 3-10B**). Finally, I compared the two lists and isolated 83 proteins that shared dual localization in both the synapse and the nucleus (**Fig. 3-10B and S7**), including the importins.

Of the 83 proteins, more than one-third (34/83) have been reported to undergo stimulus-driven nucleocytoplasmic shuttling, with several known to associate with IPO7 or the Imp- α / β 1 import pathway, suggesting the possibility that some of these proteins may also respond to neuronal activity and undergo nuclear accumulation mediated by Imp- β 1 (**Fig. S7**). Indeed, three identified proteins, namely the AIDA (B. A. Jordan et al., 2007), ERK1 (J. S. Wiegert, Bengtson, & Bading, 2007; Zhai, Ark,

Parra-Bueno, & Yasuda, 2013) and β -catenin (Abe & Takeichi, 2007; J. Chen, Park, & Tang, 2006; M. J. Schmeisser, A. M. Grabrucker, J. Bockmann, & T. M. Boeckers, 2009) have been reported to shuttle into the nucleus from the synapto-dendritic compartment in response to neuronal activity. Furthermore, a subset of these 83 proteins are known to undergo activity-dependent nuclear shuttling in neurons, such as CDK5 (Z. Liang et al., 2015) and Ywhaz (Neasta et al., 2012a). In particular, Ywhaz is shown to interact with Imp- α 2 and Imp- α 4 (Arjomand et al., 2014a). Other proteins such as Adducin-1 (Add1) (Vukojevic et al., 2012), CASK (Gillespie & Hodge, 2013; Malik & Hodge, 2014), FMRP (Kanellopoulos, Semelidou, Kotini, Anezaki, & Skoulakis, 2012; Tian et al., 2017), GSK-3 β (Takashima, 2012), and HNRP K (Folci et al., 2014; Leal et al., 2017) are well-characterized plasticity proteins with dual functions in both the nucleus and the synapses.

Experimentally, I attempted to validate the interaction between the candidate proteins and Imp- β 1. To that end, I conducted a reverse co-IP in HEK 293T cells overexpressing GFP-tagged candidate proteins, specifically 3-Phosphoinositide Dependent Protein Kinase 1 (PDPK1), β -catenin (CTNNB1) and NDRG1 that have been reported to undergo stimulus-dependent nuclear shuttling. I detected an interaction between Imp- α and all the three candidate proteins but not with Imp- β 1, even for the positive control NF- κ B, a known cargo for classical nuclear import pathway (**Fig. 3-10C**). I reasoned that the whole cell lysis in the transfected HEK 293T cells, which includes nuclei, result in the release of Ran-GTP into the lysates. The presence of Ran-GTP, which promote Imp- α / β 1-cargo complex disassembly in the nucleus, will likely destabilise the interaction between Imp- β 1 and the Imp- α -cargo complexes in the lysates. This disruption is not relevant in synaptosomes as the Ran-GTP gradient is not present in that subcellular compartment. Nonetheless, since Imp-

$\beta 1$ is an obligate partner of Imp- α in the classical nuclear import pathway, the interaction between the candidate proteins and Imp- α indirectly illustrate that these candidate proteins are likely to interact with Imp- $\beta 1$.

Figure 3-10. Identifying activity-induced Imp-β1 interacting partners at the synapse

(A) The emPAI scores for Imp- β 1 obtained from tandem mass spectrometry (MS/MS) sequencing across three independent co-IP assays. The heatmap shows the average emPAI scores of Imp- α isoforms and IPO7 from three independent MS/MS sequencing data of Imp- β 1 co-IP of basal and stimulated synaptosomes. **(B)** A flowchart indicating the number of proteins in each filtering step (in brackets). Proteins found at least once in the three synaptic databases (199/277), MGI nuclear database (105/277) or belonging to both groups (83/277). The 83 proteins are listed in **Fig. S7**. **(C)** Lysates from transfected HEK 293T cells expressing either GFP-tagged NDRG1, β -catenin, PDPK1 or NF- κ B plasmids, after co-IP with GFP antibodies, were probed with antibodies targeted against pan Imp- α s.

Independently, the second approach I took to analyze the MS/MS sequencing data is through performing Gene Ontology (GO) analyses on the candidate protein list (277 proteins) using DAVID (Huang et al., Sherman, & Lempicki, 2009a, 2009b). Interestingly, the top GO terms for both molecular function and biological process belonged to cell adhesion and cell junction proteins (**Fig. 3-11A**). Functional annotation clustering show GO terms representing cell adhesion, cell adherens junction and cadherin-associated with cell binding enriched among the candidate proteins (**Fig. 3-11A**). It is noteworthy that these GO terms associated with cell adhesion also appeared relatively enriched for the 83 shortlisted proteins based on dual localization (**Fig. S6**). We were intrigued that many cell adhesion and cytoskeleton-associated proteins bound to Imp- β 1 at the synapse. Indeed, it has been documented that this category of proteins in neurons do undergo active nuclear import (Birbach, Verkuyl, & Matus, 2006; Grabrucker et al., 2014; Murk et al., 2012). Next, the list of cell adhesion candidate proteins defined by DAVID was analyzed using Compartments (Binder et al., 2014), a subcellular localization database, which generates an average rank for nuclear-localized proteins based on a combination of experimental evidence, text mining and software prediction. A high average rank indicates a high confidence of the proteins to be localized in the nucleus. For instance, known nuclear proteins that bind to Imp- α / β 1 such as NFAT and NF- κ B achieve a high average rank of at least 3 out of a scale of 5 and are included as positive controls, whereas mitochondrial proteins such as MT-CyB, MT-Atp8 and TIM-23 achieve a much lower rank (≤ 1) and are used as negative controls (both controls are shown as green bars in **Fig. 3-11B**). Reassuringly, many of the cell adhesion proteins in the candidate list, which are synaptic, also scored a high average rank, indicating a high probability of a nuclear presence (**Fig. 3-11B**; red fonts = annotated in nucleus; blue bars = annotated in synapse). More importantly,

the highest ranked proteins are also found in the shortlisted synapse- and nucleus- dual localizing candidate protein list from the first approach (**Fig. 3-10, S7**).

Considering the results obtained from both independent analyses, I further narrowed down the candidate list to Ywhaz, Hnrnpk, Jup, Clint1, NDRG1 and Add1, which are part of the 83 candidate proteins and achieve a high nuclear rank based on the analyses using Compartments. I next eliminated proteins that have been extensively studied for its roles in plasticity, namely the Ywhaz and Hnrnpk. Among the remaining four candidate proteins, Clint1 has not been shown to undergo stimulus driven nuclear shuttling (**Fig. S7**). I then tested both NDRG1 and Add1 by asking (1) if they undergo nuclear shuttling in neurons and (2) if they respond to neuronal activity by accumulating in the nucleus. The data show that both NDRG1 and Add1 are nuclear shuttling proteins as reflected by their nuclear accumulation following LMB treatment in the cultured hippocampal neurons (**Fig. 3-11C**). Unexpectedly, Add1 undergoes nuclear exit following glutamatergic stimulation in hippocampal neurons in culture (**Fig. 3-11D**). In contrast, NDRG1 showed activity-dependent nuclear accumulation upon glutamatergic stimulation, and thus, I proceeded to further characterize it (**Fig. 3-12**).

Figure 3-11. Cytoskeletal associated proteins were enriched based on Gene Ontology (GO) analyses on tandem mass spectrometry sequencing data

(A) Gene Ontology (GO) analyses using DAVID functional annotation tool showed the top GO term for molecular function and biological process from the candidate list (277 proteins) is cell adhesion. The top ranked GO terms for each category are plotted against their *p*-values and listed in red. **(B)** The candidate proteins from the functional annotation clustering in DAVID associated with cell adhesion are listed alongside their individual GO terms. These proteins are then scored and ranked based on the confidence for their nuclear presence using Compartments. Proteins with blue bars indicate synaptic presence while red fonts indicate nuclear presence. Green bars are control proteins (NFAT and NF- κ B as positive controls for nuclear presence while MT-CyB, MT-Atp8 and TIM-23 are mitochondrial-localized proteins as negative controls). **(C)** Cultured mouse hippocampal neurons treated with or without leptomycin B (LMB; 20 nM, 3 h) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), NDRG1 (green) or Add1 (red) and Hoechst nuclear dye (blue in merged image). The nuclear-to-cytoplasmic ratio of NDRG1 as well as normalized Add1 nuclear intensity were quantified and the group data from independent experiments were plotted. Unpaired T-test analyses were conducted between mock and LMB treated groups (N=2; ****p* < 0.001, *****p* < 0.0001). Scale bar, 10 μ m. **(D)** Cultured mouse hippocampal neurons treated with glutamate (40 mM, 5 min) or tetrodotoxin (TTX; 1 μ M, 1 h), before being fixed and immunolabeled with antibodies targeted against MAP2 (violet) and Add1 (red). The normalized Add1 nuclear intensity was quantified, and the group data is represented as bar plots. Statistical analyses performed on group data use one way-ANOVA with Tukey's post-hoc analyses (N=2; ****p* < 0.001, *****p* < 0.0001).

