Exploring the Interaction between Activity-Regulated Cytoskeleton-associated (Arc) Protein and Paraspeckles Proteins in Neuroblastoma Cells (SH-SY5Y)

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Laleh Kabiry Renani

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ABBREVIATIONS

AMPAR	–	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arc	–	Activity-regulated cytoskeleton-associated protein
BCA	–	Bicinchoninic Acid assay
BSA	–	Bovine serum albumin
CaMKII	–	Ca^{+2}/calmodulin-dependent protein kinase II
CBP	–	CREB binding protein
Cch	–	Carbamylcholine or carbachol
CDK9	–	Cycline-dependent kinase 9
Co-IP	–	Co-immunoprecipitation
CTD	–	C-terminal domain
DBHS	–	Drosophila melanogaster behavior, human splicing
DMEM	–	Dulbecco's Modified Eagle's Serum
ERK	–	Extracellular signal-regulated kinase
eRNA	–	Enhancer RNA
FBS	–	Fetal bovine serum
GKAP	–	Guanylate kinase-associated protein
HATs	–	Histone acetyltransferases
hESC	–	Embryonic stem cells
H4K12	–	Histone H4 at lysine 12
IEGs	–	Immediate early genes
IP	–	Immunoprecipitation
ICC	–	Immunocytochemistry
lncRNA	–	Long non-coding RNA
LTD	–	Long-term depression
LTM	–	Long-term memory
LTP	–	Long-term potentiation
LTSP	–	Long-term synaptic plasticity
mAchR	–	Muscarinic acetylcholine receptor
MAP2	–	Microtubule-associated protein 2

mCAT2	–	Mouse cationic amino acid transporter 2
MEK	–	Mitogen-activated protein kinase kinase
mGluR	–	Metabotropic glutamate receptor
ncRNA	–	Non-coding RNA
NEAT1	–	Nuclear paraspeckle assembly transcript 1
NES	–	Nuclear export signal
NLS	–	Nuclear localization signal
NMDAR	–	*N*-methyl-D aspartate receptor
NRD	–	Nuclear retention domain
NTD	–	N-terminal domain
PAGE	–	Polyacrylamide gel electrophoresis
PBS	–	Phosphate buffered saline
PBS-T	–	Phosphate buffered saline Tween
PML	–	Promyelocytic leukemia
PRP	–	Plasticity-related protein
PSD	–	Postsynaptic density
PSF/SFPQ	–	Polypyrimidine tract-binding protein-associated splicing factor
P54nrb	–	Nuclear RNA-binding protein 54 kDa
PSPC1	–	Paraspeckle protein component 1
RBP	–	RNA-binding protein
RIPA	–	Radioimmunoprecipitation assay
RNAPII	–	RNA polymerase II
RRM	–	RNA recognition motif
SDS	–	Sodium dodecyl sulfate
STSP	–	Short-term synaptic plasticity
TARPγ2	–	Transmembrane AMPAR regulatory proteins
TBS	–	Tris-buffered saline
TBST	–	Tris-buffered saline Tween
Tip60	–	Histone acetyltransferase KAT5

1. Summary

Our brain can be affected by various experiences that alter the activity and organization of neural circuits [1]. Studies on brain network connectivity have advanced the understanding of brain changes and adaptation in response to new experiences and memory formation [2]. Our current understanding of learning and memory processes has been dominated by the belief that synapses are the primary location for information storage in the brain [3]. Synaptic plasticity, which involves the ability of neurons to modify their connections, assists with the consolidation and storage of long-lasting memories [4]. The expression of genes such as immediate-early genes (IEGs) in neurons is dynamically altered in response to neuronal activity within the brain [5]. Mammalian activity-regulated cytoskeleton-associated protein (Arc), also known as activity-regulated gene (*Arg3.1*), is an immediate early gene induced by specific patterns of synaptic activity and is crucial for synapse development, synaptic plasticity, and memory formation [6, 7]. Arc shuttles between somato-dendritic and nuclear compartments to regulate synaptic plasticity. It was proposed that Arc functions as a hub for interactions within neurons, including both dendrites and the nucleus [8]. The molecular mechanism behind the Arc protein's role in the nucleus is not fully understood. One of the recent studies in our lab showed that Arc interacts with PSF protein, one of the main paraspeckle proteins, in the nucleus (Kanhema, not published).

Paraspeckles are a relatively newly discovered class of subnuclear bodies found in the interchromatin space of mammalian cell nuclei [9]. The key proteins that make up paraspeckles (PSF/SFPQ, NONO/P54nrb, and PSPC1) belong to the DBHS family, which stands for Drosophila melanogaster behavior, human splicing. They have a crucial role in controlling the expression of specific genes in differentiated cells by retaining RNA within the nucleus [10]. RNA-binding proteins of the DBHS (Drosophila Behavior Human Splicing) family, NONO, SFPQ, and PSPC1 have numerous roles in genome stability and transcriptional and posttranscriptional regulation [11]. Paraspeckles also are involved in the sequestration of component proteins and RNAs, with subsequent depletion in other compartments [12].

In this study, we hypothesized that Arc could interact with one or more paraspeckle proteins in the nucleus. To evaluate this, the neuroblastoma (SH-SY5Y) cells were used and treated with muscarinic cholinergic agonist, carbachol, to induce and inhibit endogenous Arc expression, and

the ERK signaling inhibitor, U0126, to inhibit Arc expression. Methods used to evaluate Arc protein-protein interactions and localization were co-immunoprecipitation (Co-IP), subcellular fractionation, and immunocytochemistry (ICC). We found that Arc co-immunoprecipitated with PSF/SFPQ and NONO/P54nrb, but not PSPC1, in the nucleus of SH-SY5Y cells. Additionally, the ICC results revealed that Arc colocalized with P54nrb in the nucleus. These findings are promising and may identify new binding partners for Arc protein related to its role in the nucleus. However, future research should test the specification of interaction between Arc and paraspeckle proteins in the nucleus by finding the binding site on Arc and paraspeckle proteins and studying the functional connection between these proteins.

2. Introduction

Numerous activities, such as reading books, learning in a classroom, or experiencing new situations, can all affect different parts of our brains. Understanding how our brains can create, learn, or adapt behaviors is an important goal in neuroscience. Modifying certain brain regions, such as the hippocampus, results in the acquisition of adaptive behavior by altering specific neurons in each area. Neurons connect with each other at locations called synapses, which are the building blocks of neural circuits. A neuron can develop hundreds of synapses on its dendrites, cell body, and axon. Synaptic transmission involves transmitting information from one neuron to another in the nervous system through synapses [13]. For a long time, it has been hypothesized that changes in the strength of synaptic connections within networks can explain the shaping of neuronal population activity that is relevant to a task [1]. One of the advancements in neurobiology over the past years has been the realization that synapses are highly flexible. Synapses can change their strength through plasticity, which can be influenced by their own activity or activity in another pathway. It is commonly known that a more profound comprehension of synaptic plasticity contributes to a better understanding of the mechanisms of memory and learning [14].

2.1.1 Synaptic Plasticity

Synaptic plasticity is the ability of neurons to change the strength or effectiveness of their synaptic connections, making it an important characteristic. A crucial feature of synaptic plasticity is the ability of neurons to incorporate new experiences into pre-existing memory traces. This plasticity can modify the neural circuit function in response to an experience and affect subsequent thoughts, moods, and behaviors (Figure 2-1). Various forms of synaptic plasticity can be classified based on the duration and type of synaptic transmission changes (enhanced or depressed) [1].

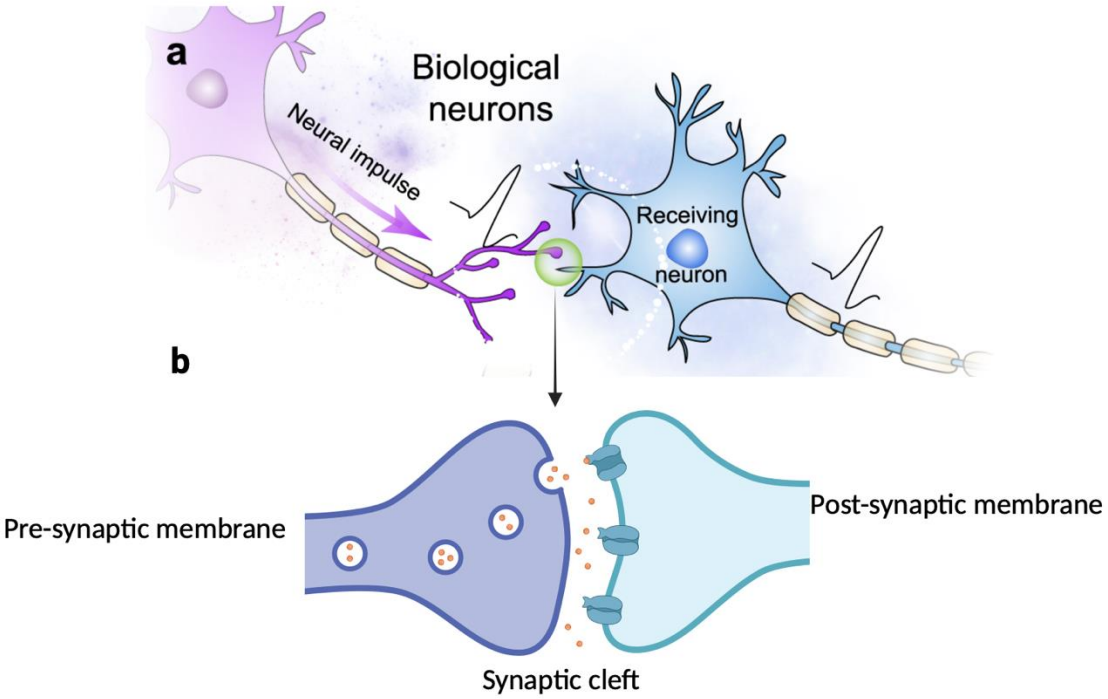


Figure 2-1: Synaptic plasticity. This figure illustrates where synaptic plasticity occurs between the neurons. a) Signal transmission between two neurons. b) Many molecular and signaling pathways control synaptic plasticity in pre- and post-synaptic membranes. The figure was adopted from [15] and made by app.biorender.com.

Short-term synaptic plasticity (STSP) and long-term synaptic plasticity (LTSP) are two categories formed based on the time scale of the stimulus-driven activity [16]. The STSP is essential for processing temporal information. Different types of STSP, including facilitation, augmentation, and potentiation, play a crucial role in handling short-term sensory inputs, changes in behavioral states, and short-term memory formation. LTSP serves as the primary mechanism for learning and memory formation [17]. Two major forms of plasticity are Hebbian, essential for learning and memory, and homeostatic plasticity, necessary for maintaining stable neuronal network activity [18].

2.1.2 Hebbian and Homeostatic plasticity

Hebbian plasticity enables neurons to alter their connections based on various experiences. According to Donald Hebb (1949), the strength of synaptic connections can be changed through repeated activation of a postsynaptic neuron by a presynaptic neuron or by engagement between neighboring neurons [19]. Siegrid Löwel has concisely expressed the concept of Hebb's Law:

“Cells that fire together wires together” [20]. As activity increases, Hebbian plasticity naturally generates a positive feedback loop [19].

Hebbian plasticity includes long-term potentiation (LTP) and long-term depression (LTD), which depend on either the molecular mediators or the loci of expression into presynaptic and postsynaptic. NMDAR (n-methyl-d-aspartate receptors)-dependent and NMDAR-independent forms are the two main categories of the molecular mediators-based form [21].

The other main receptor involved in LTP and LTD is -amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAR), which depends on Ca^{2+} influx. LTP is a stable type of synaptic plasticity and involves both homosynaptic and heterosynaptic elements [14]. Dendritic spine remodeling, including increased spine volume, stability, and clustering, is linked to LTP induction. NMDARs must be activated for LTP to occur, and several essential traits of this type of synaptic plasticity are directly derived from the NMDAR functionality [2]. It is important to note that activation of NMDARs may also result in LTD, which is linked to a persistent decrease in synaptic excitability and represents a separate type of anti-homeostatic plasticity [22]. Moreover, LTD induction is related to morphological alterations in dendritic spines, such as significant spine shrinking, which results in the abolition of dendritic spines [23]. A synaptic depression occurs when the number of synaptic vesicles ready to undergo exocytosis is lowered [24]. Several molecular mechanisms are responsible for LTP and LTD. Changes in synaptic transmission are initially caused by post-translational modifications to existing proteins, most notably changes in glutamate receptor trafficking. In contrast, later phases are caused by changes in gene expression and protein synthesis [25] (Figure 2-2).

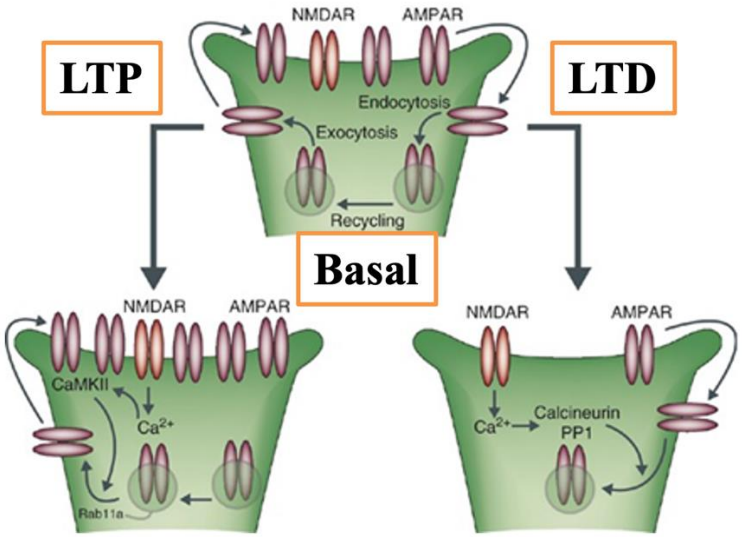


Figure 2-2: Hebbian plasticity. During the induction of LTP, Ca^{2+} flows into postsynaptic cytosol as Mg^{2+}, the blocker of NMADR is removed because of strong postsynaptic depolarization. Thus, the postsynaptic signaling, such as CaMKII, will be activated. AMPARs from a reserve pool will be integrated into the postsynaptic terminal. CaMKII will phosphorylate the cytosolic domain of the AMPARs to raise their conductance. As a result, the excitatory postsynaptic potential will rise. However, following LTD induction, phosphatases brought on by calcium influxes from NMDARs will lead to a decline in the number of AMPARs through endocytosis and synaptic depression [26, 27]. The figure was taken from Citri and C'Malenka [1].

Despite the importance of Hebbian plasticity for the proper function of the brain, it can cause a problem in terms of stability [28]. The other type of plasticity is homeostatic plasticity, which acts diametrically opposed to Hebbian plasticity. Homeostatic plasticity involves negative feedback that causes a neuron to return to its initial state, including those brought on by the Hebbian plasticity [29]. This type of plasticity can be accomplished through various processes, including synaptic scaling, changes in inhibition, and modifications to the intrinsic membrane characteristics [30]. Activity-independent spine variations may also control the potentially explosive rise in synaptic strength caused by Hebbian mechanisms. It is well known that spine sizes vary in vivo independently of activity, and the variations are inversely correlated with synapse size. As a result, the outcome of this activity-independent process might maintain homeostasis [12] (Figure 2-3).

Homeostatic plasticity is crucial because it keeps neurons from getting oversaturated, for example, during LTP or LTD, and ensures that neurons can respond to changing conditions while maintaining optimal function. Understanding how homeostatic processes could control the next excitatory drive of neurons was made by the discovery of synaptic scaling [31]. Synaptic scaling, which affects all particular neuron's synapses, is a homeostatic negative feedback process and a type of hetero-synaptic plasticity [2].

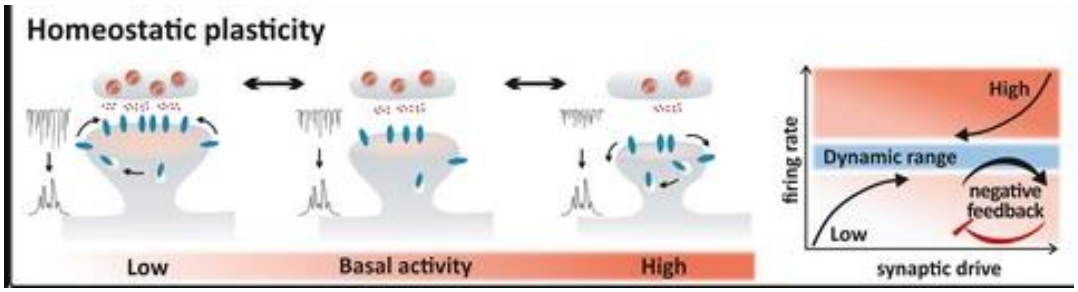


Figure 2-3: Mechanism of homeostatic plasticity. This mechanism adjusts the strength of all synapses within a given cell in response to prolonged changes in activity and keeps neuronal activity in a dynamic range. Synaptic scaling leads to AMPAR-mediated synaptic transmission enhancement and decline during low and high neuronal activity, respectively [32].

2.1.3 Molecular mechanisms of synaptic plasticity

Many findings imply that specific genes stimulated during LTP encode plasticity-related proteins (PRPs) necessary for LTP maintenance and memory formation [5]. For example, NMDAR-associated Ca^{2+} influx affects LTP stabilization by triggering intracellular signaling cascades that subsequently encourage the creation of mRNA and proteins. When NMDAR antagonists or protein creation inhibitors block these pathways, persistent LTP cannot occur, and long-term memory (LTM) formation is impaired [2, 33]. One of the synaptic-related genes is immediate early genes (IEGs) [5].