3.4.3 NDRG1 is enriched in the nucleus of the hippocampal neurons mediated by Imp- β 1 in response to stimulation

The N-myc down regulated Gene1 (NDRG1) is a synaptic protein identified in all three databases I have used for cross-referencing and it belongs to the hydrolase superfamily (Schonkeren et al., 2019). More importantly, NDRG1 has been reported to undergo nucleocytoplasmic shuttling (Kitowska & Pawelczyk, 2010; Shi, Larkin, Chen, & Sadosky, 2013) and the overexpression or knock-out studies of NDRG1 indicate the protein impacts gene expression (Cai, El-Merahbi, Loeffler, Mayer, & Sumara, 2017; Kovacevic, Chikhani, Lui, Sivagurunathan, & Richardson, 2013; Schonkeren et al., 2019). However, whether nuclear entry of NDRG1 directly modulates transcription in neurons is not known. In addition, in the peripheral nervous system, a nonsense mutation in NDRG1 is the underlying cause for peripheral neuropathy and Charcot-Marie-Tooth disease (Okuda, Kokame, & Miyata, 2008). In the central nervous system, NDRG1 is found in developing hippocampal neurons as well as in mature astrocytes of adult mice (Okuda et al., 2008), but its function remains to be defined.

Using antibodies validated against isoform-specific NDRG knockout mice (Okuda et al., 2008), I report NDRG1 presence in cultured hippocampal neurons (DIV14), specifically in the somato-dendritic compartment with a mild nuclear enrichment under basal condition (**Fig. 3-12A**). I then stimulated the cultured neurons with BDNF (25 ng/mL; 1 h) and observed a marked increase in the nuclear NDRG1 (**Fig. 3-12A**). Similarly, stimulation with glutamate (40 μ M, 5 min) also resulted in a robust nuclear enrichment of NDRG1, which is abolished in the presence of kynurenic acid, a broad-spectrum inhibitor of glutamatergic receptors (**Fig. 3-12B**). Crucially, the nuclear accumulation of NDRG1 is mediated by Imp- β 1 as the addition of INI43 prior

to and during stimulation abolishes the activity-induced nuclear accumulation of NDRG1 (**Fig. 3-12C**).

I next analyzed NDRG1 intensity in the synapto-dendritic compartment and showed that simultaneous with an increase in the nuclear NDRG1 intensity after a brief glutamate stimulation (5 min; 40 μ M), there is a corresponding reduction in the dendritic localized NDRG1 (**Fig. 3-12D**). I also measured the NDRG1 intensity that colocalized with the PSD95 positive puncta in basal and glutamatergic stimulated neurons and demonstrate a loss of NDRG1 in the post-synaptic compartment (**Fig. 3-12E**). Notably, the proportion of PSD95 positive puncta without NDRG1 signal increased upon glutamatergic stimulation and accounted for 35 % of the total quantified puncta. The cumulative frequency plot also shows basal synapses having stronger NDRG1 signal as compared to the synapses in the glutamatergic stimulated neurons (**Fig. 3-12E**). Lastly, to validate the presence of NDRG1 at the synapse and its association with Imp- β 1, I performed reverse co-IP experiments using NDRG1 antibody on both the basal and the stimulated synaptosomes. I detected the interaction between NDRG1 and the Imp- α/β 1 nuclear adaptor complex in synaptosomes (**Fig. 3-12F**).

In summary, this proteomic study identified a collection of synaptic proteins that undergo Imp- β 1 mediated nuclear translocation upon neuronal stimulation. A subset of these synaptic proteins has cell adhesion properties, of which NDRG1 is shown to directly interact with Imp- β 1 to undergo activity-dependent nuclear import following glutamatergic stimulation.

Figure 3-12. Imp- β 1 association with NDRG1 at the synapse and undergoes Imp- β 1 mediated nuclear accumulation following glutamatergic stimulation

(E)

(F)

(A) Cultured mouse hippocampal neurons stimulated with BDNF (25 ng/mL, 1 h) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), NDRG1 (green) and Hoechst nuclear dye (blue in merged image). The nuclear-to-cytoplasmic ratio of NDRG1 were quantified and the group data from independent experiments were plotted. Statistical analyses performed on group data use unpaired T-test ($N=2$; **** $p < 0.0001$). Scale bar, 2 μ m. **(B)** Cultured mouse hippocampal neurons stimulated with glutamate (Glut; 40 μ M, 5 min) in the presence or absence of kynurenic acid (Kyn; 1 mM, 5 min) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), NDRG1 (green) and Hoechst nuclear dye (blue in merged image). The nuclear-to-cytoplasmic ratio of NDRG1 were quantified and the group data from independent experiments were plotted. Statistical analyses performed on group data use one way-ANOVA with Tukey's post-hoc analyses ($N=2$; **** $p < 0.0001$). Scale bar, 2 μ m. **(C)** Cultured mouse hippocampal neurons stimulated with glutamate (Glut; 40 μ M, 5 min), in the presence or absence of INI43 (5 μ M, 1 h), before fixing and immunolabeling with antibodies targeted against MAP2 (cyan), NDRG1 (green) and Hoechst nuclear dye (blue in merged image). The nuclear-to-cytoplasmic ratio of NDRG1 were quantified and the group data from independent experiments were plotted. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses ($N=2$; *** $p < 0.001$, **** $p < 0.0001$). Scale bar, 2 μ m. **(D)** Cultured mouse hippocampal neurons stimulated with glutamate (Glut; 40 μ M, 5 min) before fixing and immunolabeling with antibodies targeted against MAP2 (cyan) and NDRG1 (green). The fold change of dendritic NDRG1 in stimulated against basal neurons were quantified and the group data from independent experiments were plotted (bracketed numbers show number of data points). Unpaired T-test analysis was conducted between basal and stimulated groups ($N=2$; ** $p < 0.01$). **(E)** Cultured mouse

hippocampal neurons stimulated with glutamate (Glut; 40 μ M, 5 min) before fixing and immunolabeling with antibodies targeted against MAP2 (blue), PSD95 (red) and NDRG1 (green). The intensity of NDRG1 that colocalized with PSD95 (synaptic NDRG1) were quantified and binned. The percentage of each binned intensity category in basal and stimulated groups was quantified (N=2; Basal: n=85, Stimulated: n=115). Scale bar, 2 μ m. **(F)** Co-IP using NDRG1 antibodies were performed in basal and stimulated synaptosomes, and the immunoblots were probed with antibodies targeted against NDRG1, Imp- β 1, Imp- α isoforms and light chain (LCh).

Chapter 4. Discussion

4.1. Direct evidence showing importin- β 1 role in activity-dependent plasticity

Our results confirm the activity-dependent movement of soluble proteins through the association with Imp- β 1 is critical for transcription modulation underlying long-term plasticity. Despite its implied involvement, the role of Imp- β 1 in nuclear import during transcription-dependent plasticity has never been directly tested through loss-of-function studies. This is in part because a homozygous deletion of Imp- β 1 in mice is embryonic lethal (Miura et al., 2006). To circumvent any long-term cellular dysfunction due to the manipulations of Imp- β 1 protein expression, we employed INI43, a small molecule inhibitor, to block Imp- β 1 nuclear entry just before and during neuronal stimulation (van der Watt et al., 2016). This allows the study of Imp- β 1 function when neurons are actively firing action potentials. Our results on INI43 inhibiting Imp- β 1 nuclear entry in neurons is consistent with other published studies and this inhibition is selective for Imp- β 1 mediated nuclear import as activity-dependent entry of NFATc3, but not CRTC1, is impacted (Ch'ng et al., 2015; Wild et al., 2019). Moreover, in line with our hypothesis that Imp- β 1 plays an important role in neuronal plasticity, the disruption of Imp- β 1 function impaired multiple nuclear events associated with long-term plasticity, including CREB phosphorylation, JunB nuclear accumulation, induction of canonical activity-regulated genes expression and the maintenance of late-LTP, while leaving the early-LTP intact. This is not surprising as unlike late-LTP, early-LTP is independent of transcription and protein synthesis, and relies predominantly on local kinases activity in the synapses (Huang, 1998). Interestingly, our data also indicate the disruption of Imp- β 1 function impaired LTD.