2.2 Immediate early genes and their roles in synaptic plasticity

Immediate early genes (IEG) are rapidly responding genes transcribed in response to synaptic input and neuronal firing. Minutes after neuronal activation, the RNA transcripts of various IEGs, including *Arc*, *egr-1*, *narp*, and *homer1a*, are transcribed [5, 34]. Within the IEGs, the activity-cytoskeleton-associated (Arc) protein, also known as activity-regulated gene (*Arg3.1*), has been identified as a regulator of long-term synaptic plasticity, including LTP and LTD, and homeostasis scaling. It is known that all these phenomena require Arc synthesis, but the direction of synaptic change remains unknown [35].

2.2.1 Activity-regulated gene (Arc/Arg3.1) and its protein

The Dietmar Kuhl and Paul Worley labs independently identified the actively regulated cytoskeleton-associated (Arc) protein in 1995 [36, 37]. Mammalian Arc and its mRNA induction are well known for their exceptional importance in information processing, learning, and memory [6]. Conversely, dysregulation of Arc expression can significantly negatively affect normal brain function by affecting the Arc-dependent plasticity [38].

Human memory-related brain regions contain the highest concentration of Arc, which may be related to synaptic plasticity and memory consolidation [39]. Arc mRNA and protein accumulate in active dendrites receiving high-frequency stimulation. Following hyperactivity induction, Arc mRNA transfer between cells results in Arc accumulation in its origin and neighboring neurons [40]. Upon hyperexpression, Arc protein assembles into virus-like capsids containing mostly Arc mRNA in local dendritic compartments. These capsids can transport mRNA to neighboring cells and exit neurons enveloped in extracellular vesicles in addition to being a key regulating synaptic protein [41].

Interestingly, in capsid “infected” neurons, stimulation of metabotropic glutamate receptors (mGluR) promotes Arc mRNA translation [41]. Following NMDA receptor stimulation in glutamate neurons, Arc mRNA was markedly increased in the nucleus before being transferred to the dendrites for translation [42]. The mouse model was used to study the glutamatergic neural network’s spine architecture and overall stability, and it was discovered that both relied on Arc expression [6, 43]. Accordingly, it was verified that Arc expression boosts spine density but, more

commonly, lowers synaptic efficiency by decreasing surface GluR1 [44]. The NMDA receptor and extracellular signal-regulated kinase (ERK) are necessary for creating the Arc-protein found at the synaptic connections of neurons [45]. Additionally, it was found that NMDA receptor activation controls the level of the Arc protein [46], an endogenous retroviral product with known activity-dependent expression in neuronal synapses, which is necessary for synaptic modulation [47].

2.2.2 Arc as a flexible hub for synaptic plasticity

The evidence of studies on the structure and function of Arc suggest that Arc can regulate several cellular processes through Arc-binding partner complexes in the postsynaptic compartment and the neuronal nucleus [48]. Additionally, it was stated that Arc is a flexible hub with two significant domains, N- and C-terminal domains (NTD and CTD), that have opposing charges and are located on each side of a central, disordered hinge region. Based on structural investigations, the NTD is predicted to have an antiparallel coiled-coil structure [49]. The NTD mediates Arc's attachment to the phospholipid membrane. Like the capsid domain of retroviral Gag polyprotein, the CTD is a tandem domain comprising two lobes, the N- and C-lobes (NL and CL) [50] (Figure 2-4A).

Various postsynaptic proteins, such as Stargazin, guanylate kinase-associated protein (GKAP), and GluN2A, interact with the mammalian Arc NL via its particular hydrophobic peptide binding pocket. These findings imply that protein-protein interactions and domain interactions control oligomerization to determine the Arc activity state [51, 52]. Additionally, Arc has three domains related to nuclear trafficking, including a non-canonical nuclear localization signal (NLS), the nuclear retention domain (NRD), and a nuclear export signal (NES). Each of these domains is involved in different functions of Arc through binding to various proteins [53] (Figure 2-4B).

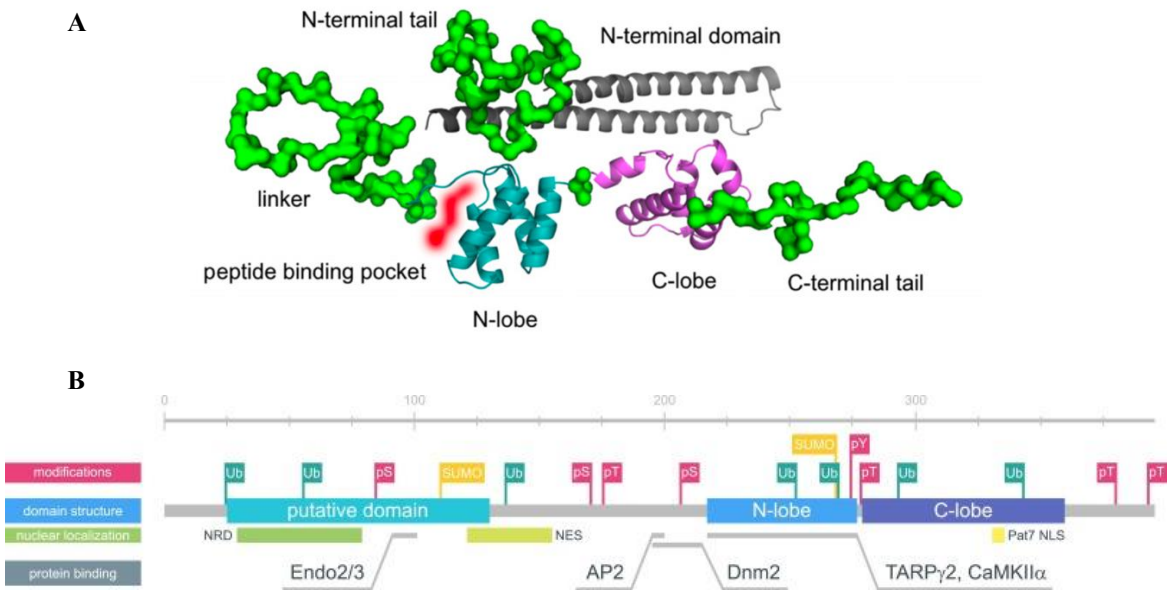


Figure 2-4: Structure of Arc protein with different binding regions. A) A hybrid model of full-length Arc illustrating different domains of Arc [54]. B) Linear structure of Arc with features, different domains, their binding proteins regions and function, and briefly known post-translational modifications [8]. NRD: nuclear retention domain; NES, nuclear export signal; NLS: nuclear localization signal.

2.2.3 Role of Arc in Hebbian Plasticity

While Arc expression is not necessary for learning or the development of short-term memory, it is crucial for LTM consolidation. Arc knockout mice display decreased spatial and fear memory consolidation [5]. Studies demonstrated that the location of Arc formation at the spine on dendrites undergoes morphological changes that increase their density and the fraction of thin spines aligning with the endocytosis of AMPA receptors [55]. Additionally, Arc stabilized nascent F-actin in the dendritic spines [54]. Through these ways, Arc is involved in consolidating long-term memory and LTD. One of the mechanisms for the induction of LTD is Arc-dependent AMPAR endocytosis. The development of mGluR-dependent LTD requires the fast induction of Arc translation by mGluRs, suggesting that Arc protein is crucial for both input-specific synaptic plasticity and cell-wide synaptic scaling [5, 35].

2.2.4 Arc involvement in homeostatic scaling

For cellular synaptic scaling in response to neuronal activity, Arc expression seems to be necessary. The induction of Arc by neuronal activity and synaptic AMPAR endocytosis gives this IEG a role in this process. Arc KO neurons (neurons with inhibited Arc expression) display a lack of homeostatic AMPAR scaling [5]. The Arc protein is also thought to work by interacting with other post-synaptic proteins. When the interaction between Arc and endophilin/dynamin is compromised, the effect of Arc on AMPAR regulation is lost. However, Arc appears to have no direct interactions with AMPAR. Arc-dependent AMPAR synaptic scaling can only occur when Arc interacts with TARPγ2 (Stargazin) [5]. Despite these findings, it is still being determined how Arc can be involved in homeostatic scaling by converting early-to-late-LTP or LTD. Together with Arc nuclear localization, Arc might perform other tasks, including its role in the nucleus [53].

2.2.5 Arc function in the nucleus

Arc protein increases quickly in the cytoplasm of activated neuronal cells. It accumulates slowly in the nucleus, causing the ratio of nuclear to cytoplasmic Arc to rise beyond baseline after 4 hours [8]. Arc associates biochemically in the nucleus with promyelocytic leukemia nuclear bodies (PML-NB), where the epigenetic regulation of gene transcription occurs [56]. Most mammalian cell nuclei include PML-NB complexes, which control nuclear transcription through different mechanisms, such as containing transcription factors and the activity of histone deacetylases. These nuclear bodies may regulate GluA1 by CREB-binding protein (CBP), which promotes CRE-dependent transcription [53]. Nuclear Arc also reduces the transcription of the GluA1 subunit of AMPA receptor, which homeostatically downscales the excitatory synaptic transmission [56].

Additionally, Arc strongly binds to βSpIVS5, a nuclear spectrin isoform, in PML bodies. According to studies, the C-terminal region of Arc (rat amino acids 228–380 of 396) and two spectrin repeats share 20% of their amino acid sequences. Several types of spectrin repeats coordinate cytoskeletal connections and can operate as a “switchboard” for interactions with other proteins [51].

The relationship between Arc and chromatin and its interaction with histone marks was studied in cultured hippocampus and cortical neurons. Fluorescent microscopy tests showed Arc interaction

with dynamic chromatin and Arc association with histone markers for active enhancers and active transcription [56]. This implies that the Arc interacts with many proteins to control various cellular functions and play a crucial role in the nucleus. However, it is unknown how its nuclear distribution impacts synaptic plasticity.

2.2.6 Arc-binding proteins in the nucleus

Arc showed that it has an epigenetic regulation on gene expression through its binding partners in the nucleus [56]. Some evidence demonstrates an interaction between Arc and Tip60, a histone-acetyltransferase, and with a chromatin remodeling complex component, nuclear PML bodies. Hippocampal neurons that express Arc cause the development of endogenous Tip60 speckles. Moreover, ectopically expressed Arc recruits Tip60 to PML bodies and induces enhanced acetylation of lysine 12 on histone 4 (H4K12), a memory-related histone mark that deteriorates with aging and one of Tip60's substrates. This effect is blocked by overexpression of dominant negative Tip60. Arc expression levels correlate with the acetylation level of H4K12 [8, 57]. The acetylation of histones by acetyltransferases (HATs) is a significant epigenetic alteration investigated in neurons. Only a tiny subset of the many proteins in nuclear PML bodies have HAT activity [58]. These include Tip60, p300, and the CREB binding protein (CBP), which have been linked to learning and memory [57].

Furthermore, overexpression experiments in heterologous cells demonstrate that Arc and Tip60 coimmunoprecipitate and form a complex with βSpIVS5 cultured hippocampal neurons. Studies suggest that nuclear Arc also may facilitate learning-induced gene expression by opening chromatin. In conclusion, evidence suggests that nuclear Arc probably regulates chromatin and transcriptional responses by multiple mechanisms. However, the nuclear actions of Arc have not been defined at the molecular or functional level [8, 56, 57].

It was also determined that CBP exists near activity-regulated genes such as transcription of *arc/arc3.1* and plays a role in the activity-regulated increase in RNA polymerase II (RNAPII) binding at enhancers. Additionally, enhancer-RNA (eRNA) synthesis seems initiated near enhancer centers where CBP and RNAPII bind together. Moreover, an intact *Arc* gene promoter appears to be necessary for enhancer-RNA (eRNA) synthesis [59]. Arc enhancer needs an unknown factor for its transcription activity without its promoter. Moreover, chromatin looping

prevents eRNAs from activating nonspecific target genes [60]. Accordingly, and with Arc's contribution to chromatin remolding, it may be possible that Arc is involved in gene transcription through its regulation effects on eRNA or ncRNa. One of the newly discovered nuclear bodies, the paraspeckle, is highly enriched in ncRNA.

2.3 Paraspeckles protein

The cell nucleus is a highly organized structure. This nuclear organization is associated with genome maintenance and gene expression control and thus influences growth, development, and cellular proliferation. Moreover, the lack of proper nuclear organization mainly links to diseases such as acute promyelocytic leukemia due to the loss of subnuclear promyelocytic leukemia (PML) bodies. Various subnuclear bodies contain unique nuclear proteins and nucleic acids correlated with the nuclear organization. In the interchromatin area, many subnuclear bodies, including splicing factor-enriched nuclear speckles, Cajal bodies, and PML bodies, are found [10].

One type of granule assembled by liquid-liquid separation is a nuclear paraspeckle [12], first identified by Fox and colleagues in 2002 [61]. Paraspeckles are newly identified subnuclear/ribonucleoprotein bodies [62]. A proteomic investigation of isolated human nucleoli found 271 proteins, 30% of which were new. It was demonstrated that some of these recently discovered novel proteins were not enriched in nucleoli but instead were found to be diffusely distributed inside the nucleoplasm and concentrated in five to twenty sub-nuclear foci. These foci did not immediately overlap with markers for any previously identified subnuclear structure. Since the foci were seen in the interchromatin area close to nuclear speckles, but separately from them, they were named "paraspeckles" [9].

Paraspeckles are observed only in mammalian cell nuclei, including transformed and primary cell lines, embryonic fibroblasts, tissues, and tumorigenic biopsies. They have dynamic structures with erratic, sausage-like shapes and sizes ranging from about 0.5 to 1.0μm. Additionally, their numbers vary within cell populations and depending on cell type. Interestingly, to date, only one type of mammalian cell has been reported to be free of paraspeckles—human embryonic stem cells (hESC) [63].

2.3.1 Paraspeckle components

Several protein and RNA components are known to be enriched in paraspeckles and RNA-binding proteins (RBP), with reported roles in mRNA processing [9, 10]. Thus, paraspeckles are found as potential hubs for co- and post-transcriptional processes [64]. Paraspeckle proteins are defined by their colocalization in subnuclear foci with a member of the mammalian DBHS (Drosophila melanogaster behavior, human splicing) protein family. Three members of the DBHS protein family, including PSPC1 (paraspeckle protein component 1), P54NRB/NONO (nuclear RNA-binding protein 54 kDa, also known as NONO), and PSF/SFPQ (polypyrimidine tract-binding protein-associated splicing factor, also known as SFPQ) [63], are the most well studied intrinsic protein components of paraspeckles [10] (Figure 2-5).

Figure 2-5: Protein domains of SFPQ, NONO, and PSPC1, three members of the DBHS family. Dashed boxes are related to low-complexity domains and uncharacterized DNA binding domains in SFPQ. Amino acid boundaries in H. sapiens are indicated in numbers [64].

DBHS proteins have a nuclear localization signal at their C-terminus and are regarded mainly as nuclear factors. These proteins are dynamic within the nucleus, which means they cycle between the nucleoplasm, paraspeckles, and the nucleolus under normal conditions and accumulate within perinucleolar cap structures when RNA Pol II transcription is inhibited [62]. The DBHS family proteins are called ‘‘core paraspeckle proteins’ [63]. Reported interactions between all members of this family suggest that they exist as either homo- or heterodimers in vivo [65, 66]. They share >50% sequence identity within two N-terminal RNP-type RNA recognition motifs and a C-terminal coiled- coil domain. One of these RNP-type RNA recognition motifs and the coiled-coil domain (which mediates dimerization) are required for PSPC1 to be targeted to paraspeckles [10, 66]. This finding explains the discovery of PSPC1 in the nucleolar proteome. Knockdown of either of the two highly expressed DBHS proteins (P54NRB/NONO and PSF/ SFPQ) in HeLa cells results in a paraspeckle loss [67]. In contrast, the knockdown of the less abundant DBHS

protein PSPC1 in HeLa cells does not affect paraspeckles [67]. Thus, highly expressed DBHS protein dimers are at the core of paraspeckle structural integrity [10].

Most paraspeckle proteins have roles in Pol II transcription and RNA processing, including the members of the DBHS protein family, several transcription factors, and a co-transcriptional splicing factor [9]. There are two specific paraspeckle RNA components, each belonging to different classes: Ctn is regulated within paraspeckles and is implicated in the control of gene expression by RNA nuclear retention, whereas the abundant nuclear ncRNA, NEAT1, is an architectural paraspeckle component essential for their formation and maintenance [9].

2.3.2 Paraspeckle RNAs

After the initial discovery of paraspeckles, some evidence indicated that paraspeckles are RNA-protein bodies and contain RNAs, of which the lncRNA NEAT1 is the most studied [68] (Figure 2-5B). In this regard, the nuclear bodies have been recognized to be sensitive to transcriptional inhibition and Rnase treatment, identifying that certain ribonucleic acids possibly play a structural role [63]. Thus, they may have RNA-related functions such as processing or transport [9, 10, 69]. The A-to-I edited CTN RNA, a long isoform transcribed from mCAT2 (mouse cationic amino acid transporter 2), was the first identified RNA component of paraspeckles [63].

Accordingly, paraspeckles were first degraded after cell treatment with RNAse [70]. Second, all major paraspeckle proteins were established to contain RNA binding motifs associated with their described functions in RNA processing. PSPC1 showed that it needs its RNA-biding domain for the paraspeckle targeting [61]. Eventually, studies demonstrated that dissembling and reassembling of paraspeckles occur in the absence and restoration of active Pol II transcription, respectively. This suggests that paraspeckle formation may depend on RNA production [10].

Additionally, two paraspeckle-associated RNA species have been identified, providing great clues to understanding paraspeckle formation and function [9]. Moreover, studies reported that NEAT1 plays architectural roles during paraspeckle formation. These studies demonstrated that paraspeckles are disintegrated upon depletion of NEAT1 transcripts by an antisense oligonucleotide or siRNA, and their protein components become evenly distributed throughout the

nucleoplasm [63]. In general, Figure (2-6) shows different components of paraspeckles as well as their substructure.

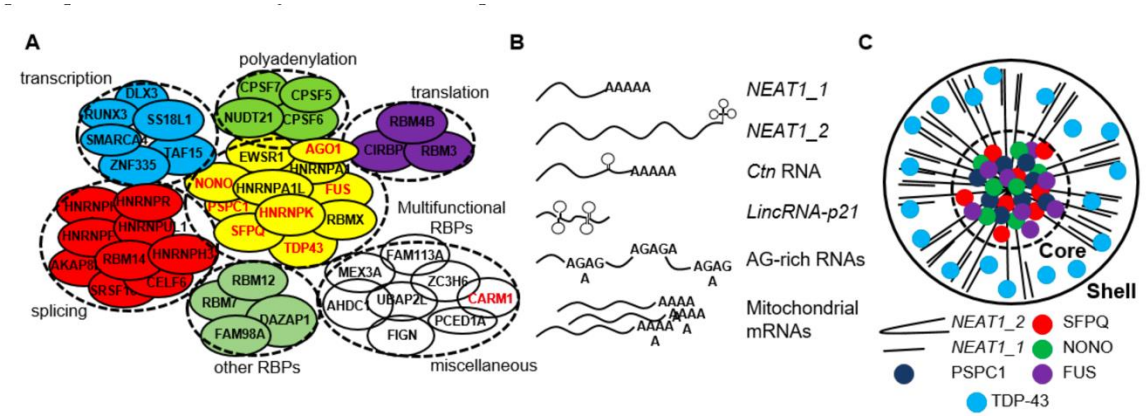


Figure 2-6: Protein components of Paraspeckle. A) This scheme shows various types of paraspeckle proteins with their different roles. NONO, PSPC1, and SFPQ are the main paraspeckle proteins in the middle. B) Different paraspeckle RNA components with newly identified lincRNA-p21 and mitochondrial mRNAs. C) Paraspeckle substructure [64].

2.3.3 Paraspeckle formation

Following an understanding of the main paraspeckle protein and RNA components, discussing paraspeckle formation is possible. The model for paraspeckle formation first starts with the production of NEAT1 transcripts in daughter nuclei after cell division [71]. The *NEAT1* gene produces short and long isoforms named NEAT1_1 (3.7 kb) and NEAT1_2 (23 kb). Both isoforms are single exon RNAs with different modes of terminal end processing. While NEAT1_1 is polyadenylated, NEAT1_2 has a structure similar to a triple helix t-RNA at its 3¢end, shared with 129 other lncRNAs in vertebrates [64].

Paraspeckles showed that they cannot form without the production of NEAT1 RNA. Additionally, paraspeckle has been observed with abundant DHBS proteins. After NEAT1 is transcribed, it forms complexes with DBHS proteins, which build up the paraspeckle particle. The NEAT1 RNA-DBHS protein complex makes a dynamic structural scaffold where paraspeckles can exchange with a pool of DBHS proteins in the nucleoplasm. Thus, the oligomerization propensity of DBHS proteins may be involved in the paraspeckle structural framework [65, 66] (Figure 2-7).

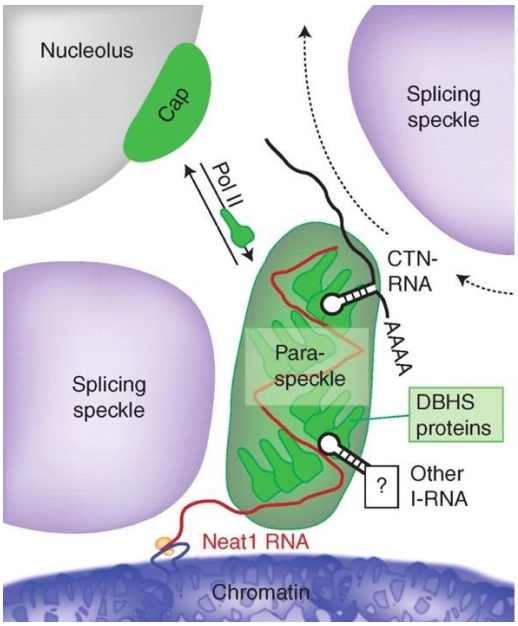


Figure 2-7: Model for paraspeckle formation. As it is clear, the formation of paraspeckles occurs near the NEAT1 gene locus in the interchromatin space. First, NEAT1 RNA interacts with DBHS protein. Second, other RNA species, such as A-to-I edited mRNA, were regulated in paraspeckles and elsewhere in the nucleus through interaction with DBHS proteins. Inhibition of RNA Pol II transcription results in the accumulation of DBHS proteins at perinuclear caps [9].

The first paraspeckles form very close to the NEAT1 gene locus, and paraspeckles also remain closely associated with the NEAT1 gene in interphase. This location may be the production site for RNA species regulated in paraspeckles. Additionally, paraspeckle proteins are defined by the co-localization of SFPQ, NONO, or PSPC1 with the long noncoding RNA NEAT1. Both SFPQ and NONO are necessary for paraspeckle formation and integrity, as siRNA knockdown of both proteins showed impaired paraspeckle formation [62]. While the precise functional role of paraspeckles is unknown, the generally identified role for paraspeckles is that their gene expression increases under stress conditions. Moreover, paraspeckles contribute to gene regulation by sequestering a subset of nuclear proteins, including DBHS proteins [62]. Interestingly, the association between nuclear paraspeckles and cytoplasmic P-bodies is linked to the control of gene expression through mRNA storage [68].

2.3.4 The function of different paraspeckle proteins

PSPC1

It was initially unexpected that the accumulation of PSPC1 was not identified in nucleoli. Instead, it was discovered in a proteomic screen for nucleolar components. However, investigating PSPC1's dynamic properties showed its nucleolar connection. Additionally, freshly divided cells that had not yet started transcription again after cell division showed a peri-nucleolar concentration of PSPC1. In addition to its coiled-coil domain, the minimum PSPC1 segment directed to paraspeckles has at least one RNA recognition motif (RRM) motif [9].

SFPQ/PSF

SFPQ is an abundant nuclear RNA-Binding Protein (RBP) that has been identified to have roles in neurodegenerative diseases. Due to its capacity to interact with various protein and nucleic acid partners, SFPQ has been referred to as a "multifunctional" protein. Some of the described nuclear functions for SFPQ include paraspeckle creation, alternative splicing, transcriptional control, DNA damage repair, and genome stability maintenance [72]. Moreover, according to the cellular environment, SFPQ has been demonstrated to function as both a coactivator and a corepressor in the context of transcriptional regulation. Thus, it plays various cellular roles in the nucleus, cell body, and axon of different types of neurons that are directly related to neuronal development, differentiation, proliferation, and maintenance. Additionally, it has been demonstrated that in mouse brains, SFPQ polymers attach to and stabilize the long pre-mRNA in neurons, attracting cyclin-dependent kinase 9 (CDK9) and activating RNA polymerase II [72].

NONO/P54nrb

The other paraspeckle protein, NONO/P54nrb, which has a nuclear localization signal (NLS) at its C-terminus, is found in the nucleus of most mammalian cells and is mainly distributed in the subnuclear domain known as paraspeckles. New research strongly suggests that NONO has additional roles in carcinogenesis, including controlling cell proliferation, apoptosis, migration, and DNA damage repair [73]. NONO interactions are controlled mainly by the interactors and post-translational modifications that affect its structure. The precise mechanism governing NONO's transcriptional control still needs extensive further research. However, the majority of

gene regulation processes, such as pre-mRNA splicing, transcription activation and termination, nuclear retention of damaged RNA, DNA unwinding, double-strand break repair, and preserving proper circadian clock activities, are all carried out by NONO [74].

Additionally, NONO interacts with other promoters of numerous transcriptionally active genes, including rhodopsin, oct4, TORCs (transducer of regulated CREB), and AR (androgen receptor). This stimulates transcription, a process frequently linked to a synergistic impact with other promoters. NONO occasionally attaches to a suppressor to avoid transcription activation [73] (Figure 2-8).

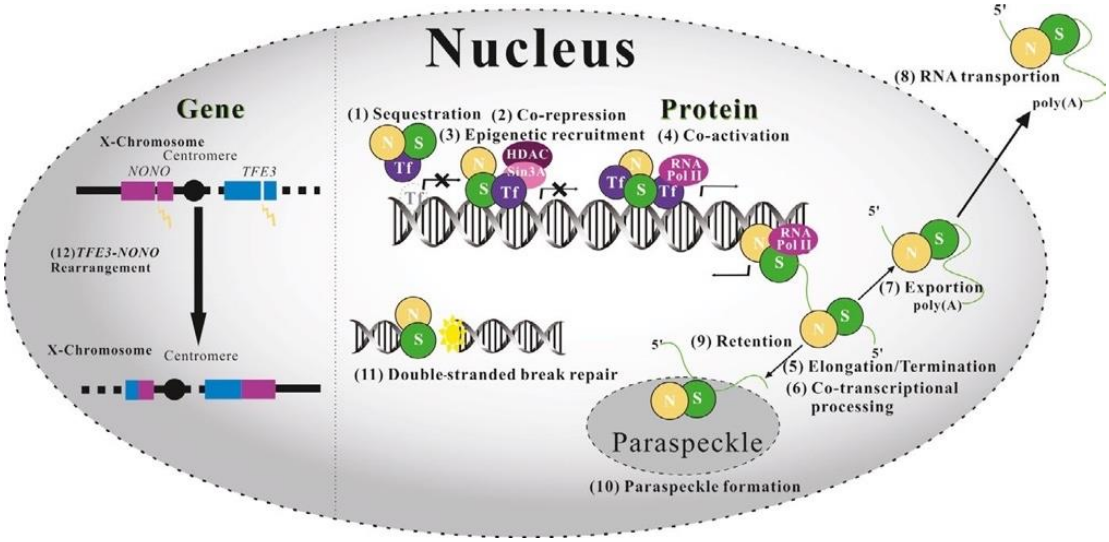


Figure 2-8: Schematic illustration of the NONO and SFPQ protein function. The small green and orange spheres represent the DBHS proteins SFPQ (S) and NONO (N). SFPQ and NONO can block transcription factors from binding to specific promoters (1), serve as co-repressors there (2), and trigger epigenetic silencing when combined with repressors (3). Through (5) elongation to termination, co-activation of transcription is linked to both SFPQ and NONO in step four. To allow co-transcriptional processing, messenger ribonucleoprotein (mRNA) export, and cytosolic trafficking, SFPQ and NONO continue to be linked to nascent mRNA (6-10). Additionally engaged in fixing double-stranded breaks are SFPQ, NONO, and PSPC1 (11). TFE3-NONO rearrangement (12) [73].

2.3.5 Paraspeckle in neurons

The non-coding RNAs (ncRNAs) do not code for any proteins and play crucial roles in every aspect of cellular life. Long ncRNAs (lncRNAs) are thought to be essential for the evolution of primate brains. Therefore, it is not unexpected that lncRNAs are particularly prevalent in the central nervous system (CNS), with at least 40% of tissue-specific lncRNAs expressed in the brain. The lncRNAs typically exhibit unique tissue and cell-type specific expression patterns. Numerous neuro-specific processes, such as neuronal plasticity, synaptic transmission, neurogenesis, brain development, and aging, are regulated by lncRNAs. The Nuclear Paraspeckle Assembly Transcript 1 (NEAT1; ubiquitous, highly expressed nuclear-retained regulatory lncRNA) significantly functions in normal and pathological cellular physiology. The diverse range of roles that lncRNAs play in transcriptional and epigenetic mechanisms includes the recruitment of transcription factors and chromatin-modifying complexes to specific nuclear and genomic sites, alternative splicing, and other post-transcriptional RNA modifications through the formation of nuclear domains containing RNA-processing factors, nuclear-cytoplasmic shuttling, and translational regulation. The lncRNAs organize nuclear architecture by building lncRNA-protein complexes that scaffold protein components of the transcription machinery and alter the chromatin state [75] (Figure 2-8).

Numerous epigenetic mechanisms, including chromatin remodeling, which can change gene expression patterns and even trigger alternative splicing, have been proposed to explain the fantastic capacity of neurons to regulate gene expression dynamically. The fact that failure of chromatin-modifying enzymes results in severe memory impairment demonstrates the significance of chromatin modification in learning and memory. However, there needs to be more information about the exact molecular mechanisms and proteins involved in chromatin remodeling related to gene expression. Regarding this, the identified roles for nuclear Arc and paraspeckle proteins provide new insights that Arc may contribute to different aspects of gene regulation, such as mRNA processing and chromatin remolding through interaction with paraspeckle proteins.

3. Aims

While some studies have reported nuclear Arc function, the molecular mechanisms through which nuclear Arc can play these roles still need to be clarified. Identifying nuclear Arc binding partners is one way to address this issue.

The overall aim of this study was to investigate the possible interaction between endogenous Arc protein and paraspeckle proteins in neuroblastoma (SH-SY5Y) cells.

Specific aims:

1: Does Arc and three main paraspeckle proteins (P54nrb; PSPC1; PSF/SFPQ) express in SH-SY5Y cells?

2: Does carbachol and U0126 regulate Arc and paraspeckle protein expression?

3: Is there a difference in Arc and paraspeckle protein expression between the cytoplasm and nucleus?

4: Does Arc interact with all three main paraspeckle proteins in neuroblastoma cells?

5: Is there a connection between Arc binding to functionally distinct nuclear proteins-paraspeckle (PSF/SFPQ, P54nrb/NONO, PSPC1)?

6: Does Arc co-localize with paraspeckle proteins in the nucleus?

4. Materials and Methods

Figure (4-1) summarizes the methods used in this study. All the steps are explained in detail in the following.

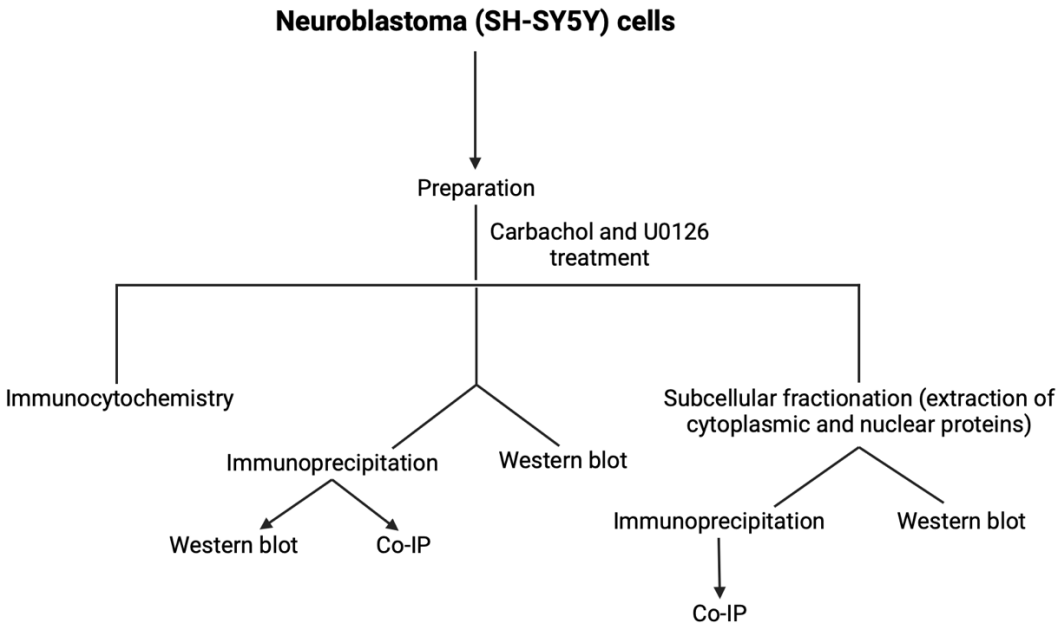


Figure 4-1: method outline.

4.1 Cell culture and sample preparation

4.1.1 Neuroblastoma (SH-SY5Y) cell line

Human neuroblastoma-derived cell lines were used in this study. This cell line, known as SH-SY5Y, is derived from the SH-SY subclone of the original SK-N-SH human neuroblastoma cell line. The parental SK-N-SH cell line was first established in 1970. It was derived from metastatic cells found in the bone marrow aspirate of a four-year-old female of unknown ethnicity. This cell line can synthesize neurotransmitters, express neural markers, and differentiate into various mature human neuronal phenotypes by adding specific compounds. The SH-SY5Y cell line can express a catecholaminergic phenotype with the ability to synthesize dopamine and noradrenaline [76]

(Figure 4-2). Previous studies show that SH-SY5Y cells are widely used for studying neurodegenerative disorders [77] and can represent various proteins, such as endogenous Arc [78].

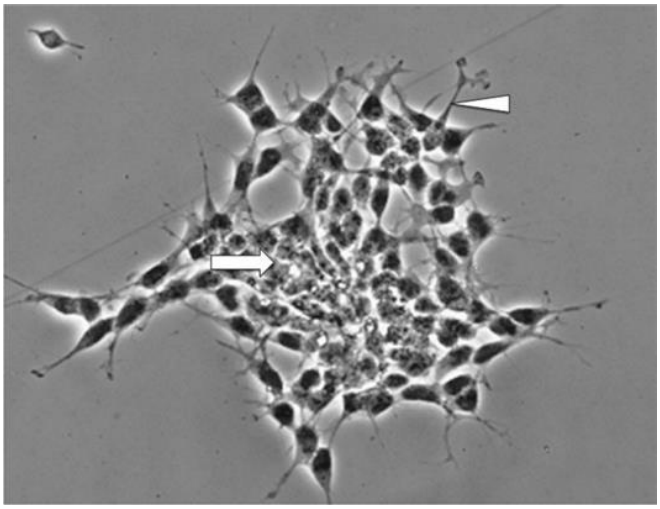


Figure 4-2: Undifferentiated SH-SY5Y cells. The cells can be seen extending short neurites at the cluster's edges (arrowhead). Cells frequently form clumps of spherical cells atop one another and tend to proliferate in clusters (arrow) [79].