4.1.1 Imp- β 1 mediated nuclear import is critical for CREB phosphorylation

The induction of activity-regulated genes expression is known to be mediated by calcium signaling and various downstream calcium-dependent kinases such as CamKs and ERKs (van Haasteren, Li, Muda, Susini, & Schlegel, 1999; West et al., 2001). These kinases, in turn, modulate the phosphorylation profiles of the transcription factors, for instance CREB, to turn on the expression of activity-regulated genes. As reviewed by Moens *et al.* (2007), the phosphorylation of CREB (S133) can be achieved through multiple kinases that are stimulus-dependent (Johannessen & Moens, 2007). Although some of these kinases, such as PKA, can enter the nucleus via passive diffusion, other kinases have been reported to engage the nuclear adaptor proteins for its nuclear entry. For instance, CamKIV binds to Imp- α (Lemrow et al., 2004) while MAPK binds to IPO7 to shuttle into the nucleus. Of note, both Imps- α and IPO7 heterodimerize with Imp- β 1, and thus, supporting the involvement of Imp- β 1 in the nuclear entry of these kinases and the subsequent induction of activity-regulated genes expression (Chook & Süel, 2011).

There is also the hypothesis that the kinases are constitutively localized in the nucleus and the calcium transients arriving at the nucleus will activate these kinases, thereby phosphorylating CREB (Hardingham et al., 2001; Wayman, Lee, Tokumitsu, Silva, & Soderling, 2008). Given that the blockade of Imp- β 1 mediated nuclear entry in our study occurred 30 min prior to the stimulation, it implies that some of the crucial kinases for CREB phosphorylation have yet to reside in the nucleus. Instead, they only enter the nucleus in response to activity. Indeed, there are kinases that have been reported to undergo activity-induced nuclear shuttling in neurons including γ CamKII (Huan Ma et al., 2014).

4.1.2 Importin- β 1 is crucial for LTD induction

Interestingly, our data also open up the possibility of Imp- β 1 mediated nuclear entry regulating LTD as Imp- β 1 accumulates in the nucleus following synapse specific stimulation associated with LTD. Moreover, we also show the blockade of Imp- β 1 mediated nuclear entry negatively impacted LTD induction. In support of this idea, several reports have shown that LTD is transcription-dependent (Kauderer & Kandel, 2000; Kemp et al., 2013; O'Riordan et al., 2006), with certain forms of LTD, such as DHPG-mediated and mGluR-LTD, driving transcription of activity-regulated genes (Lindecke et al., 2006), the activation of NF- κ B nuclear signaling and DNA binding (O'Riordan et al., 2006; H. Wang & Zhuo, 2012). Furthermore, in other brain regions such as cerebellar Purkinje cells, LTD is transcription-dependent and requires the activation of myocyte enhancer factor-2 (MEF2) (Andzelm, Vanness, Greenberg, & Linden, 2019; Napolitano et al., 1999).

In our hippocampal slice recordings, we observed a failure in the induction of LTD when Imp- β 1 function is blocked. This impairment is not due to a loss of synaptic transmission since INI43 is well-tolerated and does not alter baseline synaptic transmission even after 180 min post-stimulation. Hence, it is likely that the loss of Imp- β 1 function impairs the signaling mechanisms required for the induction LTD. For example, it is known that the Protein Phosphatase 2A (PP2A) is critical for LTD induction through the dephosphorylation of GluA1 subunit of the AMPAR (Mauna, Miyamae, Pulli, & Thiels, 2011). Neurons express an endogenous PP2A inhibitor (I_2^{PP2A}) which encodes a canonical NLS and is basally sequestered in the nucleus. A loss of Imp- β 1 function would result in the cytoplasmic retention of the I_2^{PP2A} , which in turns, lead to the inhibition of PP2A activity on AMPARs, thereby impairing LTD induction (Arif et al., 2014).

Alternatively, the pre-existing association of the target synaptic proteins with the nuclear adaptor proteins including Imp- β 1 may be crucial for the induction of LTD. Indeed, proteins from our target list that associated with Imp- β 1, such as GSK3 β , Camk2 α or MAPK, are all required for the induction of LTD since small molecule inhibition of these plasticity proteins blocks LTD induction and abolishes late-LTD (Peineau et al., 2007; Sajikumar, Navakkode, & Frey, 2007).

4.2 Local translation of importin- β 1 in synapto-dendritic compartment response to stimulation

Establishing the importance of activity-driven Imp- β 1 nuclear import during long-term plasticity encourages us to further characterize Imp- β 1 nuclear signaling in response to activity. Imp- β 1 is localized in different subcellular compartments and our data show the activity-induced shuttling of Imp- β 1 from the synapto-dendritic compartment to the nucleus. Moreover, there is an increasing number of synapto-dendritic proteins reported to undergo activity-dependent retrograde movement to the nucleus (Brigidi et al., 2019; W. A. Herbst & K. C. Martin, 2017; A. F. Lim et al., 2017; Marcello, Di Luca, & Gardoni, 2018; Parra-Damas & Saura, 2019). Since many signaling cascades at the synapto-dendritic compartment are activity-dependent and are highly regulated locally to enable rapid response to the stimuli, we reasoned that the synapto-dendritic Imp- β 1 is likely to be regulated by local mechanisms.

In this paper, I provide evidence showing the local translation of synapto-dendritic Imp- β 1 upon neuronal stimulation. I report the presence of at least three distinct Imp- β 1 mRNA isoforms in the synaptosomes and using Puro-PLA in the presence of CHX, I observed activity-dependent increase in the Imp- β 1-ribosome complex in the synapto-dendritic compartments (David et al., 2012; tom Dieck et al., 2015).

4.2.1 Local translation models in neurons

At present, there are two models to describe the localization of translation in the synapto-dendritic compartment. First model is the spine specific model, which states that upon activation, the transcripts and translational machineries will enter the stimulated spines and the translation occurs within the stimulated spines (Rangaraju et al., 2017). In contrast, the second model, known as the clustered plasticity model, states that following stimulation, translation will occur in the dendritic shaft, but the complexes move closer to the most stimulated spines. The newly synthesized proteins in the dendritic shaft will then diffuse and be “captured” by all the stimulated spines within 50 μm from the site of synthesis (Rangaraju et al., 2017).

I attempted to determine which model does the activity-induced local Imp- β 1 translation follows. While I observed a trend towards synaptic localization (Group IV puncta; **Fig. 3-8B**) in response to bicuculline stimulation, this difference is nonetheless, insignificant. We reasoned that the bicuculline-induced action potential bursting affects only a subset of the neurons in the culture and this likely diluted our ability to detect a robust change in the quantified samples. In addition, it is possible that the neuronal activity increases the pool of newly synthesized Imp- β 1 proteins by increasing the number of ribosomes attached to each Imp- β 1 transcript. Indeed, it has been recently reported that the Imp- β 1 transcripts in the neuropils can be translated by both monosomes and the polysomes (Biever et al., 2020). If the activity-induced Imp- β 1 translation is through polysomes, the increase in translational machinery might not be detected in the quantification (**Fig. 3-8B**) as I counted the number of Imp- β 1-ribosome puncta without taking into account of their intensity. Given the limited resolution of the micrographs, the multiple translational machinery in one transcript might not be

resolved and would be considered as one punctum although the intensity signal might be higher.

4.2.2 A model reflecting the local regulation of importins in the synapses in response to neuronal stimulation

Collectively, our data, together with the published report by Jeffrey and colleagues (2009), argue that importins' function at the synapse is highly regulated and is likely shaped by activity. Our updated model predicts the synaptic stimulation first triggers the activation of NMDARs through the phosphorylation of the NLS in the cytoplasmic tail of NR1-1a subunit by protein kinase C. This in turns releases Imp- α 1 anchored at the site. In parallel to Imp- α 1 release, Imp- β 1 mRNA is loaded onto ribosomes and translated in the synapto-dendritic compartment. The overall increase in local importins concentration will enhance the formation of different Imp- β 1 heterodimers, including those with Imp- α and IPO7. Consequently, it facilitates the binding to different synaptic proteins for their retrograde movement and entry into the nucleus (**Fig. 4-1**). Interestingly, a recent report identifying translating mRNAs in the neuropil detected the presence of different importin mRNAs including Imp- β 1, suggesting that local translation of other importins, apart from Imp- β 1, can also occur at the synapses (Biever et al., 2020).

Figure 4-1. Model for activity-dependent regulation of importins at synapo-dendritic compartment

Following synaptic stimulation, Imp-α is released from its NMDAR anchor. Simultaneously, Imp-β1 is locally translated in the synapo-dendritic compartment. Consequently, more Imp-α/β1/cargoes or Imp-β1/7/cargoes are formed and potentially shuttled back to the nucleus to modulate transcription underlying long-term plasticity.