Since the early 1980s, this cell line has been frequently employed as a model for neurons since it exhibits numerous biochemical and functional characteristics associated with neurons. Three distinct phenotypes of cells are present in the SK-N-SH cell line: neuronal (N-type), Schwannian (S type), and intermediate (I type). The SH-SY5Y cell line is a N-type neuroblast-like cell line that is relatively homogenous. This cell line expresses the opioid, muscarinic, and nerve growth factor receptors. These cells can multiply in culture for extended periods of time without becoming contaminated, which is necessary for creating an in vitro cell model [80].

4.1.2 Cell culture cultivation and collection of SH-SY5Y cells

Neuroblastoma (SH-SY5Y) cells were grown in T75 flask included Dulbeccos Modified Eagle Medium (DMEM, Sigma, D6429) with 10% Fetal Bovine Serum (FBS, Sigma F7524) and 1% v/v Penicillin/Streptomycin (P/S, Gibco 15140-122) as supplements and incubated in Hera cell incubator at 37°C aerated with 5% CO_2. Based on the number of passages and when the confluency became about 90%, the cells were ready for splitting. So, **first,** the media was aspirated, and the

cells were washed with 10 mL of Dulbecco's phosphate-buffered saline (DPBS, Sigma D8537). **Second**, 2 mL 0,25% Trypsin-EDTA (Sigma, T4049) was added and incubated at 37°C aerated with 5% CO2 for 3 minutes to detach the cells from the flask wall (trypsinization). **Third**, 5 mL DMEM was added to stop the trypsinization, and the cells were transferred to the 15 mL tube and pelleted at 800g and 25°C for 4 minutes. After that, DMEM was removed, the pallet resuspended by adding 1 mL of DMEM, and it was ready for the next passaging.

4.1.3 Cell treatment by Carbachol and U0126

Carbamylcholine chloride (Carbachol) is a parasympathomimetic and an antagonist for both the muscarinic and nicotinic receptors. It can activate mAchr-coupled ERK, thus inducing transient Arc mRNA expression through mAchR activation in SH-SY5Y cells (Figure 4-3). Conversely, U0126 is a specific MAP kinase (MEK) inhibitor, an upstream ERK/MAPK activation regulator. It can inhibit Arc mRNA and protein expression [81, 82]. Treatments were done using carbamylcholine chloride (carbachol, Sigma, C4382) and U0126 (Promega).

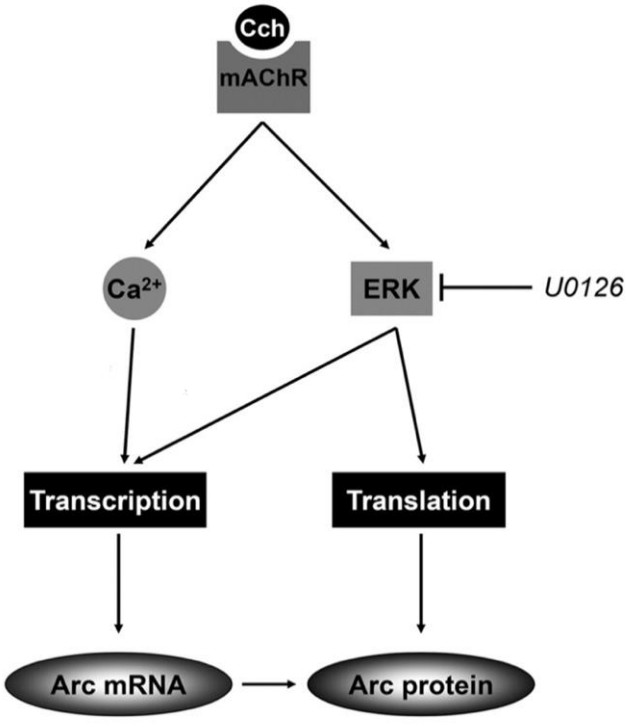


Figure 4-3: Arc expression regulation mediated by mAchR. Arc transcription is activated by mAchR stimulation by Cch via two distinct mechanisms: calcium release and ERK activation. Cch-induced ERK activation also modulates the translation of Arc mRNA. Additionally, Arc mRNA translation is affected by Cch-induced ERK activation. Conversely, adding U0126 can decrease the Arc transcription and Arc mRNA translation as it blocks the upstream kinase MEK and indirectly inhibits the ERK pathways [81].

SH-SY5Y cells were grown on poly-D-lysine coated 10 mm Petri dishes, including DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. After the confluency became approximately 90%, one petri dish was treated with 20 mM U0126 and incubated for 30 minutes at 37°C. After that, 100 mM carbachol was added to this petri dish and incubated for 1 hour at 37°C. One petri dish was treated with 10 mM carbachol, and another petri dish was treated with 20 mM U0126; both were incubated for 1 hour at 37°C. One petri dish was chosen as a control, so it was not treated. Then, 1 mL 0.25% Trypsin-EDTA (Sigma-Aldrich, T4049) was added and incubated for 3 minutes at 37°C until trypsinization occurred. 3 mL DMEM was added to stop the trypsinization, and the cells were transferred to 4 separate 15 mL tubes labeled with the type of treatment and pelleted at 800 g and 25°C for 4 minutes. The DMEM was discarded, and the cell pellet was resuspended in 1 mL of ice-cold DPBS. The cells were then pelleted by centrifugation (10000 rpm, 4 minutes), and the supernatant was removed. The collected cell pellets were frozen at -80° and used for the following experiments. Figure (4-4) shows the whole procedure.

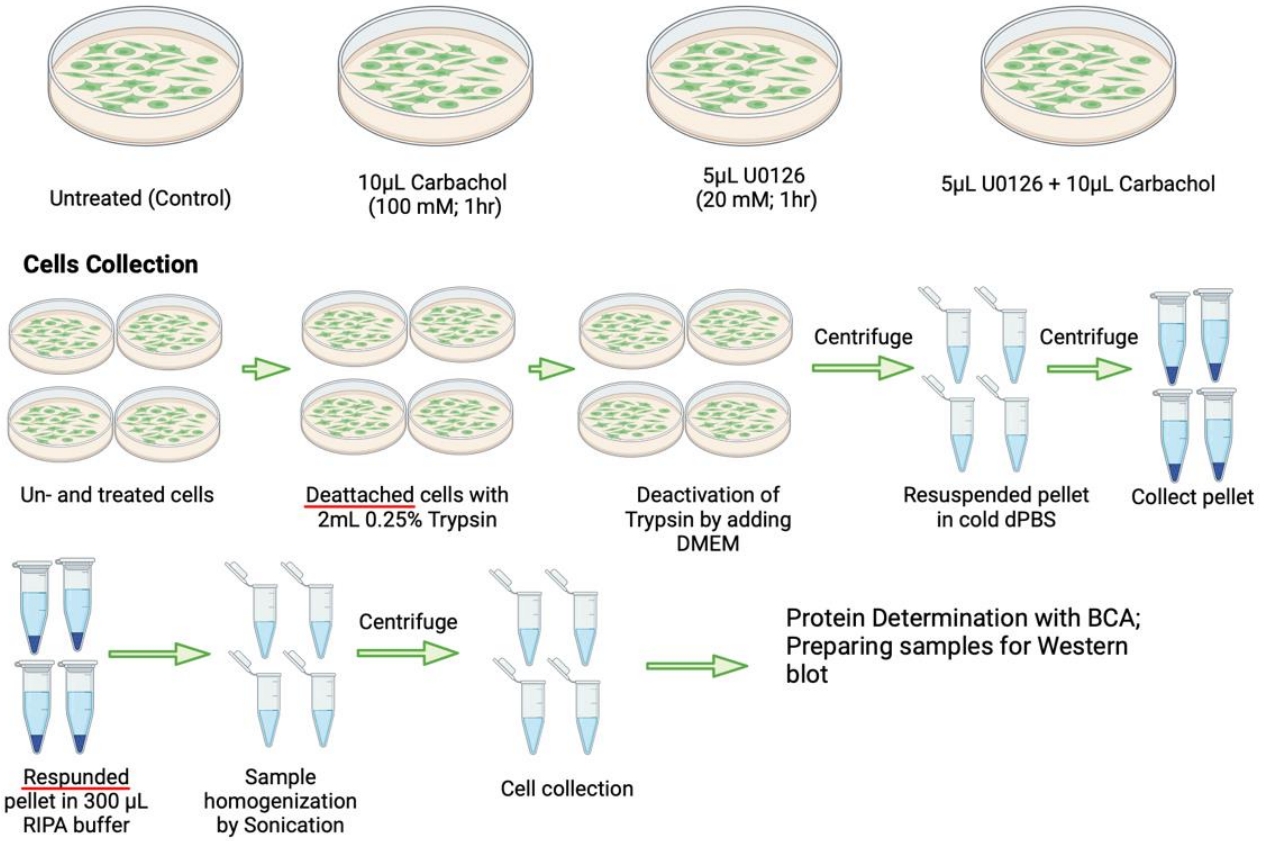


Figure 4-4: The procedure for cell treatment and collection. All steps of cell treatment were done under the laminar flow hood (Thermofisher) to avoid any kind of contamination. The figure is created in Birender.com.

4.2 Cell lysis and Protein concentration determination

4.2.1 Cell lysis

300 µL Radio Immunoprecipitation Assay (RIPA) buffer (Abcam) supplemented with 1x protease inhibitor was added to the collected pellets of treated and untreated cells, followed by sonication (40% amplitude, 30 sec on/10 sec off) using Ultrasonic homogenizer model 150 V7T from Biologics. Then, lysates were centrifuged at 10000 g for 10 min at 4°C; the supernatants were collected in different tubes and stored at -80°C. The concentrations of each group of protein samples were determined through bicinchoninic acid protein assay (BCA).

4.2.2 Protein concentrations determination

Determining protein concentration after protein extraction and before any other procedure is crucial because it provides information about a biochemical pathway. BCA protein assay is applied to measure total protein in a biological sample. BCA is a critical reagent that produces a purple-colored product, which is analyzed regarding a standard curve to measure protein concentration.

For this reason, the Pierce BCA assay kit (Thermo Fischer Scientific, 23225) was used. Based on the standard curve generated using bovine serum albumin (BSA) at the concentrations of 0.625, 0.125, 0.250, 0.5, 1, and 2 ug/uL, respectively, protein concentrations were determined. BSA solutions included mixing Reagent A and B at 50:1 (v/v). A 96-well plate was used for diluting protein samples by 1:39 (v/v) in working solution and incubated for 30 min at 37°C with gentle shaking. Absorbance measurement was done by SpectraMAX Plus 384 microplate readers from Molecular Devices.

4.3 Subcellular protein fractionation

Subcellular fractionation was done to extract separate cytoplasmic and nuclear protein fractions from the total lysate. The Thermofisher NE-PER nuclear and cytoplasmic extraction kit (78835) was used for this. This kit contains two cytoplasmic extraction reagents (CER I, II) and one nuclear extraction reagent (NER). The whole process is shown in Figure (4-5). Also, the protocol can be found on the company's website (www.thermofisher.com).

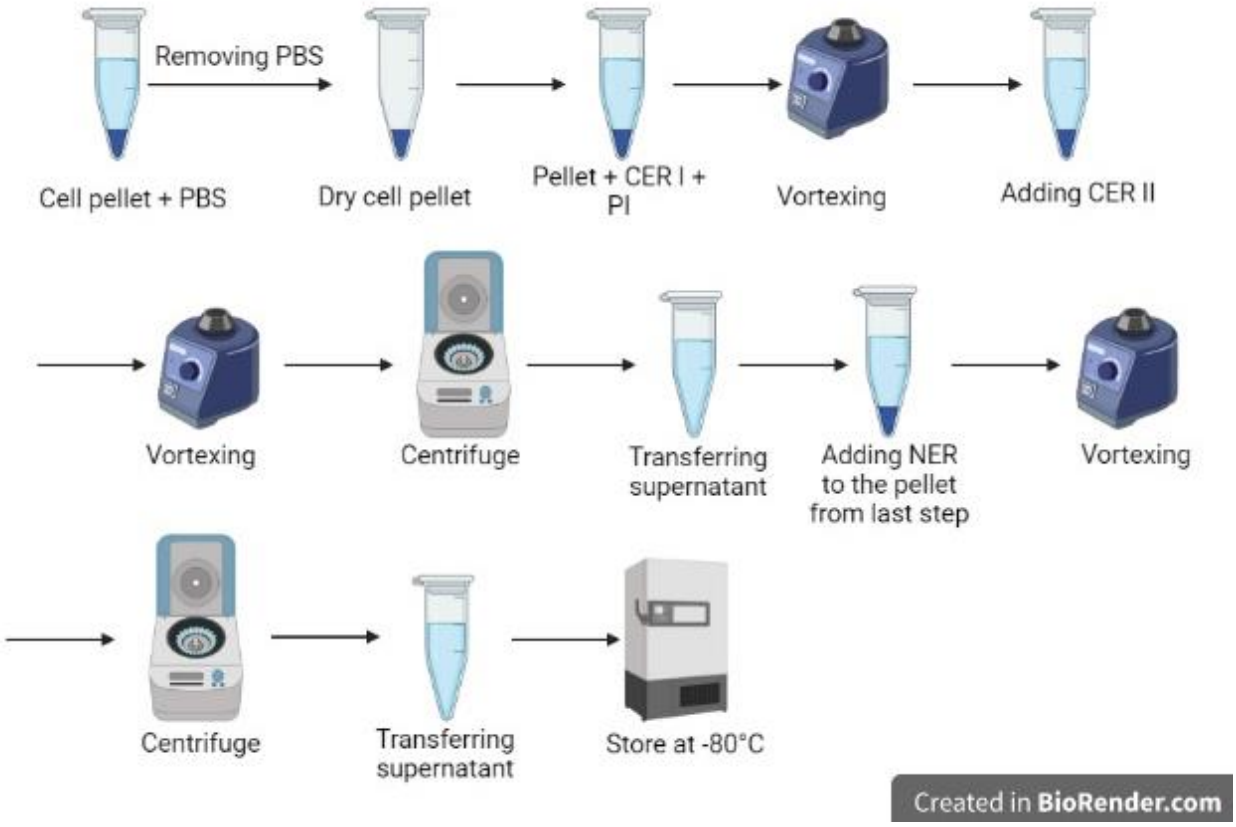


Figure 4-5: Subcellular protein fractionation process. Based on the kit guidance, for the 20 µL cell pallet, 200 µL of CER I, 11 µL CER II, and 100 µL NER reagents were added. 2 µL and 1 µL of PI were added to CER I and NER, respectively. It was unnecessary to add PI to CER II. The highest vortexing setting and a maximum microcentrifuge speed (~16000 × g) were applied. The cell samples were kept on ice during the process, and the centrifuge temperature was set at 4°C. *CER*: Cytoplasmic Extraction Reagent; *NER*: Nuclear Extraction Reagent; *PI*: Protease Inhibitor.

4.4 SDS-PAGE and Western Blot

Western blot is an important, widely used technique that enables researchers to identify specific target proteins within a mixture of proteins extracted from cells. This technique has three different main parts, including 1) protein separation based on their size, 2) protein transferring to a solid support, and 3) identifying target protein by using a proper primary and secondary antibody. After cell sample collection, the SDS-PAGE method was applied to separate proteins by mass. Western blot techniques were used to identify target proteins within the samples.

4.4.1 SDS-PAGE

Polyacrylamide gel electrophoresis (SDS-PAGE) is a method to separate proteins based on their molecular weight. In this method, sodium dodecyl sulphate (SDS) and polyacrylamide gel are used to eliminate the effect of the structure and charge, and proteins are separated only based on polypeptide chain length. SDS is a detergent that strongly affects protein structure and can denature it. The combination of SDS and a reducing agent that cleaves disulfide bonds results in proteins unfolding into linear chains with negative charge proportional to the polypeptide chain length. By using polyacrylamide, a mesh-like matrix is formed, which is suitable for the separation of proteins. SDS-PAGE is an easy, inexpensive, and relatively accurate method that separates proteins based on length.

In this study, 1.5 mm 10% polyacrylamide gels were made using Mini-Protean System 3 (BioRad). Before using samples, 2X sample buffer supplemented with 100 mM Dithiothreitol (DTT, Sigma, D9779) was added to 20 µg of protein samples, and they were incubated at 95°C for 5 min. The running buffer (1X Tris-Glysine SDS; BioRad) was poured into the electrophorator. Then, the gel was placed inside the electrophorator, which was connected to the power supply. Protein samples and PageRuler Plus as a marker were loaded into each well. Running electrophoresis was started at a low constant voltage (80 V) to separate the gel, and then a higher constant voltage (100 V) was applied to stack the gel.

Table 4-1: Composition of sodium dodecyl sulfate (SDS) polyacrylamide gel

Compounds	Resolving ge (10%)	Stacking gel (4%)
ddH_2O	1.91 mL	3.59 mL
30% Acrylamide/Bis (BioRad)	2.5 mL	0.67 mL
1.5M Tris-HCL pH 8.8	2.9 mL	-
0.5M Tris-HCL pH 6.8	-	0.63 mL
10% SDS (Sigma, L4509)	150 µL	50 µL
10% Ammonium persulfate (APS, Sigma, A3678)	50 µL	50 µL
Tetramethylendiamine (TEMED, Sigma, T9281)	15 µL	15 µL

4.4.2 Western blotting

After protein separation, the western blot technique was used. Before transferring a gel to a solid support, nitrocellulose membranes (BioRad), two sponges, and two filter papers were soaked in a cold transfer buffer (1X Tris-Glysine; BioRad). A transfer sandwich was created and relocated to the transfer apparatus poured with cold transfer buffer, and the apparatus was kept on ice. The voltage for this step was 100 V.

After 1 hour, the membrane was soaked in Ponceau to ensure that protein transfer was successful. Then, Ponceua was washed with 1X TBS (Tris-Buffered Saline). The membrane was blocked with 5% non-fat dry milk in 1X TBST (Tris-Buffered Saline Tween-20) for 1 hour at room temperature. The proper primary antibody made in 3% BSA and TBST was added, and the membrane was incubated overnight at 4°C on a shaker. Following, the membrane was washed three times with TBST for 5 minutes each and on a shaker. The proper secondary antibody (conjugated to horseradish peroxidase, HRP) made in TBST was added, and the membrane was incubated for 1 hour at room temperature with gentle agitation. Again, the membrane was washed three times with TBST for 5 minutes each. Enhanced chemiluminescence (ECL, BioRad; the ratio of 1:1 of two ECL reagents was mixed together) was added to visualize the proteins separated on the membrane. Imaging was applied using Chemi Doc ^{TM} XBS+ and Image software (Figure 4-6).

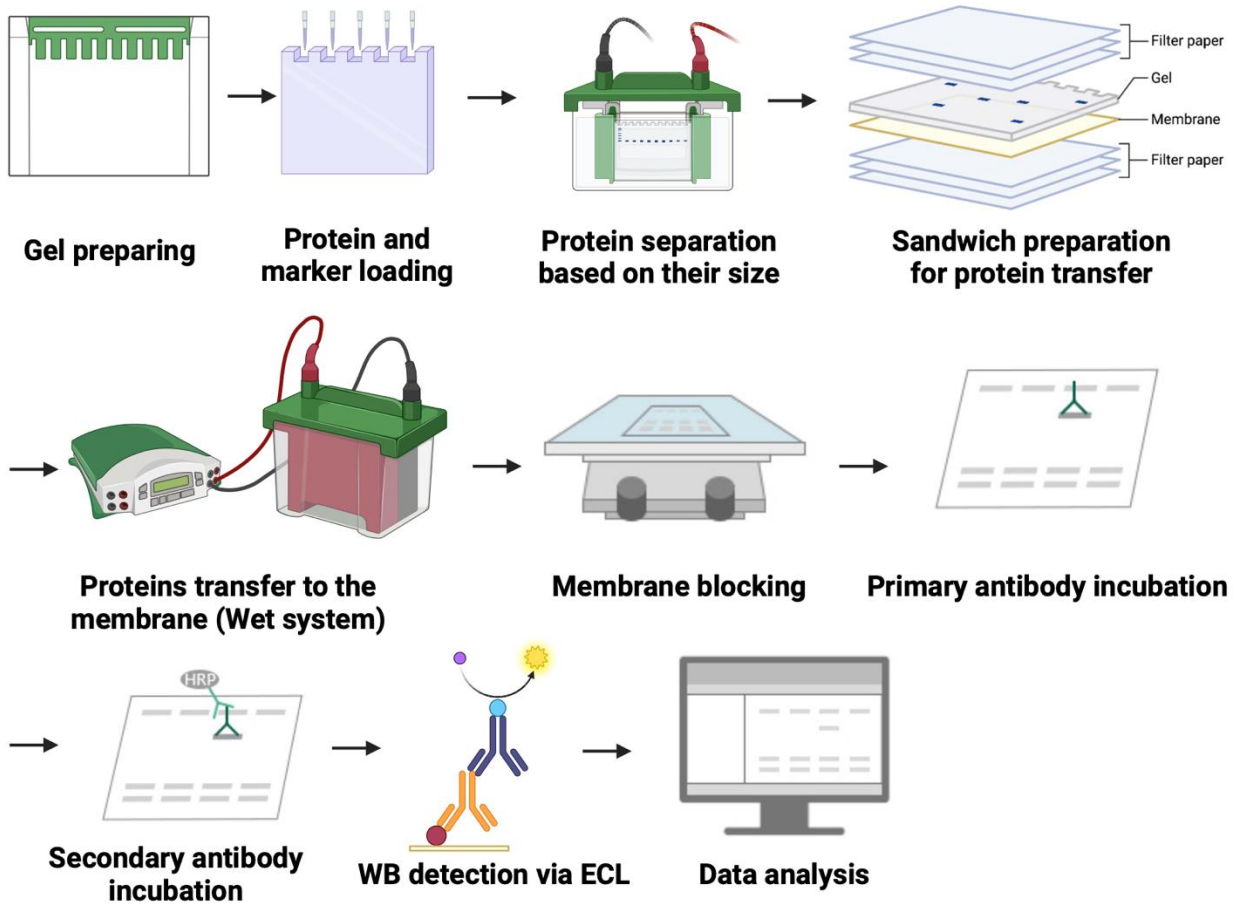


Figure 4-6: SDS-PAGE and Western Blot procedures. SDS polyacrylamide gel electrophoresis was applied for protein separation based on their sizes. The wet system was used for transferring proteins from the gel to the nitrocellulose membrane. After membrane blocking, proper primary and secondary antibodies were added to the membrane, and WB detection was done using ECL. *WB*: Western blot. The Figure is created in Biorender.com.

4.5 Immunoprecipitation and co-IP

Immunoprecipitation (IP) is a technique that allows the purification of a target protein from the rest of a sample using a specific antibody. Following IP, co-IP determines protein-protein interactions between a purified protein from IP and additional proteins identified by western blotting. IP mainly works with antibodies bound to protein A, Protein G, or a mix called protein A/G. These are two bacterial proteins that bind to antibodies.

The immunoprecipitation method was used to purify Arc protein within samples. For this, first, 20 µg Sepharose G fast flow beads (GE Healthcare) were washed with cold 1X PBS. After

centrifugation, PBS was removed. Then, 2 µg anti-Arc primary antibody (monoclonal Arc-C7 Mouse; SantaCruz) was added to the beads. 2 µg IgG mouse antibody was added to a different tube as a negative control. The volume of each tube was adjusted to 300 µl with PBS-T. The complex of the antibody bead was incubated for 30 min on a rotator at RT. The beads were rinsed three times with PBS-T, and then 250 µg of treated and untreated lysate were added. The antibody-bead complex with sample lysates was incubated overnight at 4°C on a rotator. Next, the complex was washed three times with PBS-T and prepared for SDS-PAGE, followed by Western blotting (Figure 4-7).

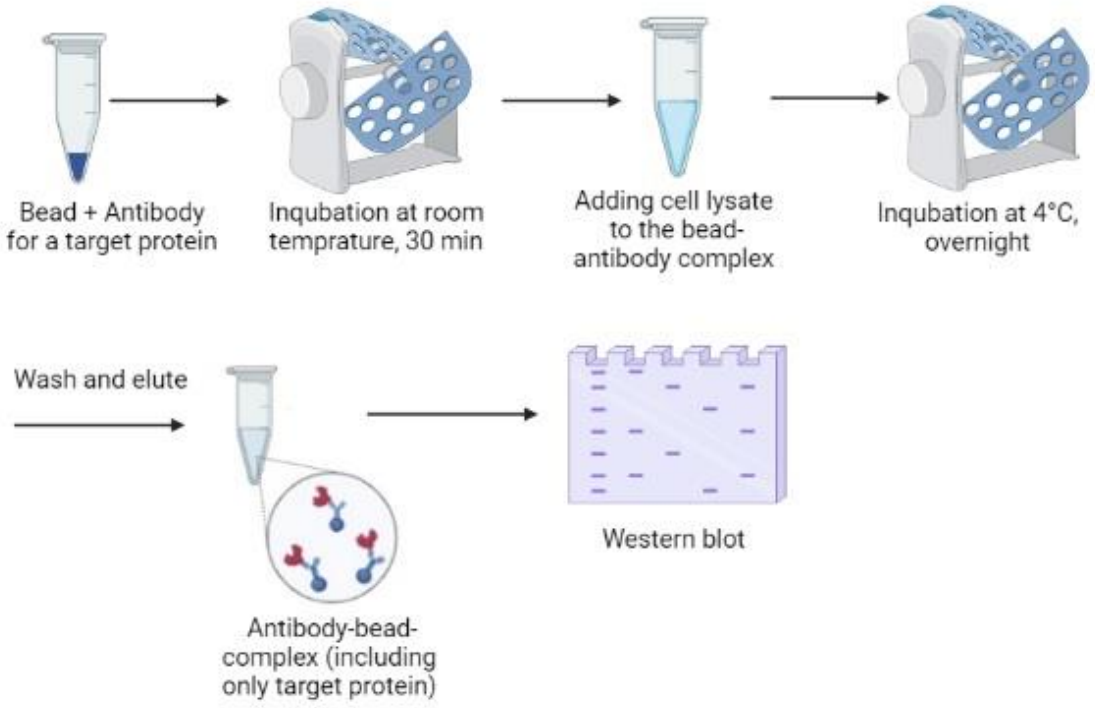


Figure 4-7: Immunoprecipitation principle. After adding beads and antibodies, the mixtures were rotated to allow antibodies to bind to the beads. The bead-antibody complex was given enough time to bind to target proteins inside the total cell lysates. Finally, target proteins inside the antibody-bead complex were purified from total lysate and were used for WB analysis. The Figure is created in Biorender.com.

4.6 Immunocytochemistry

4.6.1 Preparing coverslips

18 mm coverslips (marienfield-superior, Lauda-Konigshofen, Germany) were put into a 50 ml conical tube was purred with 1 N HNO3 (Sigma-Aldrich, St. Louis, Mo) and sonicated for 15 min in an ultrasonic bath (SONOREX TK20; BANDLIN electronic GmbH and Co KG, Berlin, Germany). The acid was aspirated, coverslips were rinsed with autoclaved milli-Q water, and again were sonicated for 15 min. After washing again, the coverslips were autoclaved at 121°C for 20 min. Following, the coverslips were treated with 100% ethanol and were dried and kept in a lightproof box in a biological safety cabinet.

4.6.2 Cell seeding, fixation, and permeabilization

SH-SY5Y cells were seeded with a 2% density in 1.5 ml DMEM on coverslips within the 12-well plates. When the cells reached ~80% confluency, DMEM was aspirated and 1.5 ml 37°C and substituted with preheated DPBS. Fixation was applied using a paraformaldehyde solution (4% paraformaldehyde, 4% sucrose, 1X PBS) for 20 min at RT. Then, 4% PFA was rinsed, and the coverslips were kept in 1x PBS in 14°C.

The next step was permeabilization with 0.1% Triton in 1X PBS for 5 min in the new 12-well plate containing fresh 1X PBS. The Triton was soaked out, rinsed, and washed with PBS for 5 min. The PBS was removed, and the coverslips were rewashed with PBS for 10 min. All these steps were done at RT. Finally, the cells were blocked with 5% GS (Goat Serum; 300 µl for each coverslip) in PBS for 1 hour.

After blocking, cells were diluted in blocking solution (Anti MAP2 in 5% GS) and incubated at 4°C overnight. 3 times washing with 1X PBS was applied, followed by adding a secondary antibody (donkey anti-chicken) in blocking solution and incubated in the dark for 1 hour at RT. The washing step was repeated, and the coverslips were mounted using DAPI (Thermo Fisher Scientific) or Nucleu blue to see the nucleus.

4.6.3 Fluorescence cell imaging

Fluorescence imaging was performed using Plan-ApoChromat 60x (Carl Zeiss AG), EC plan Neofluar 60x (Carl Zeiss AG) objectives, and Axiocam 503 mono digital camera controlled by ZEN pro software (Carl Zeiss AG). The captured fluorescence images were analyzed using ImageJ/Fiji (Figure 4-8).

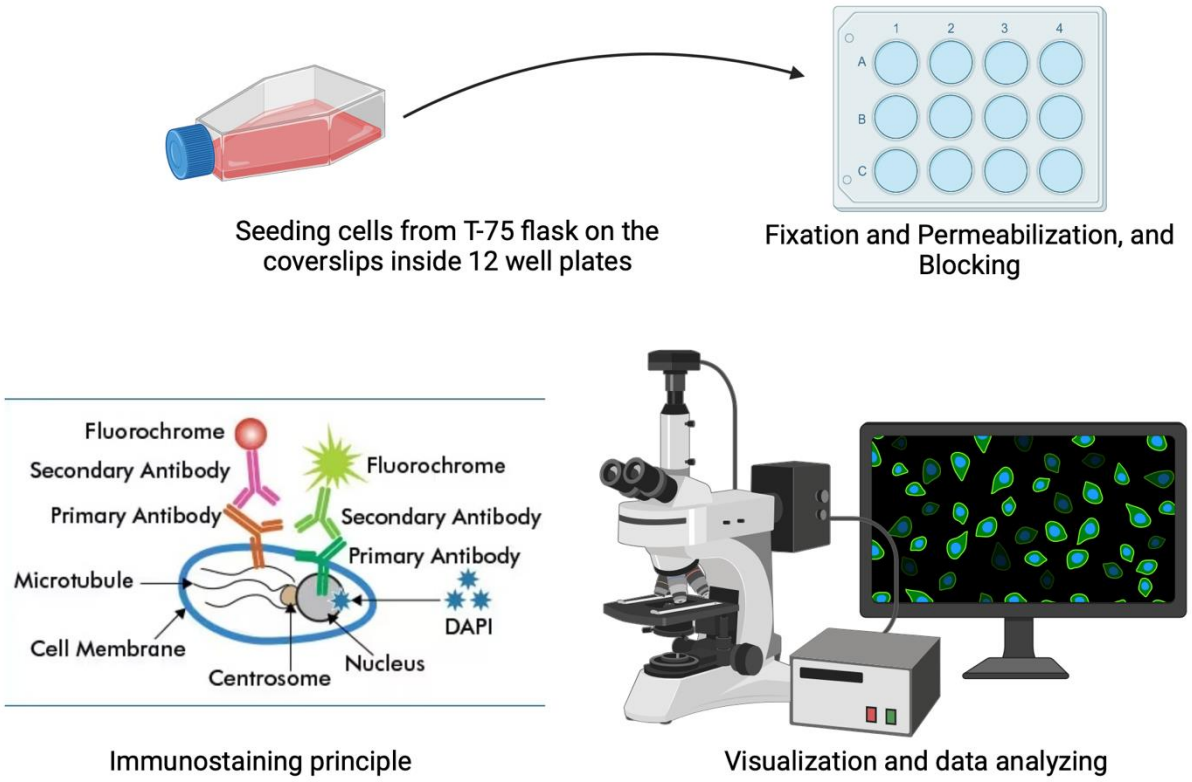


Figure 4-8: ICC procedure. SH-SY5Y cells were seeded on the prepared coverslips within the 12 well plates. After cell fixation, permeabilization, and blocking, the proper immunostaining protocol was applied to detect the target proteins inside the nucleus. Finally, the samples were analyzed using confocal microscopy. The figure is created on Biorender.com.

5. Results

5.1 Effect of carbachol and U0126 on Arc and paraspeckle proteins

The effects of carbachol and U0126 on Arc and paraspeckle protein expressions in neuroblastoma cells were investigated. After collecting Western Blots data (3 groups for each protein), the band intensities were measured using ImageJ software. These data were then imported in GraphPad Prism 9.3.0 (GraphPad Software, Inc.) as mean +/- S.E.M for statistical analysis and bar graphs. An ordinary one-way ANOVA test with multiple comparisons was applied to detect whether the results were significant. The significance rate was set to $p < 0.05$.

5.1.1 Carbachol and U0126 can regulate Arc expression in SH-SY5Y cells

Results showed that the SH-SY5Y cells were responsive to cholinergic stimulation by Cch and expressed endogenous Arc protein through ERK pathway signaling more than the control samples. Endogenous Arc was induced after 1 h treatment with Cch (Figure 5-1). However, SH-SY5Y cells almost showed no endogenous Arc expression after 30 min treatment by U0126 (inhibitor of ERK/MEK signaling pathway) compared to control lysates. Moreover, samples treated with both Cch and U0126 show that endogenous Arc expression was increased compared to the control, while the amount of Arc was less than the Cch group due to the inhibitory effect of U0126. This demonstrates that the endogenous Arc expression pathway was affected by the Cch treatment more strongly than the U0126 treatment. Figure (5-1) shows the obtained bands and a graph for these groups of samples.

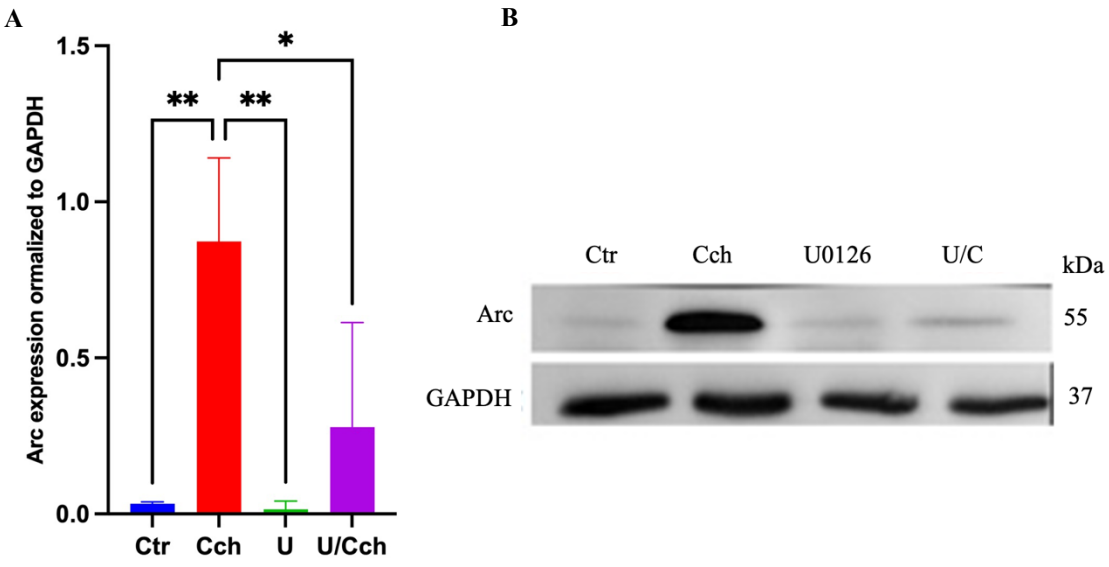


Figure 5-1: Carbachol and U0126 regulate Arc expression. A) Densitometric analysis of Western blots shows Arc protein expression in SH-SY5Y cells resulting from Cch (red box), U0126 (green box), and U/Cch (purple box) treatment. Arc levels were normalized to GAPDH, and relative expression was obtained as bar graphs. Mean + S.E.M for each Arc protein is displayed. Asterisks determine the significant increase or decrease relative to Ctr (ordinary one-way ANOVA with multiple comparisons, $p < 0.05$; n = 3). B) Representative Western blots show Arc expression treated differently. *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126.