4.2.3 Non-conventional role of importin- β 1

It is also possible that Imp- β 1 may have other local but uncharacterized role that might impact on the long-lasting synaptic plasticity. For instance, importins have shown to affect reverse phase separation (Guo et al., 2018). Specifically, Imp- β 2 binding to PY-NLS, as well as Imp- α / β 1 binding to cognate NLS inserted to RNA binding protein FUS, prevents liquid-liquid phase separation of the FUS, indicating Imp- β 1 role in phase separation (Yoshizawa et al., 2018). As a matter of fact, PSD and the organization of active zone are proposed to be formed via phase separation (X. Chen, Wu, Wu, & Zhang, 2020; McDonald, Fetter, & Shen, 2020; Milovanovic & De Camilli, 2017). Since neuronal stimulation causes synaptic plasticity which includes structural changes in the PSD, the increased Imp- β 1 protein level might be a local response for modulating these structural changes.

4.3 Imp- β 1 association with synaptic cargoes

In this study, we decided to cast a wide net to identify additional synaptic proteins that bound to Imp- β 1 in the stimulated synaptosomes. The MS/MS sequencing and immunoblots of Imp- β 1 co-IP assay in the basal and stimulated synaptosomes showed Imp- β 1 association with IPO7 and all Imp- α s except Imp- α 2 (**Fig. 3-9, 3-10**). Since the Imp- β 1 co-IP generated a diverse target list, stringent selection criteria were necessary to remove background noise. Among the list of candidate proteins, at least three proteins, β -catenin, AIDA and ERK1 are previously identified as synapse-to-nucleus signaling proteins in response to activity (B. A. Jordan et al., 2007; M. J. Schmeisser et al., 2009; Zhai et al., 2013).

Interestingly, functional annotation clustering of the target list showed the enrichment for GO terms associated with cell-cell adhesion. Perhaps, this is not surprising given that the nucleocytoplasmic shuttling of cell adhesion proteins or

cytoskeletal proteins have been well-documented in many cell types. Specifically in the neurons, a subset of these proteins respond to different stimulation by undergoing nuclear translocation, such as Shank3, which is a synapse-to-nucleus signaling protein implicated in Autism Spectrum Disorder (Grabrucker et al., 2014; Jiang & Ehlers, 2013), Vimentin, an Imp- β 1 interactor implicating in Alzheimer's disease (Levin et al., 2009; Perlson et al., 2005), Profilin, which involves in Fragile X syndrome (Birbach et al., 2006; Michaelsen-Preusse et al., 2016; Murk et al., 2012), and Afadin, where its deletion leads to hydrocephalus (VanLeeuwen et al., 2014; Yamamoto et al., 2013). Incredibly, many of these dual-localized proteins does not only help in maintaining the structural integrity of the different subcellular compartments, they also function as transcriptional modulators capable of regulating genes expression (Kumeta, Yoshimura, Hejna, & Takeyasu, 2012).

Chapter 5. Conclusion and future directions

5.1 Importin- β 1 role in activity-dependent plasticity

In this study, we provide direct evidence demonstrating Imp- β 1 role in transcription-dependent plasticity by using INI43 to block Imp- β 1 function specifically during activity. However, a caveat in this study is that the specificity of INI43 to Imp- β 1 mediated nuclear entry has not been directly investigated, albeit multiple studies have hinted its specificity through overexpression rescue experiments or by using siRNA to complement INI43 effects. Specifically, in this study, I showed only the nuclear entry of Imp- β 1 cargoes, including NFAT and JunB, are affected by INI43 treatment but not the nuclear entry of Imp- β 1 independent cargo, CRTC1. To further strengthen our data, future studies can inhibit Imp- β 1 function with other methods such as using validated siRNA targeted against Imp- β 1 and determine if the nuclear events underlying transcription-dependent plasticity as well as LTP are impaired.

5.1.1 Imp- β 1 role in each signaling event underlying activity-dependent plasticity

Although our study provides evidence ascertaining the importance of Imp- β 1 function in activity-dependent plasticity, the data does not allow us to differentiate if Imp- β 1 mediated nuclear entry is needed for each of the individual nuclear signaling event, that is the phosphorylation of CREB, the expression of activity-regulated genes and JunB nuclear localization. Perhaps, it is a consequential effect, that is, since the phosphorylation of CREB is affected, its downstream signaling events are thus, affected. Similarly, I also did not investigate if Imp- β 1 function is critical for the subsequent delayed gene expression required for late-LTP in this study. To address these limitations, future studies can apply the small molecule inhibitor, INI43, at different time points corresponding to the different events to pinpoint when exactly is Imp- β 1 mediated nuclear entry needed. In addition, these studies can co-apply INI43

with for instance, a constitutively active CREB or nuclear targeted JunB individually to delineate the effect of INI43 for each specific nuclear event. This could also help in overcoming the challenge of overlapping window period of some of these events.

5.1.2 Importin-β1 role in LTD

Unlike LTP, the role of Imp-β1 in LTD is less clear. We hypothesized that the failure in the nuclear retention of I_2^{PP2A} , due to the blockade of Imp-β1 mediated nuclear import, inhibited PP2A in the synaptic sites, thereby impairing LTD induction. To that end, future studies can probe the localization of I_2^{PP2A} , the activity of PP2A and the phosphorylation profile of AMPAR following INI43 incubation.

Of note, PP2A can also function in the nucleus to dephosphorylate CREB (S133), approximately 20 min after the induction of LTD (Mauna et al., 2011). Crucially, PP2A has been reported to bind to Imp-β1 (Lubert & Sarge, 2003). In addition, there are synapse-to-nucleus signaling proteins, specifically, CREB2, that is reported to undergo nuclear accumulation following LTD stimulation using Imp-α (K.-O. Lai, Y. Zhao, T. H. Ch'ng, & K. C. Martin, 2008). These suggest that Imp-β1 mediated nuclear entry is needed, not only for the induction of LTD, but also in other processes required to maintain LTD. Hence, future studies can investigate when and how Imp-β1 mediated nuclear entry is implicated in the maintenance of LTD by applying INI43 at different time point.

5.2 Function of locally translated importin-β1 at synapto-dendritic compartment

In this study, I showed the activity-dependent nuclear shuttling of synapto-dendritic Imp-β1 through live imaging and independently, uncovered an enhanced local translation of Imp-β1 in the synapto-dendritic compartments in response to activity. However, the question remains; is the nuclear shuttling of specifically the newly

synthesized synapto-dendritic pool of Imp- β 1 required for the induction of the nuclear signaling events underlying long-term plasticity.

This question can be tackled in three steps. First, to demonstrate the newly translated Imp- β 1 at the synapto-dendritic compartment undergo nuclear shuttling, future studies can employ microfluidic chambers to compartmentalize the dendrites from the soma and perfuse only the dendritic compartments with labeled amino acids. Subsequently, by co-staining for Imp- β 1, the data would indicate if there were nuclear accumulation of newly synthesized synapto-dendritic Imp- β 1 in response to activity.

Second, to demonstrate the retrograde nuclear shuttling of Imp- β 1 from the synapto-dendritic compartments is required to induce these nuclear signaling, future studies can use the same microfluidic chambers set up and perfuse only the dendritic compartments with INI43. This halt the retrograde movement of Imp- β 1 from the dendritic compartment to the nucleus. Similarly, the studies can also apply INI43 locally to the dendritic regions of brain slices using for instance, Pico spritzer, and ask if late-LTP and/or LTD is impaired. These data would reveal if the nuclear translocation of synapto-dendritic localized Imp- β 1 is required for long-term plasticity.

Third, to show the local translation of Imp- β 1 is required for the induction of the nuclear signaling events underlying long-term plasticity, these studies can add anti-sense oligonucleotides targeting Imp- β 1 transcripts to the dendritic compartments and asked if it impacted the nuclear signaling and/or long-term plasticity. However, a major challenge for all these compartmentalized experiments would be to restrict the spread of the pharmacological treatments and the oligonucleotides to the soma in the neurons.

5.3 Candidate approach to identify potential synapse-to-nucleus signaling proteins

5.3.1 NDRG1 function in synapse-to-nucleus signaling and transcription-dependent plasticity

I selected one of the synaptic cell adhesion proteins, NDRG1 for further studies and validated its dual localization in the synapse and the nucleus. Moreover, the characterization shows NDRG1 undergoes Imp- β 1 mediated nuclear accumulation during glutamatergic stimulation with a corresponding loss in the synapto-dendritic compartment. Future studies are needed to ascertain its synapse-to-nucleus signaling role. In particular, these studies can first provide direct evidence on the movement of the synapto-dendritic localized NDRG1 into the nucleus following activity via live cell imaging. Subsequently, these studies can explore the role of nuclear NDRG1 including the types of genes that are expressed following the activity-dependent nuclear translocation of NDRG1. Finally, using either a knock-out model or small molecule inhibitor, these studies can block the functions of NDRG1 and determine if transcription-dependent plasticity is affected. Since NDRG1 is recognized as an important therapeutic target for multiple cancers (K. C. Park, Paluncic, Kovacevic, & Richardson, 2020), the characterization of NDRG1 role in transcription-dependent plasticity is crucial as these chemotherapies targeted against NDRG1 might affect the patients' cognitive abilities.