5.1.2 Carbachol and U0126 do not have any regulation effects on paraspeckle protein expressions in SH-SY5Y cells

First, the Western blot results demonstrated that SH-SY5Y cells express paraspeckle proteins. Thus, these cells are the proper cell line for this study. Second, as shown by Figure (5-2) and based on the data obtained from Western blots and quantitative analysis, treating SH-SY5Y cells with carbachol or U0216 did not show any differences compared to the control group. The amount of expression for all target paraspeckle proteins in different treatment groups (1 h by Cch, 30 min by U0126, and 1 h by U/Cch) was similar to the control group in total lysate. This can determine that paraspeckle proteins use different pathways for their expression than Arc protein. This pathway is also not responsive to cholinergic stimulation by carbachol or the inhibitory effect of U0126.

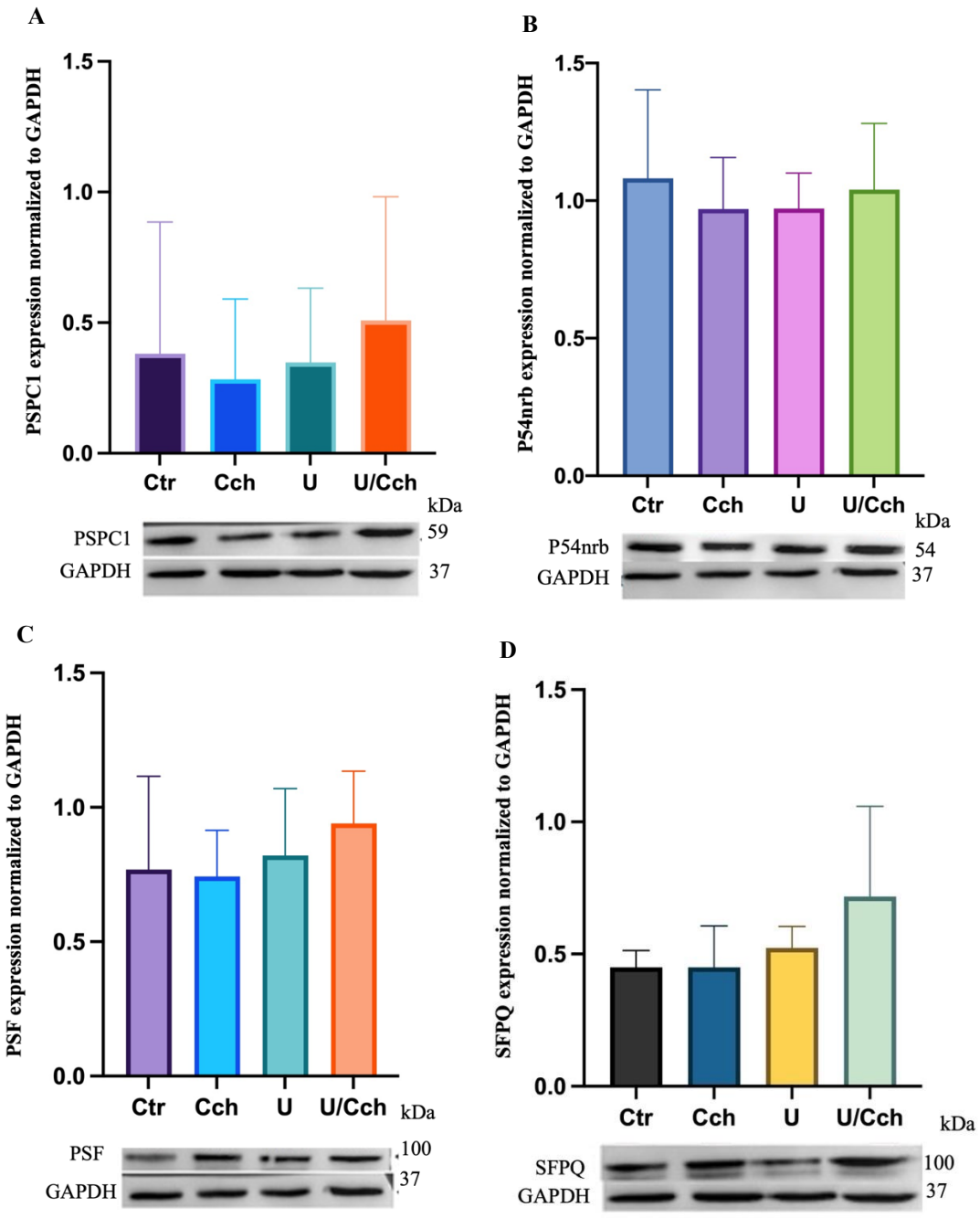
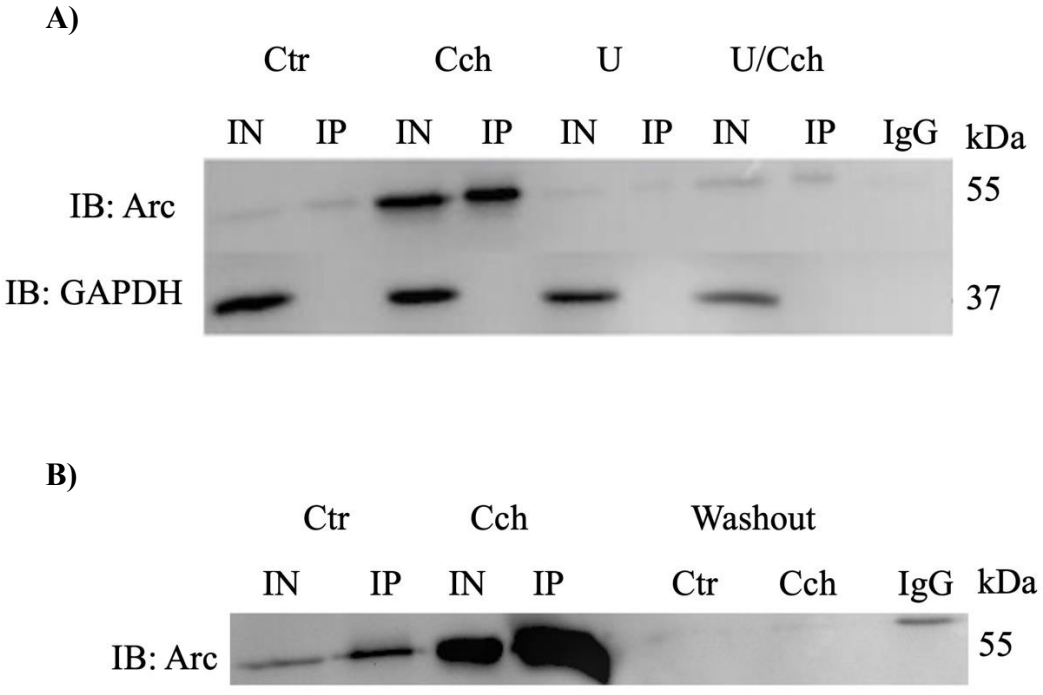


Figure 5-2: Effect of Carbachol and U0126 on paraspeckle protein expression. Densitometric analysis of Western blots shows Arc protein expression in SH-SY5Y cells resulting from Cch (carbachol), U (U0126), and U/Cch treatment, and Ctr (untreated). Mean + S.E.M for each paraspeckle protein is displayed. The ordinary one-way ANOVA with multiple comparisons did not show significant differences between different treatments for each paraspeckle protein ($p < 0.05$; n = 3); PSPC1 (A), P54nrb (B), PSF (C), and SFPQ (D). All values were normalized to GAPDH loading control from the same lane. *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126. Representative Western blots are shown below each panel.

5.2 Immunoprecipitation (IP) results

5.2.1 Arc was effectively purified by using antibodies from SH-SY5Y cells

After determining the regulation of Arc expression via carbachol and U0126 treatment, Arc protein was purified using a mouse anti-Arc antibody (Arc-C7, Santa Cruz) (Figure 5-3). The volume of 250 µg of cell lysate from different treated and untreated samples was used for IP. For establishing proper purification of Arc protein, 40 µg of total lysates with or without treatment were used as positive control/input (IN). Negative controls were applied using IgG to determine that the primary antibody was specific for the Arc protein and GAPDH as it does not bind to Arc.



Representative western blot of Arc protein. The explanation will be on the next page.

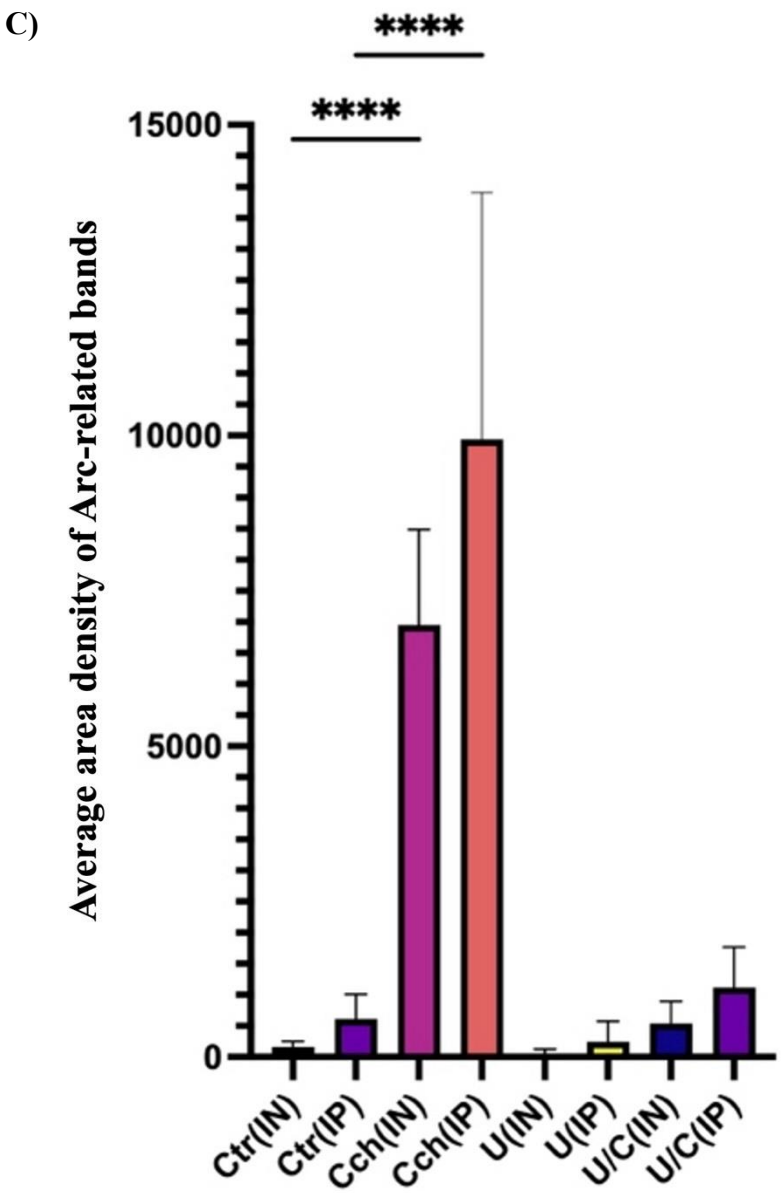
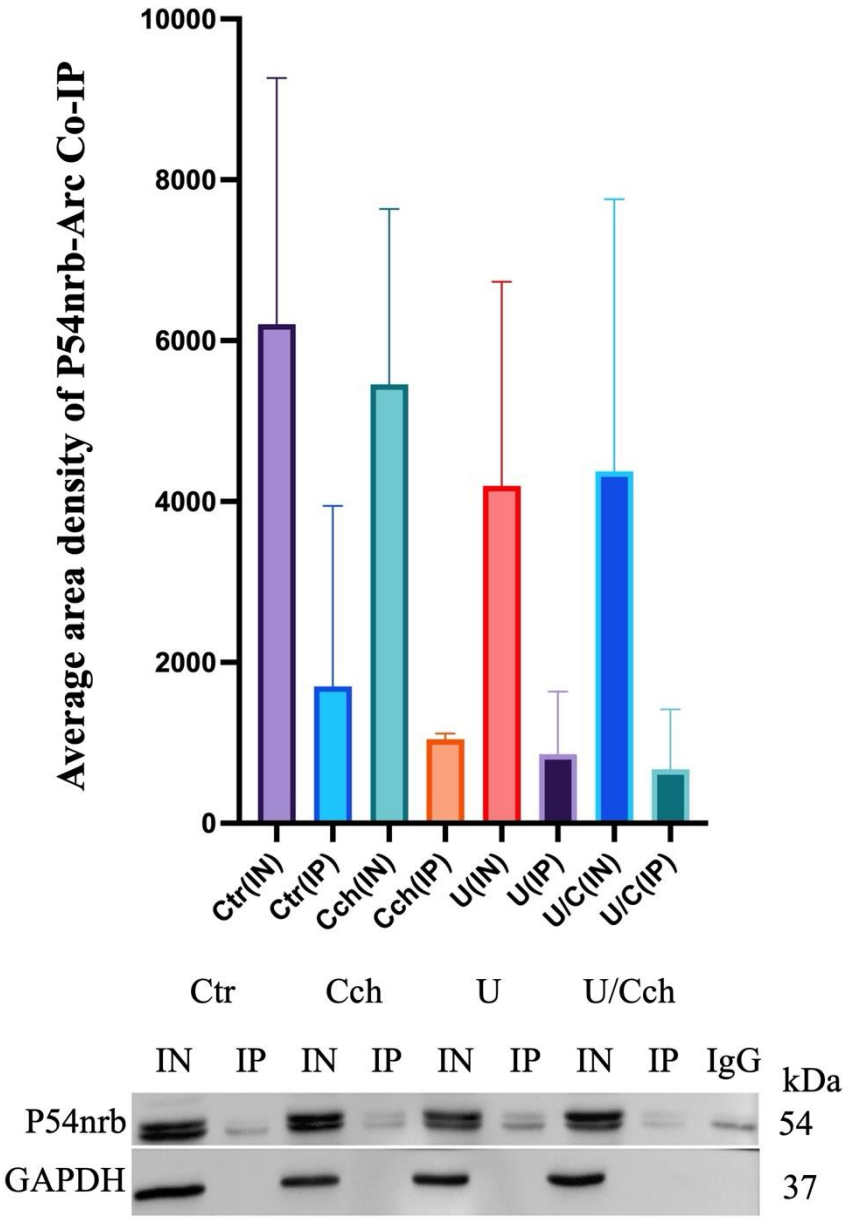


Figure 5-3: Arc immunoprecipitation from treated and untreated SH-SY5Y cells. A) Representative western blot of Arc protein that was immunoprecipitated (IP) from treated and untreated SH-SY5Y cells using an Arc or immunoglobulin G (IgG) antibody. Input (IN) is 40% total lysate. B) Representative western blot of the same samples treated by Cch and washout samples to detect that the IP procedure was done correctly, as there are no detectable bands in washout samples. C) Densitometric analysis of Western blots and mean + S.E.M for Arc protein is displayed. Quantification of Arc protein IP showing significant enrichment of Arc protein using an Arc antibody. Arc protein was specifically pulled down in the IP together with Cch-induced Arc expression (ordinary one-way ANOVA with multiple comparisons: Ctr (IN) vs. Cch (IN), $p < 0.0001$; Ctr (IN) vs. Ctr (IP), $p = 0.0001$). Data are presented as the bands' mean intensity (n = 5). *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126.