5.3.2 Other potential synapse-to-nucleus signaling proteins

In the preliminary characterization, I observed the activity-dependent nuclear exit of Add1 following stimulation. The activity-dependent nuclear exit mechanism has been reported for several signaling proteins including CtBP1 (Ivanova et al., 2015), HDAC4 (Schlumm, Mauceri, Freitag, & Bading, 2013) and FOXO3 (Dick & Bading,

2010), where their nuclear export increase transcription. Notably, nuclear Add1 interacts with RNA polymerase II and zinc-finger protein 331, where zinc-finger protein 331 is thought to act as a transcriptional repressor (C.-M. Liu, Hsu, Lin, & Chen, 2017; Meiboom et al., 2003). Accordingly, the nuclear exit of Add1 might free RNA polymerase II for transcription. This renders Add1 a promising signaling protein and future studies can further characterize it by elucidating its nuclear role in neurons and if blocking its nuclear exit, using LMB (**Fig. 3-11C**), would impair transcription-dependent plasticity. On the similar vein, Stxbp1, one of the 83 shortlisted candidate protein, has also been indirectly reported to exit the nucleus to the cytoplasm following glutamatergic stimulation (**Fig. S7**) (Zhang et al., 2011).

Lastly, among the 83 shortlisted proteins, there are 20 proteins, excluding Stxbp1, Add1 and NDRG1, that undergo stimulus dependent nuclear translocation in non-neuronal cells (indicated as Y in **Fig. S7**). Specifically, since synaptic activity causes an increase in the intracellular calcium levels in neurons and given that Cask is reported to undergo calcium-dependent nuclear accumulation in non-neuronal cells (**Fig. S7**), future studies can characterize Cask as a potential synapse-to-nucleus signaling (Ojeh, Pekovic, Jahoda, & Määttä, 2008; Schuh, Uldrijan, Gambaryan, Roethlein, & Neyses, 2003).

Chapter 6. References

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Chapter 7. Appendix

Figure S1. Importin- β 1 (3E9) antibody is specific and is suitable for co-IP assay

Lysates from transfected HEK 293T cells with HA-tagged human Imp- β 1 plasmids and lysates from Imp- β 1 IP experiments in non-transfected HEK 293T cells were probed with antibodies targeted against Imp- β 1 (3E9) and HA protein tag.

Figure S2. 3' UTR of importin- β 1 full transcript (NM_008379.3)

Long isoform	Short isoform	PolyA signal
tctggtaccactgggat	tgatgacctgagacccccactggaaatctccaatcttttgaaaaacccggaagtgaggagtgtgcacggat	
gctgaatgcttggaatgagaggatgagtgagtgaggcttga	aaacaccacactgaaaatcctgccacagccaacagcagcagcg	
ctgttagacatgagctaagtaagcactgagttttagaaaacataacatcagccatcttgaacagagaaacaatggatttacttac		
ttatataaaacaaaaaagctgtctctccaggggaaggctagaactagcttctgtcttgccggtgccgaatgggtgactgatttg		
ggagaggaggacctgggttcacctgtggggtgtgtgtgaaaggcagcctggccctgctactgaggagaaagatggagcccg		
gtcttgagcccaccttactgtaccgttgacttggtactgtctatgtgccttgagaaggcgcgtgggggatttcacagtctgagaat		
gagtgtgtgtgagtgaggcggtaccacattctcaacttcaagtcattgcagtttcttttccagaaaacaaaggggttgatgttg		
atctcataaactaactgaagttctggctactgatgcagcacaagagacgtgttttttttaaatgaggaaagacgctgttttaggttg		
gtttggtttcattttaattactctagggaaaacactgacgaatggcagcgtctctgtctgatctttcatcaaggcgcttctctgtac		
tatggttcagctgtgaatgtagctatggggggaggggggagaaaggagaactctggagtttagaggatatagaaaaataaaagtaca		
gtttgtacaagaaaaatgcagacttcaaagacagaaaaagccacaacccaaacaaaatttaaatgcttgaaattggcatcataa		
acaagctctctctgacttgtattgtggcagtcggaatgccccagaaaactgtgccaaagagtttagaaaacaaatgtacaataaa		
agtaaatcacactcaaaacgcgaacttcaggttaactatgtgaattgcgaatgacgaaaatttaattgtgaacgatctgataaaata		
accatttggaact	gcttggccttctgtcccttctttgactgactgcaatgcggtattggtctcttctgtcactccaaacaaactaacc	
actaagacagtggtgtgtatccgtatacacagcatcacacactaaatacaactgttgtgatgaaaatggcaccaggaagaag		
gctctatcttccactgaagttgaagattagtaaaatgggtgcagatggtgaccctgctcgacgcttagtaatttttaagtgtggt		
gtgctgtgattatataaaatcagaattatcttctttacaagggaacaaggcaccctgagtttagagctttcagcaataagaag		
catttcagtaagctggccctcttggtcgtgcagatagtaggagattagctggcgtttgaatatgctgctttaaaaaacagtagaca		
ccgaggaagtttttattgcttctggttagtggtgaagaaacacaagaccctcattccatttccattttagtcaaaaggtattg		
gcttaggtttctccgtgctcctttcatccctcctgctgagcctgttctaagtgggattggctccgcttgcctgtccgtacgaaatg		
cagcgtctgtagtagctctttacagggtatcagatattgtgccttttggtgccaggtcagagtcaaagtgccgatctgatctgtgaac		
cagtgtaaaaaaagtatgtgaaggagaaatgctccattatgagcaaagctcttaggagccaagccctcgggagaagcccag		
gcctctgaacgcctgtttccatcttagctgtttttcagatttactaaccattcctgatactagatagggttggaaacagggttaggg		
aagggggcagaaatattgggggaaaggaaaaacaatgtgatcattcctcagtgctacccatttctgtcctgtgtgggtgcttagcg		
agacagcagaggatgaagtacaccgagaacctgatgaaaacaagacctgcctgtgtttgtcgtgttctgttccaggaagcagt		
tgacaagtgtgttactaatctttctccagatgcattcagtgctcgagaggaggaaatgggctgattggattgtggtcttggtgcct		
tgctgttctattctgcactggttacgcattttaactccttttagttaccttcagctactgtttctatggtttgggtgtctgtcctaatcag		
ttcttaccacataaggcaaatgaatgcgaatgcattctgtccactcactgtctgggccttcccaccctgagcttggcacattcctt		
caagaatgtagttaccgtctgcttggaagatgtcagtgcaaatgtgaagaaaatgggcatcggaatagacttgggtttgccacaagt		
ggcatctgcctgcaccaattacattgggttaattttgcttttcatttttctcagttaccccagtcctccctctgttgaaatgttgagat		
ttagctgtcagaggcaactgtgatcctgccttagggactggagaagtggagggacttgctcagtatggtgttctaattgttcagaacca		
agcagctctccttacttactatttaagatgtgtgtagttttagcttagaagaagttcaaatccatccactttgatagcatcaatctgta		
attggcattcagaatgcccttgcatgccagctgtgaaggcattaaagacgtgttaaaacacttgagcccaactttattaacaaaaa		
ctgataaccaccaccttattcttgaataaagtcgcttatttttattttcaacattt		

Figure S3. qPCR melt curve plots for primers targeting different importin- β isoforms

The melt curves for the qPCR primers used to detect Imp- β 1 isoforms in purified synaptosomes.

Figure S4. Corresponding reduction in dendritic importin- β 1 following KCl and glycine induced Imp- β 1 nuclear accumulation

Cultured mouse hippocampal neurons treated with KCl (50 mM, 5 min) + glycine (100 μ M, 5 min), tetrodotoxin (TTX; 1 μ M, 1 h) and APV (40 μ M, 1 h) in Mg^{2+} free aCSF, before being fixed and immunolabeled with antibodies targeted against MAP2 (violet) and Imp- β 1 (red). The normalized dendritic Imp- β 1 staining against MAP2 staining were quantified and the group data is represented as bar plots and indicated in the table. Statistical analyses performed on group data use one way-ANOVA with Dunn's post-hoc analyses (N=3; * $p < 0.05$, **** $p < 0.0001$).

Figure S5. Importin- α antibodies are specific for its isoforms

Lysates from transfected HEK 293T cells with Flag-tagged Imp- $\alpha1$, - $\alpha2$, - $\alpha3$ and - $\alpha6$, as well as photoactivatable GFP-tagged Imp- $\alpha4$ plasmids were probed with antibodies targeted against individual Imp- α isoforms. Red arrows indicate the overexpressed Imp- α .

Figure S6. Gene ontologies on the shortlisted 83 candidate proteins based on dual localization analyses.