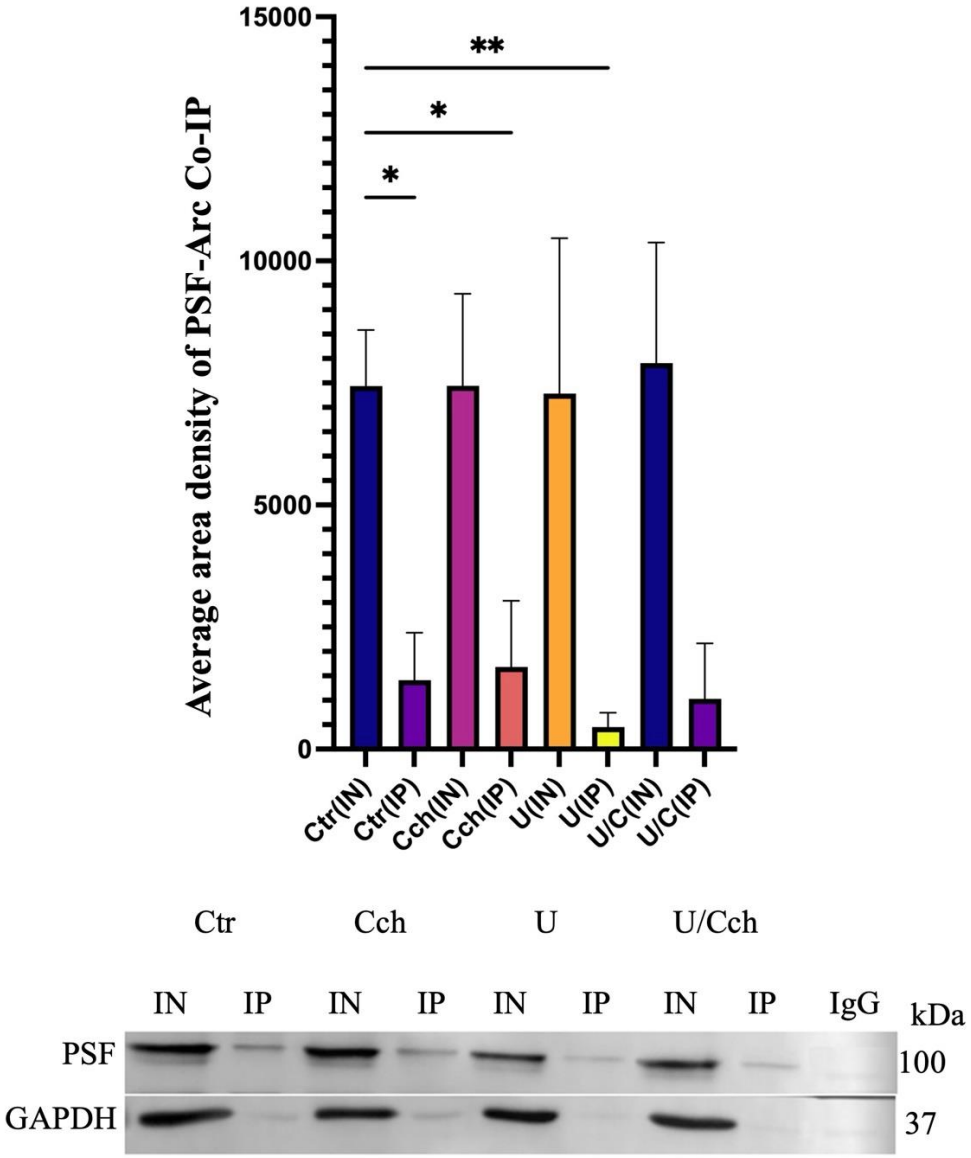
Based on (Figure 5-1A), First, Arc was detected in both input and IP samples for control and treated samples by Cch, U0126, and U/Cch; however, there was no band in the IgG sample. It means that the primary antibody can only bind to Arc. Furthermore, as GAPDH does not bind to Arc, there were no detectable bands for IP samples (figure 5-3B). Both demonstrated that the IP protocol appropriately worked, and Arc protein was effectively purified from lysate cells. Second, purified Arc protein showed different expression levels after treatment by Cch, U0126, and U/Cch (Figure 5-3C). Thus, the amount of purified Arc after 1h treatment by Cch was enormously higher than the control group. In the U0126 group, much less amount of purified Arc (near to zero) was detected compared to the control group. Moreover, Arc expression increased after 1 hour treatment by U/Cch, resulting in more detectable purified Arc than the control group. However, this increase was less intense than the effect of Cch.

5.2.2 Arc coimmunoprecipitated with P54nrb, PSF, and SFPQ but not with PSPC1

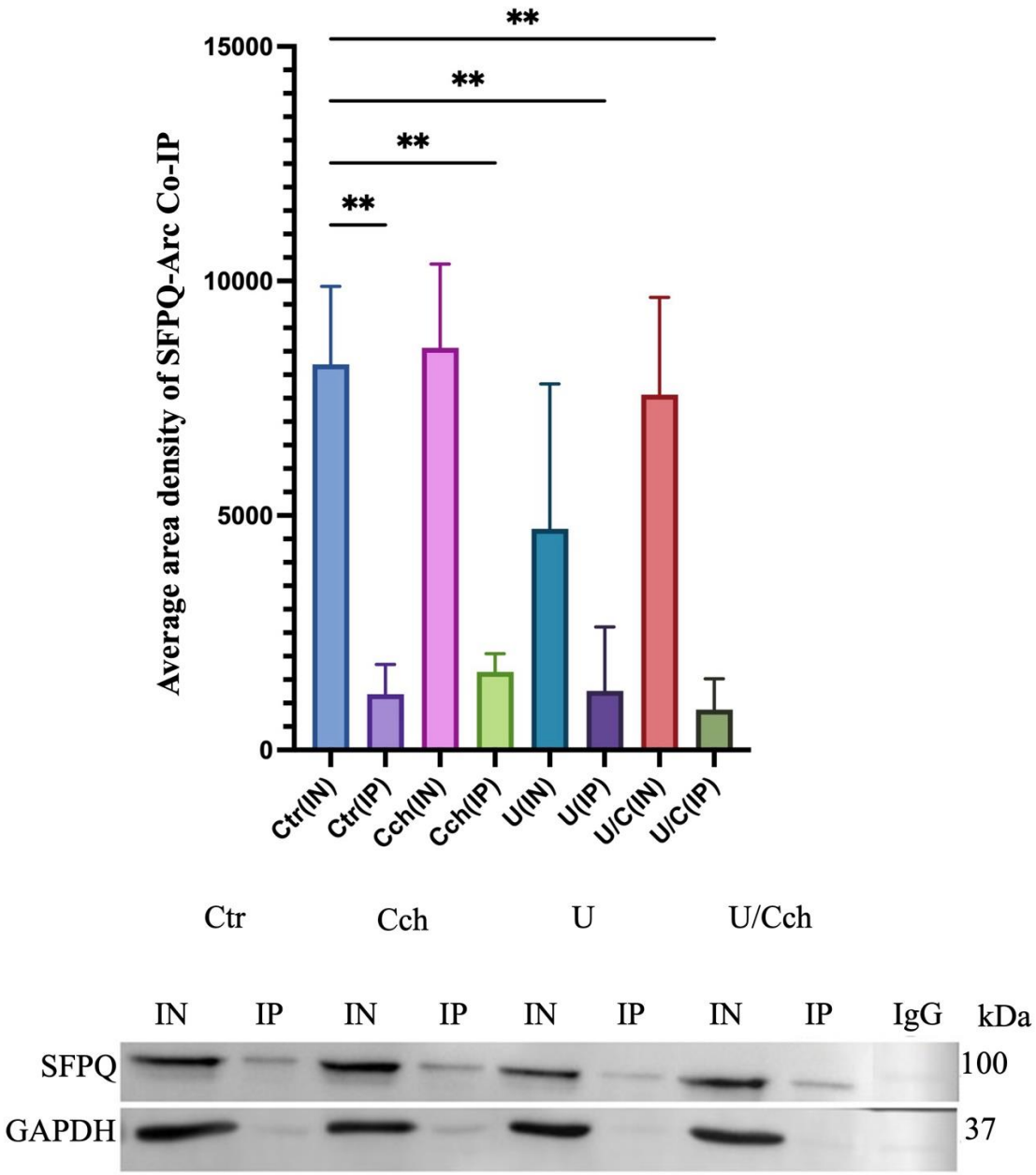
The coimmunoprecipitation (Co-IP) method is a way through which it can be possible to investigate a possible interaction between two different proteins. However, this method is only partially qualified to show the specific protein-protein interaction, but can be a good start for this type of study. Accordingly, first, we purified endogenous Arc protein from the total lysate; we applied Western blot and used the same blots for Co-IP. Second, different primary antibodies specified for paraspeckle proteins were applied (Rabbit anti-nmt55/p54nrb antibody, Abcam; mouse anti-PSF/PSF (D-8), Santa Cruz; mouse anti-SFPQ, Santa Cruz; rabbit anti-PSPC1, Abcam). Finally, we investigated the blot with different primary antibodies to determine the possible interaction between Arc and paraspeckle proteins. Similar to the IP method, positive control was input, and GAPDH and IgG were negative controls for the whole process. The results showed that Arc can coimmunoprecipitate with three paraspeckle P54nrb, PSF, and SFPQ proteins (Figure 5-4, A-C) but not PSPC1 (Figure 5-4D).



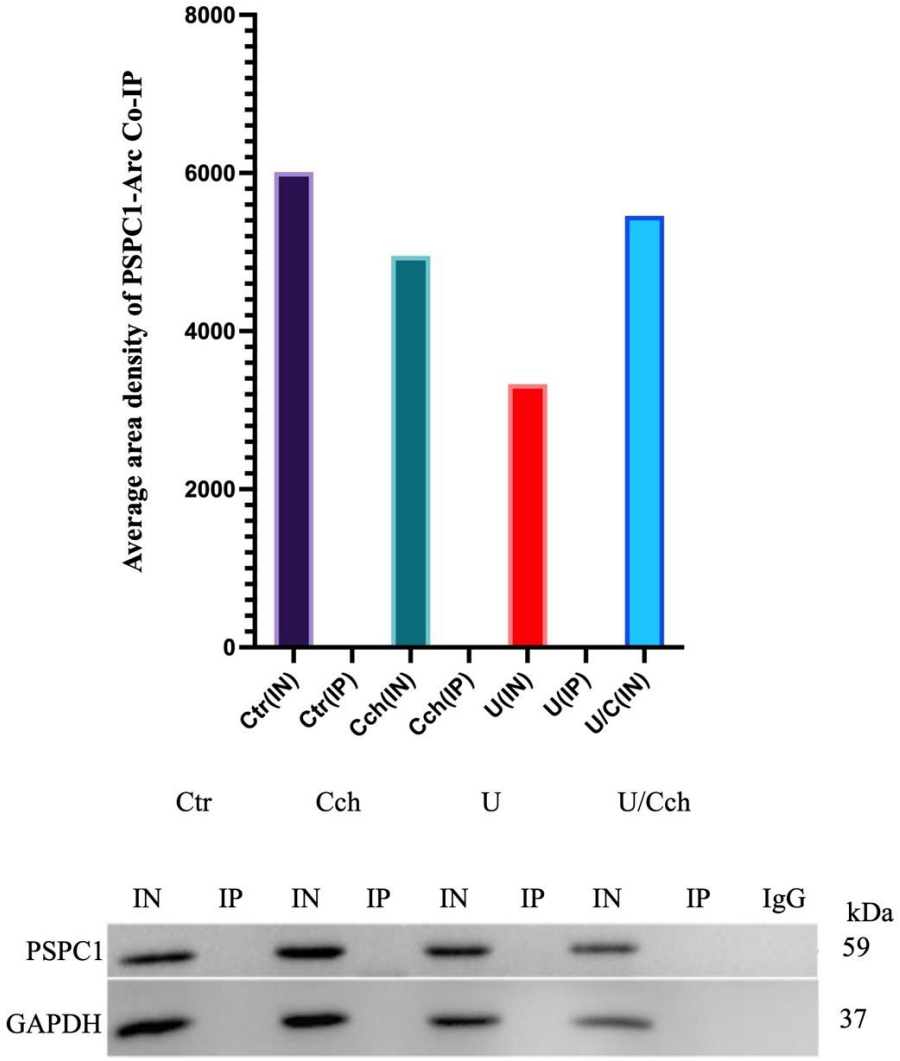
A) Arc-P54nrb Co-IP graph and Western blot (explanation comes in following)



B) Arc-PSF Co-IP graph and Western blot (explanation comes in following)



C) Arc-SFPQ Co-IP graph and Western blot (explanation comes in following)



D) Arc-PSPC1 Co-IP graph and Western blot (explanation comes in following)

Figure 5-4. Interaction between Arc and paraspeckle proteins in SH-SY5Y cells. The graphs (A, B, and C) show that Arc could coimmunoprecipitate with P54nrb (A), PSF (B) and SFPQ (C). Densitometric analysis of Western blots and mean + S.E.M for each protein are displayed. Ordinary one-way ANOVA with multiple comparisons was done for Arc-PSF and Arc-SFPQ. It showed that there is a significant interaction between Arc with both PSF ($p < 0.05$, n = 3) and SFPQ ($p < 0.05$, n = 3). The effects of Cch and U on the regulation of Arc expression were significant. D) There was no interaction between Arc and PSPC1 (n = 1). Representative western blots on the bottom of each graph demonstrate a considerable difference between IN and IP for each group of treated or untreated cells. Undetectable bands for IgG and GAPDH prove that both could not bind to Arc or paraspeckle proteins. Detectable bands in IP groups show the interaction between Arc and paraspeckle proteins. *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126.

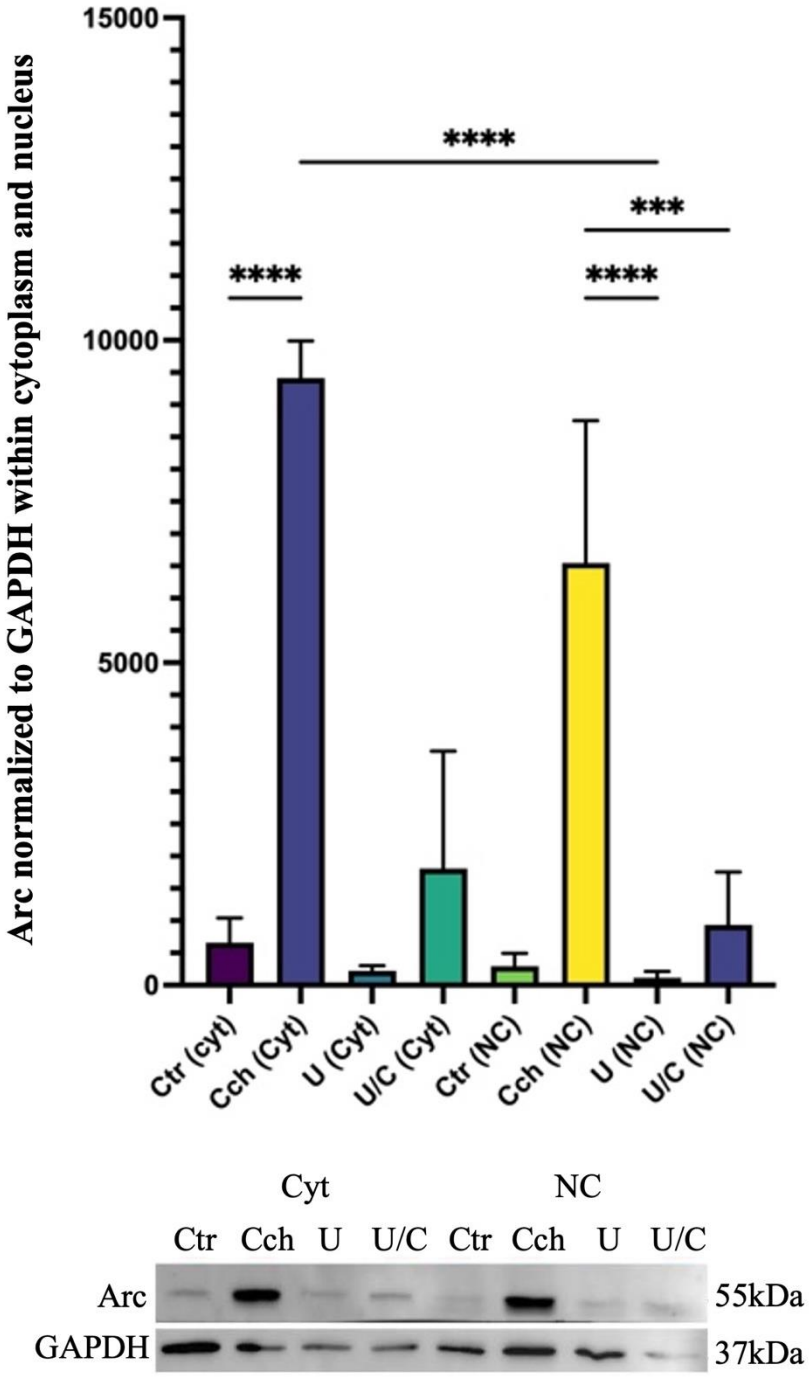
These results prove that treatment with carbachol, U0126, or both first has no regulation effect on paraspeckle protein expressions. Second, the differences between IP results for treated cells with carbachol and controls illustrated the carbachol-induced expression of endogenous Arc. Because, after carbachol treatment, the amount of effectively purified Arc was more than that of the control group. There were also differences between coimmunoprecipitated Arc treated by carbachol and control with P54nrb, PSF, and SFPQ. More purified Arc means a stronger interaction between Arc and paraspeckle proteins. At the same time, the results after treatment by U0126 were contradictory. Co-IP results for groups treated by U0126 demonstrated less Arc protein to interact with paraspeckle proteins. Third, Arc did not interact with PSPC1, as it could not coimmunoprecipitate with PSPC1.