GO terms for BP	P-Value	Genes
NLS-bearing protein import into nucleus	5.63E-05	KPNA6, KPNA4, KPNB1, KPNA1
negative regulation of neuron apoptotic process	8.20E-05	GPI1, HSPH1, HSP90AB1, PDPK1, STXBP1, ITSN1, UBE2V2
protein import into nucleus	1.82E-04	KPNA6, KPNA4, IPO7, KPNB1, KPNA1
regulation of synaptic vesicle priming	4.10E-04	STX1B, STXBP1, STX1A
membrane fusion	5.11E-04	STX1B, NSFL1C, STX6, STX1A
intracellular protein transport	6.09E-04	STX1B, CDK5, STX6, CASK, IPO7, KPNB1, STX1A
exocytosis	0.001267	CADPS2, SCRNI, CDK5, STXBP1, STX1A
synaptic vesicle fusion to presynaptic active zone membrane	0.001742	STX1B, STXBP1, STX1A
positive regulation of calcium ion-dependent exocytosis	0.00258	CDK5, STXBP1, STX1A
peptidyl-serine phosphorylation	0.003005	GSK3B, CDK5, PDPK1, CAMK2A, MAPK3
positive regulation of protein binding	0.00352	GSK3B, HSP90AB1, CDK5, ADD1
protein transport	0.004972	CADPS2, STX1B, STXBP1, STX6, KPNA6, KPNA4, IPO7, KPNB1, KPNA1
positive regulation of exocytosis	0.006947	CADPS2, STXBP1, STX1A
positive regulation of excitatory postsynaptic potential	0.006947	STX1B, GSK3B, STX1A
positive regulation of telomerase activity	0.006947	TCP1, CTNNB1, MAPK3
protein heterooligomerization	0.007928	STK11, JUP, CTNNB1, GRB2
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	0.007948	PSMA6, PSMA7, PSMA8
negative regulation of establishment of	0.008939	DNAJA1, HSPH1

protein localization to mitochondrion		
positive regulation of telomere maintenance via telomerase	0.009011	TCP1, CTNNB1, MAPK3
cell-cell adhesion	0.010295	TXNDC9, CLINT1, NDRG1, YWHAZ, ADD1

GO terms for MF	P-Value	Genes
protein binding	6.23E-09	GSK3B, HSP90AB1, STXBP1, FMR1, CAMK2A, ITSN1, RAPH1, IPO7, STK11, HSPH1, CHCHD3, STX6, KPNA6, KPNA4, CYC1, PCDH1, KPNA1, ANKS1B, MAPK3, CADPS2, HSP90AA1, JUP, PDPK1, CASK, YWHAZ, DNAJA1, COPS3, STX1B, NSFL1C, HNRNPK, CDK5, BIN1, CSNK2B, TCP1, GNAS, UBE2V2, CTNNB1, UBE2O, GRB2, MAPT, UTRN, KPNB1, STX1A
protein kinase binding	6.77E-09	GSK3B, HSP90AA1, HSP90AB1, JUP, PDPK1, STXBP1, YWHAZ, STX1B, RPS19, CDK5, CTNNB1, GRB2, MAPT, UTRN, NEFH
protein domain specific binding	5.56E-06	STX1B, HNRNPK, STXBP1, CSNK2B, GNAS, GRB2, MAPT, YWHAZ, KPNB1, STX1A
poly(A) RNA binding	2.72E-05	HSP90AA1, HSP90AB1, STXBP1, FMR1, RPL23, RPLP0, HUWE1, YWHAZ, ADD1, SYNCRIP, HNRNPK, RPS19, TCP1, UBE2O, GRB2, KPNB1, RPS13
cadherin binding involved in cell-cell adhesion	3.44E-05	TXNDC9, HNRNPK, HSP90AB1, JUP, CTNNB1, CLINT1, NDRG1, YWHAZ, ADD1
nuclear localization sequence binding	1.63E-04	KPNA6, KPNA4, KPNB1, KPNA1
protein transporter activity	2.86E-04	KPNA6, KPNA4, IPO7, KPNB1, KPNA1
identical protein binding	4.54E-04	HSP90AA1, AHCY, GOT1, BIN1, FMR1, STXBP1, CSNK2B, G6PDX, GRB2, YWHAZ, PSMA7
protein complex binding	0.001094	STK11, RPS19, BIN1, CTNNB1, CASK, MAPT, YWHAZ, KPNB1
ubiquitin protein ligase binding	0.0017	DNAJA1, GPI1, GSK3B, TCP1, UBE2V2, UBE2O, YWHAZ
tau protein binding	0.001734	GSK3B, HSP90AA1, BIN1
ion channel binding	0.002052	HSP90AA1, HSP90AB1, FMR1, CTNNB1, STX1A
SNARE binding	0.002945	STX1B, STXBP1, STX6, STX1A
unfolded protein binding	0.004608	DNAJA1, HSP90AA1, HSP90AB1, TCP1
nucleotide binding	0.004647	GSK3B, HNRNPA3, HSP90AA1, HSP90AB1, PDPK1, CAMK2A, CASK, AGAP3, TUBA1C, ACLY, SYNCRIP, STK11, HSPH1, CDK5, TCP1, GNAS, UBE2O, MAPK3

threonine-type endopeptidase activity	0.004698	PSMA6, PSMA7, PSMA8
ionotropic glutamate receptor binding	0.006443	GSK3B, GNAS, CTNNB1
protein phosphatase binding	0.007184	HSP90AA1, JUP, CTNNB1, GRB2
cadherin binding	0.007409	JUP, CTNNB1, NDRG1
GTPase binding	0.007409	STOML2, HSP90AA1, BIN1

The 83 shortlisted proteins based on dual localization in synapse and nucleus were analyzed using David. The top 20 Gene Ontology (GO) terms for Biological Process (BP) and Molecular Function (MF) were listed. The GO terms associated with cell adhesion were highlighted.

Figure S7. Literature search on the 83 shortlisted candidate proteins

Accession	GS	Name	Stimulated			Basal			Stimulated / Basal	Nuclear shuttling	Remarks
			IgG	Imp- β 1	Imp-B1/IgG	IgG	Imp- β 1	Imp-B1/IgG			
Q3TED3	Acly	ATP-citrate synthase	-1	0.44	-0.44	-1	0.21	-0.21	2.1		
D3Z0T1	Add1	Alpha-adducin	-1	0.28	-0.28	-1	-1	1	-0.28	Y	Contains NLS, calcium-dependent nuclear shuttling in cancer cells (Kiang & Leung, 2018)

A0A1D5RMG 4	Agap3	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 3	-1	0.44	-0.44	-1	0.04	-0.04	11	Y	Its splice variant, CRAG, contains nuclear localization signal. ROS triggered the association of CRAG with polyQ and the nuclear translocation of CRAG-polyQ complex (Qin et al., 2006). CRAG knock out mice exhibited suppressed kainic acid induced cfos expression in hippocampus. (Nagashima et al., 2019)
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A2ALT5	Ahcy	Adenosylhomocysteinas e	-1	0.44	-0.44	-1	-1	1	-0.44	Y	Nucleocytoplasmic shuttling in basal HeLa cells (Grbeša et al., 2017) Nuclear accumulation in transcriptionally active cells (Radomski, Kaufmann, & Dreyer, 1999)
Q9CZS1	Aldh1b1	Aldehyde dehydrogenase X, mitochondrial	-1	0.11	-0.11	-1	-1	1	-0.11		
S4R2J3	Anks1b	Ankyrin repeat and sterile alpha motif domain-containing protein 1B (Fragment) (AIDA)	-1	0.23	-0.23	-1	-1	1	-0.23	Y	Contains monopartite NLS, Activity-dependent synapse-to-nucleus shuttling. (Bryen A. Jordan, Brian D. Fernholz, Latika Khatri, & Edward B. Ziff, 2007)

Q3TWT5	Asah1	Acid ceramidase	-1	0.44	-0.44	-1	-1	1	-0.44		
D3Z2Y8	Bdh1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	-1	0.15	-0.15	-1	0.12	-0.12	1.25		
Q6P1B9	Bin1	Bin1 protein	-1	0.5	-0.5	-1	-1	1	-0.5		
A0A0M3HEP6	Cadps2	Calcium-dependent secretion activator 2	-1	0.12	-0.12	-1	0.06	-0.06	2		
F8WHB5	Camk2a	Calcium/calmodulin-dependent protein kinase type II subunit alpha	-1	0.16	-0.16	-1	-1	1	-0.16	Y	Contains NLS, NMDAR dependent PSD localization (Zalcman, Federman, & Romano, 2018)
O70589-2	Cask	Isoform 2 of Peripheral plasma membrane protein CASK	-1	0.44	-0.44	-1	-1	1	-0.44	Y	Calcium dependent nuclear shuttling (Ojeh et al., 2008; Schuh et al., 2003)

G3UXG7	Csnk2b	Casein kinase II subunit beta	-1	0.16	-0.16	-1	-1	1	-0.16	Y	<p>Contains NLS. FGF2 binding targeted the holoenzyme containing Csnk2b into the nucleus (Filhol et al., 2003).</p> <p>Mitogenic stimulation induces nuclear translocation (Lorenz, Pepperkok, Ansorge, & Pyerin, 1993).</p>
A0A0G2JDL3	Cdk5	Cyclin-dependent-like kinase 5	-1	0.13	-0.13	-1	-1	1	-0.13	Y	<p>Nuclear translocation in depolarized neurons (Z. Liang et al., 2015)</p> <p>Stress induces nuclear translocation in SHSY5Y cells (Song et al., 2016)</p>

F6QFL0	Chchd3	MICOS complex subunit	-1	0.32	-0.32	-1	-1	1	-0.32		
Q3UPG6	Clint1	Clathrin interactor 1	-1	0.13	-0.13	-1	-1	1	-0.13		
Q8C5W0-4	Clmn	Isoform 4 of Calmin	-1	0.44	-0.44	-1	-1	1	-0.44		
Q3TEA5	Cops3	COP9 signalosome complex subunit 3	-1	0.44	-0.44	-1	-1	1	-0.44		

Q3UZT7	Ctnnb1	Catenin beta-1	-1	0.28	-0.28	-1	-1	1	-0.28	Y	<p>NMDAR dependent synapse-to-nucleus translocation (Michael J. Schmeisser, Andreas M. Grabrucker, Juergen Bockmann, & Tobias M. Boeckers, 2009)</p> <p>NMDAR dependent activation of calpain induced cleavage of CTNNB1 at N-terminus, which in turns, accumulates in the nucleus in hippocampal neurons (Abe & Takeichi, 2007)</p>
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B7ZNF6	Ctnnd2	Catenin delta-2	-1	0.21	-0.21	-1	-1	1	0.21		Contains NLS (Koutras & Lévesque, 2011) Nuclear translocation required for NPRAP gene regulation (Koutras, Lessard, & Lévesque, 2011)
Q9D0M3-2	Cyc1	Isoform 2 of Cytochrome c1, heme protein, mitochondrial	-1	0.15	-0.15	-1	0.05	-0.05	3		
Q56A15	Cycs	Cytochrome c	-1	0.44	-0.44	-1	-1	1	-0.44	Y	Apoptotic signal, DNA damage, depletion of VDAC1 during differentiation and nitro-oxidative stress (for a protective role) drives nuclear accumulation of Cycs (González-Arzola et al., 2019)

D3YZY5	Dhodh	Dihydroorotate dehydrogenase (quinone), mitochondrial	-1	0.44	-0.44	-1	-1	1	-0.44		
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B1AXY1	Dnaja1	DnaJ homolog subfamily A member 1	-1	0.16	-0.16	-1	-1	1	-0.16	Y	<p>Mutant glucocorticoid receptor results in nuclear accumulation of Dnaja1 in Cos cells (Tang, Ramakrishnan, Thomas, & DeFranco, 1997)</p> <p>Dj2 accumulates in nucleolus with reduced staining in cytosol following prolonged heat treatment (Davis, Alevy, Chellaiah, Quinn, & Mohanakumar, 1998)</p> <p>Dj1 accumulates in nucleoli after heat shock (Terada & Mori, 2000)</p>
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D3Z6U8	FMR1	Fragile X mental retardation protein 1 homolog	-1	0.15	-0.15	-1	0.08	-0.08	1.66		Contains NLS and NES, known nucleocytoplasmic shuttling protein that shuttles mRNA out of the nucleus (Feng et al., 1997).
Q00612	G6pdx	Glucose-6-phosphate 1-dehydrogenase X	-1	0.11	-0.11	-1	-1	1	-0.11		
A0A0G2JGS8	Gabrb1	Gamma-aminobutyric acid receptor subunit beta-1 (Fragment)	-1	0.13	-0.13	-1	-1	1	-0.13		
Z4YKV1	Gnas	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	-1	0.28	-0.28	-1	-1	1	-0.28		
F7ALS6	Got1	Aspartate aminotransferase, cytoplasmic	-1	0.23	-0.23	-1	-1	1	-0.23		

A0A0U1RQ18	Gpi1	Glucose-6-phosphate isomerase	-1	0.28	-0.28	-1	-1	1	-0.28	Y	Hypoxia drives nuclear import of GPI in cancer cells (Funasaka, Yanagawa, Hogan, & Raz, 2005)
Q60631	Grb2	Growth factor receptor-bound protein 2	-1	0.14	-0.14	-1	-1	1	-0.14	Y	Accumulates in the nucleus and plasma membrane after EGF stimulation with a reduction in cytosol in A431 cells (Yamazaki et al., 2002)

Q5KU03	Gsk3b	Glycogen synthase kinase 3 beta	-1	0.28	-0.28	-1	-1	1	-0.28	Y	<p>Cell senescence (Zmijewski & Jope, 2004), pro-apoptotic stimuli (Bijur & Jope, 2001) induce nuclear translocation.</p> <p>Contains bipartite NLS, growth factor stimulation induces nuclear export while cell cycle progression induces nuclear import (Meares & Jope, 2007)</p>
Q8BG05	Hnrnpa3	Heterogeneous nuclear ribonucleoprotein A3	-1	0.11	-0.11	-1	-1	1	-0.11		<p>Nucleocytoplasmic shuttling protein for RNA export (Papadopoulou, Boukakis, Ganou, Patrino-Georgoula, & Guialis, 2012)</p>

P61979	Hnrnpk	Heterogeneous nuclear ribonucleoprotein K	-1	0.22	-0.22	-1	-1	1	-0.22	Y	<p>Hnrnpk relocalized to cytoplasm during VSV infection (Pettit Kneller, Connor, & Lyles, 2009).</p> <p>Serum stimulation or activated pERK drives phosphorylated Hnrnpk into cytosol (Habelhah et al., 2001).</p> <p>BDNF activity drives HnrnpK dendritic accumulation in hippocampal neurons (Morais, 2014).</p>
A0PJ91	Hsp90aa1	Hsp90aa1 protein	-1	0.44	-0.44	-1	-1	1	-0.44	Y	<p>HSP90 interacts with Imp-β1 for glucocorticoid receptor nuclear import (Echeverría et al., 2009)</p>
E9Q3D6	Hsp90ab1	Heat shock protein HSP 90-beta	-1	0.44	-0.44	-1	-1	1	-0.44		

											<p>B-naphthoflavone induces aryl hydrocarbon receptor complexing with HSP90 to enter nucleus in HeLa cells (Tsuji et al., 2014)</p> <p>Hypoxia drives nuclear entry of HSP90 (Katschinski et al., 2004)</p>
A0A0J9YTZ7	Hsph1	Heat shock protein 105 kDa (Fragment)	-1	0.32	-0.32	-1	-1	1	-0.32	Y	<p>The β-splice variant of Hsph1 shuttles to the nucleus following stress to induce HSP70 expression (Stetler et al., 2010).</p> <p>Nuclear entry dependent on Imp-β1 cargo (Saito, Yamagishi, & Hatayama, 2007).</p>

A2AFQ0	Huwe1	E3 ubiquitin-protein ligase HUWE1	-1	0.44	-0.44	-1	-1	1	-0.44		
Q9EPL8	Ipo7	Importin-7	-1	0.42	-0.42	-1	0.05	-0.05	8.4	Y	
F6U8U1	Itsn1	Intersectin-1	-1	0.44	-0.44	-1	-1	1	-0.44		LMB traps Itsn1 in the nucleus, Imp- α cargo (Alvisi et al., 2018)
Q02257	Jup	Junction plakoglobin	-1	0.28	-0.28	-1	-1	1	-0.28	Y	Inadequate MAPK activity results in APC cytoplasmic sequestration along with Jup nuclear accumulation in cancer cells (Hildesheim, Salvador, Hollander, & Fornace, 2005)
Q60960	Kpna1	Importin subunit alpha-5	-1	0.23	-0.23	-1	0.055	-0.055	4.09	Y	

O35343	Kpna4	Importin subunit alpha-3	-1	0.29	-0.29	-1	0.06	0.13	-2.26	Y	
Q4FJZ2	Kpna6	Importin subunit alpha-6	-1	0.27	-0.27	-1	0.15	-0.15	1.74	Y	
Q3TFE8	Kpnb1	Importin subunit beta-1	-1	1.16	-1.16	-1	0.93	-0.93	1.25	Y	
D3Z3G6	MAPK3	Mitogen-activated protein kinase	-1	0.15	-0.15	-1	0.02	-0.02	7.5	Y	<p>Synapse-to-nucleus signaling proteins dependent on synaptic activation (Zhai et al., 2013).</p> <p>Nuclear entry dependent on Imp-β1 (Perlson et al., 2006) and IPO7 (Lorenzen et al., 2001).</p>

Q3UH19	Mapt	Microtubule-associated protein	-1	0.68	-0.68	-1	-1	1	-0.68	Y	<p>Contains NLS (Alonso et al., 2010)</p> <p>Disrupted microtubules (Siano et al., 2019), oxidative and heat stress (Sultan et al., 2011) induce nuclear translocation of Tau in neurons.</p>
E9PVF3	Ndrp1	N-myc downregulated gene 1	-1	0.21	-0.21	-1	-1	1	-0.21	Y	<p>DNA damage drives nuclear translocation of NDRG1 in p53 dependent manner (Kitowska & Pawelczyk, 2010)</p> <p>Hypoxia induces nuclear accumulation of NDRG1 in human trophoblasts (Shi et al., 2013)</p>

Q80TQ3	Nefh	Neurofilament heavy polypeptide	-1	0.44	-0.44	-1	-1	1	-0.44		
F6V7K3	Npepps	Puromycin-sensitive aminopeptidase	-1	0.12	-0.12	-1	-1	1	-0.12		
A2AT02	Nsfl1c	NSFL1 cofactor p47	-1	0.44	-0.44	-1	-1	1	-0.44		

E0CXD4	Pcdh1	Protocadherin-1	-1	0.28	-0.28	-1	0.08	-0.08	3.5	Y	<p>ICD of α-Pcdh and γ-Pcdh undergo nuclear shuttling in cell lines (Haas, Frank, Véron, & Kemler, 2005; Hambsch, Grinevich, Seeburg, & Schwarz, 2005)</p> <p>Processing of human Pcdh (Fat1) releases C-terminal that shuttles into the nucleus in cell lines (Magg, Schreiner, Solis, Bade, & Hofer, 2005)</p>
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F2Z4A5	Pdpk1	3-phosphoinositide-dependent protein kinase 1	-1	0.26	-0.26	-1	-1	1	-0.26	Y	<p>Contains NLS. IGF1, insulin, nerve growth factor and activated src kinase stimulates nuclear localization of Pdpk1. PI3K signaling modulates nucleocytoplasmic regulation of Pdpk1. (Kikani, Dong, & Liu, 2005; M. A. Lim, Kikani, Wick, & Dong, 2003; Scheid, Parsons, & Woodgett, 2005)</p> <p>Stimulated EP4 receptors by prostaglandins drives nuclear translocation of Pdpk1 in human eosinophils (Sturm et al., 2015)</p>
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A2AS44	Pkp4	Plakophilin-4	-1	0.28	-0.28	-1	-1	1	-0.28		
Q9Z2M7	Pmm2	Phosphomannomutase 2	-1	0.12	-0.12	-1	-1	1	-0.12		
A0A087WRA7	Ppp1r7	Protein phosphatase 1 regulatory subunit 7	-1	0.25	-0.25	-1	-1	1	-0.25		
Q9QUM9	PsmA6	Proteasome subunit alpha type-6	-1	0.13	-0.13	-1	-1	1	-0.13		
Q9Z2U0	PsmA7	Proteasome subunit alpha type-7	-1	0.12	-0.12	-1	-1	1	-0.12		Interacts with Imp- α 2 (Arjomand et al., 2014b)
Q9CWH6	PsmA8	Proteasome subunit alpha type-7-like	-1	0.18	-0.18	-1	-1	1	-0.18		
F2Z3U3	Raph1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	-1	0.44	-0.44	-1	-1	1	-0.44		

A0A1B0GSG5	Rnh1	Ribonuclease inhibitor	-1	0.44	-0.44	-1	-1	1	-0.44	Y	Cellular stress induces nuclear accumulation of Rnh1 in HeLa cells (Pizzo et al., 2013)
Q3TDK6	Rogdi	Protein rogdi homolog	-1	0.13	-0.13	-1	-1	1	-0.13		Suggested as presynaptic to nucleus shuttling protein (Riemann, Wallrafen, & Dresbach, 2017)
P62830	Rpl23	60S ribosomal protein L23	-1	0.41	-0.41	-1	-1	1	-0.41		
D3YVM5	Rplp0	60S acidic ribosomal protein P0 (Fragment)	-1	0.23	-0.29	-1	-1	1	-0.29		
P62301	Rps13	40S ribosomal protein S13	-1	0.49	-0.49	-1	-1	1	-0.49		
S4R223	Rps19	40S ribosomal protein S19	-1	0.54	-0.54	-1	0.22	-0.22	2.49		

A0A0N4SWD0	Scrn1	Secernin-1 (Fragment)	-1	0.28	-0.28	-1	-1	1	-0.28		
Q8BJU0-2	Sgta	Isoform 2 of Small glutamine-rich tetratricopeptide repeat-containing protein alpha	-1	0.1	-0.1	-1	-1	1	-0.1		

Q3V4A1	Stk11	Serine/threonine-protein kinase STK11	-1	0.28	-0.28	-1	-1	1	-0.28	Y	<p>Antroalbol H induces nuclear translocation of Stk11 in adipocytes (F. Wang et al., 2019)</p> <p>Metabolic stress induces Stk11 nuclear exit via exportin 7 in astrocytes (H. J. Liang et al., 2015)</p> <p>Imp-α cargo. Stk11 mostly in the nucleus and the binding of Strad and Mo25 modulate its nuclear exit (Dorfman & Macara, 2008)</p>
A2AG41	Stoml2	Stomatin-like protein 2, mitochondrial	-1	0.17	-0.17	-1	-1	1	-0.17		

Q5D0A4	Stx1a	Syntaxin 1A	-1	0.83	-0.83	-1	-1	1	-0.83		
P61264	Stx1b	Syntaxin-1B	-1	0.22	-0.22	-1	-1	1	-0.22		Nucleocytoplasmic shuttling proteins that is Ran dependent in neurons (Pereira et al., 2008).
Q9JKK1-2	Stx6	Isoform 2 of Syntaxin-6	-1	0.13	-0.13	-1	-1	1	-0.13		
A0A140LHJ4	Stxbp1	Syntaxin-binding protein 1	-1	0.45	-0.45	-1	0.28	-0.28	1.61	Y	Contains NLS (Sharma et al., 2005) Kainate acid and glutamate stimulation induce nuclear exit in neurons (Wan et al., 2010; Zhang et al., 2011)

G3UZ48	Syncrip	Heterogeneous nuclear ribonucleoprotein	-1	0.44	-0.44	-1	0.32	-0.32	1.38	Y	LMB traps Syncrip in cultured cortical neurons (Smart, 2017) Nuclear Syncrip redistributes to cytoplasmic stress granules following stressors such as PMA, thapsigargin, arsenite and heat shock (Quaresma et al., 2009)
W0TYI4	Tcp1	T-complex protein 1	-1	0.18	-0.18	-1	-1	1	-0.18		
Q3TIZ0	Tuba1c	Tubulin alpha chain	-1	4.85	-4.85	-1	-1	1	-4.85		
A0A0A6YVV3	Txndc9	Thioredoxin domain-containing protein 9	-1	0.44	-0.44	-1	-1	1	-0.44		

Q6ZPJ3	Ube2o	(E3-independent) E2 ubiquitin-conjugating enzyme UBE2O	-1	0.44	-0.44	-1	-1	1	-0.44		Interacts with several Imp- β members with IPO7 achieving the highest score (Mashtalir et al., 2014)
B2KF55	Ube2v2	Ubiquitin-conjugating enzyme E2 variant 2	-1	0.28	-0.28	-1	0.24	-0.24	1.17		
Q99PL6-2	Ubxn6	Isoform 2 of UBX domain-containing protein 6	-1	0.44	-0.44	-1	-1	1	-0.44		
O08614	Utrn	Utrophin	-1	0.44	-0.44	-1	-1	1	-0.44		

D3YXN6	Ywhaz	14-3-3 protein zeta/delta	-1	0.28	-0.28	-1	-1	1	-0.28	Y	<p>Forskolin increases RACK1 binding with Ywhaz, essential for RACK1 nuclear import and PKA-dependent BDNF transcription. cAMP/PKA mediated nuclear accumulation of Ywhaz in SHSY5Y cells (Neasta et al., 2012b)</p> <p>Interacts with Imp-α2 and Imp-α4 (Arjomand et al., 2014b)</p>
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83 shortlisted candidate proteins that have an enhanced association with Imp- β 1 following synaptic stimulation in purified synaptosomes. The emPAI scores are reflected in each condition and the proteins that are known to undergo nuclear shuttling are denoted; Y – nuclear shuttling in

response to stimulus in non-neuronal cells, **Y** – nuclear shuttling in response to stimulus in neurons, and **Y** – known synapse-to-nucleus signaling protein.