5.3 Extraction of nuclear and cytoplasmic proteins from SH-SY5Y cells

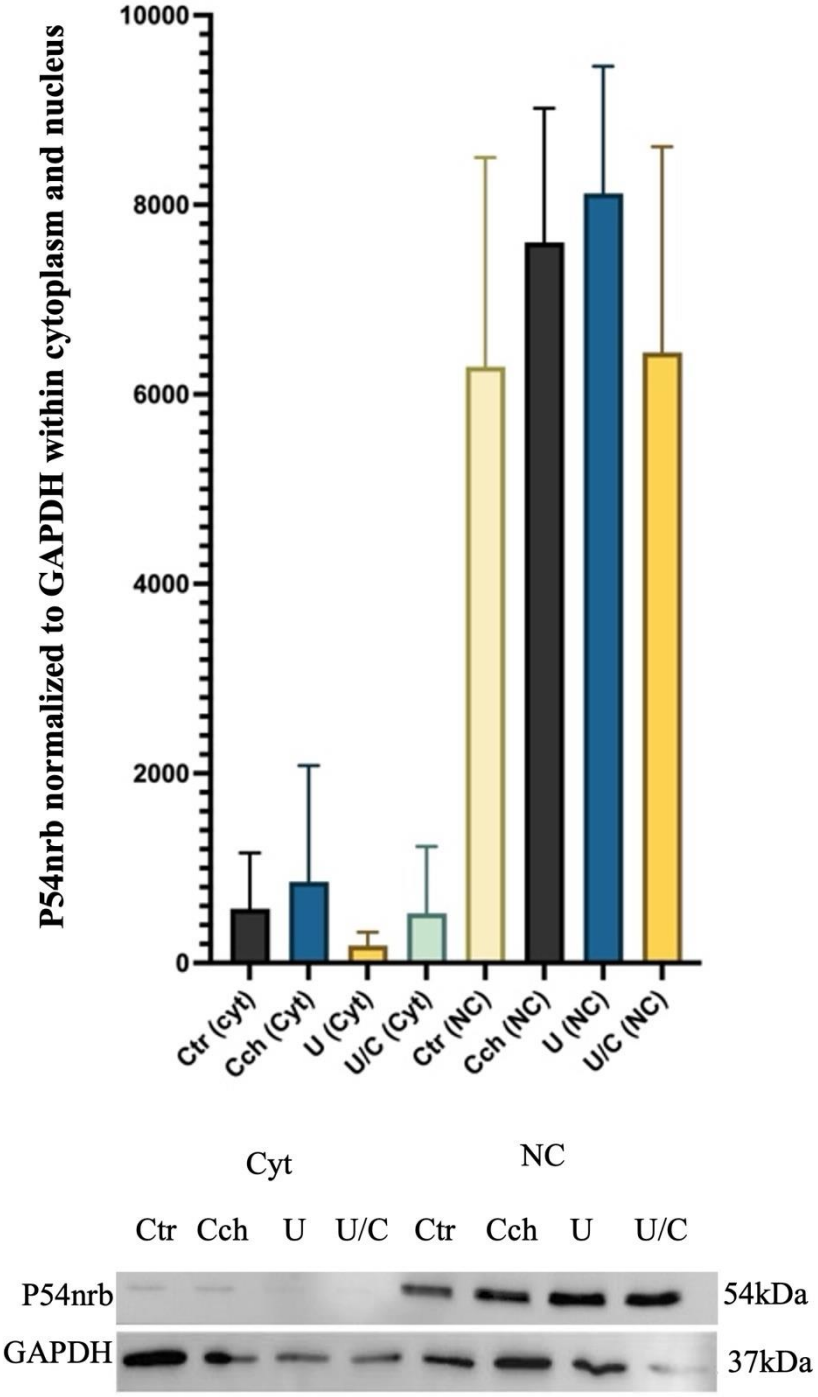
The process of subcellular fractionation was done based on the process mentioned in part (4.3, method and material) for extracting nuclear and cytoplasmic proteins from SH-SY5Y. The main focus of this study was on the nuclear Arc. Moreover, paraspeckle proteins are located mainly in the nucleus. So, nuclear Arc and paraspeckle proteins should be extracted from SH-SY5Y cells to prove the specification of interactions between nuclear Arc and paraspeckle proteins.

5.3.1 Detecting nuclear and cytoplasmic Arc and paraspeckle proteins after treatment by carbachol and U0126

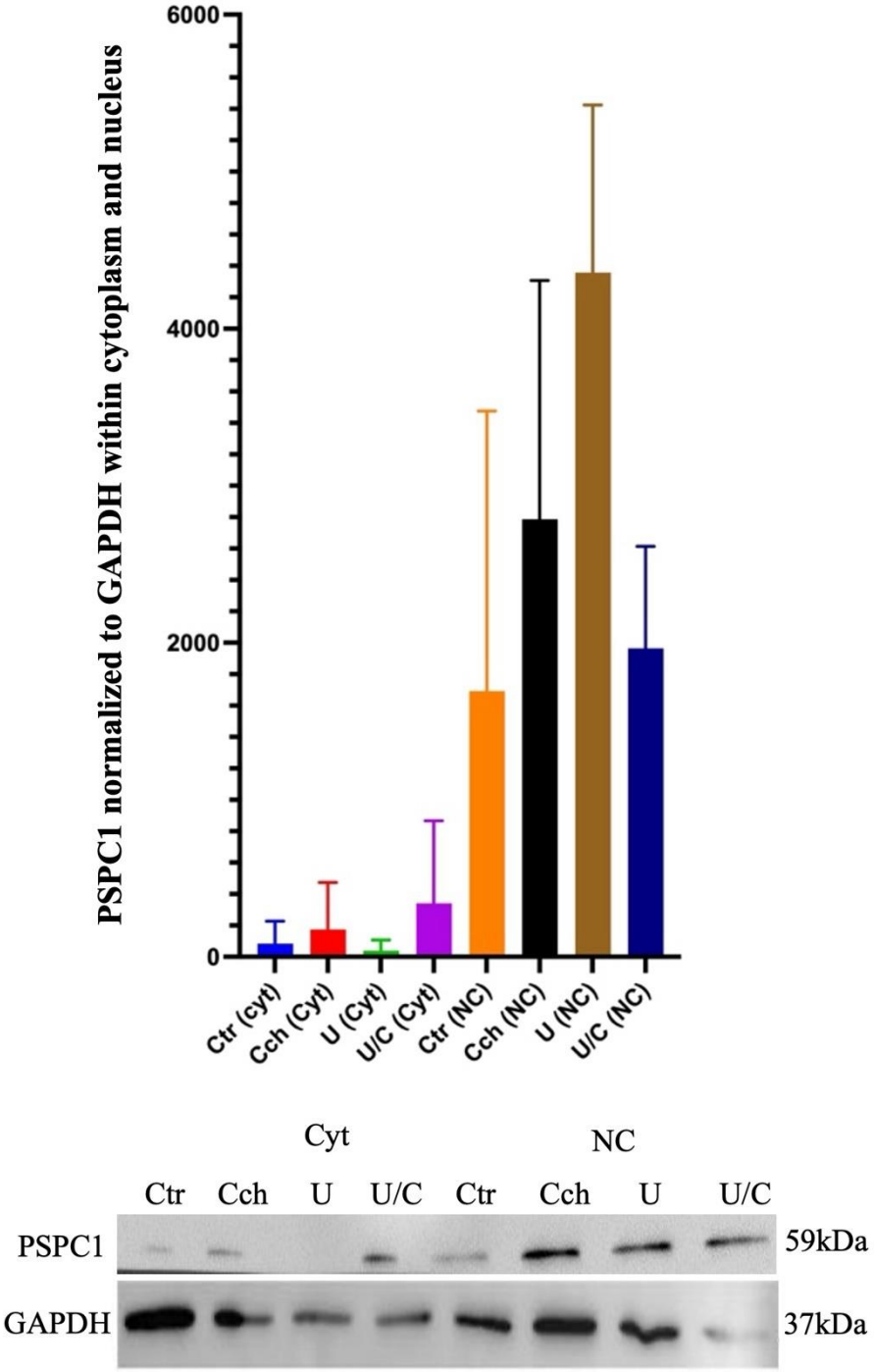
After extraction of nuclear and cytoplasmic proteins from SH-SY5Y cells, for differentiated nuclear Arc from cytoplasmic ones, samples were used for doing Western blot. Besides that, different treatment effects by carbachol and U0126 on nuclear and cytoplasmic Arc and paraspeckle proteins were investigated. For this, the same antibodies used for total lysate were applied. Similarly, GAPDH was used to normalize the amount of each protein. Figure (5-5) illustrates the results of this experiment.



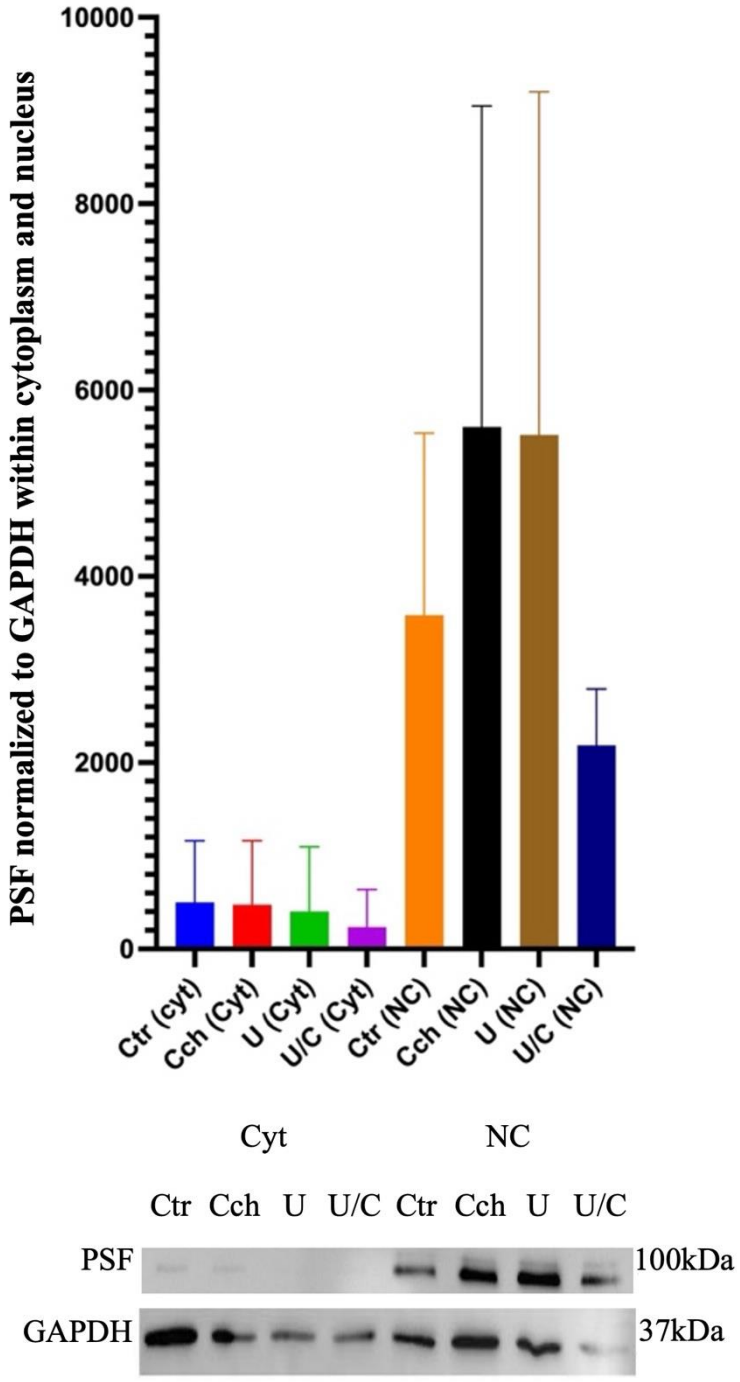
A) Graph and Western blot related to subcellar fractions of Arc protein. (explanation comes in following)



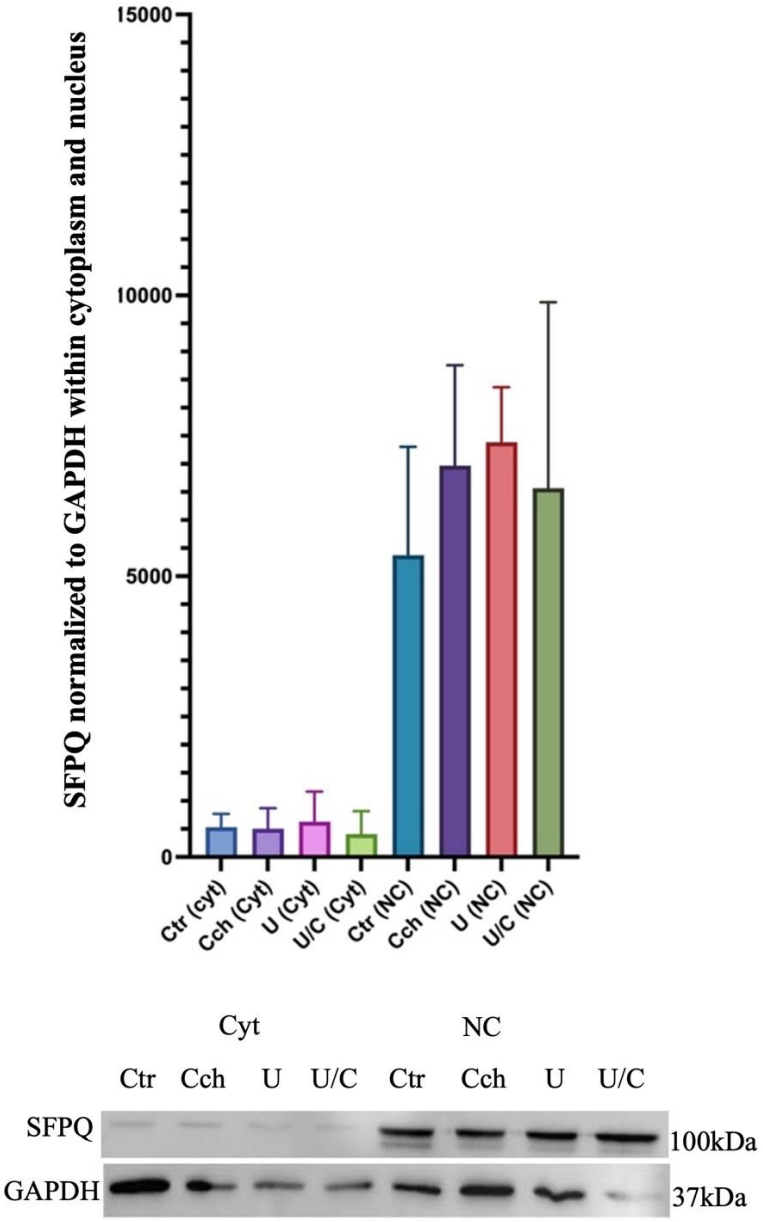
B) Graph and Western blot related to subcellar fractions of P54nrb protein. (explanation comes in following)



C) Graph and Western blot related to subcellar fractions of PSPC1 protein. (explanation comes in following)



D) Graph and Western blot related to subcellar fractions of PSF protein. (explanation comes in following)



E) Graph and Western blot related to subcellar fractions of SFPQ protein. (explanation comes in following)

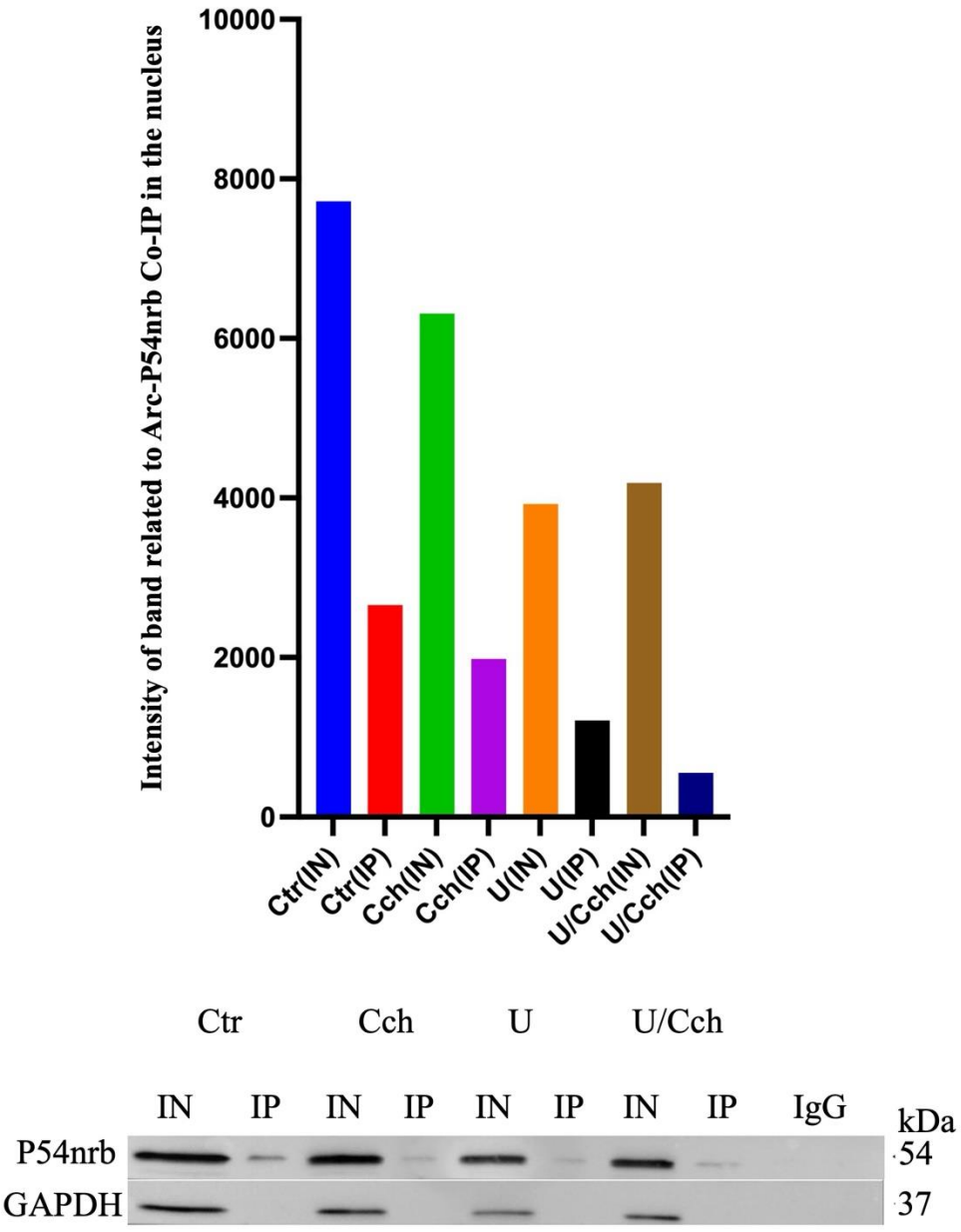
Figure 5-5: Effect of carbachol and U0126 treatment on nuclear and cytoplasmic proteins. Densitometric analysis of Western blots and mean + S.E.M for each protein are displayed. Ordinary one-way ANOVA with multiple comparisons shows that Arc expressed more in the cytoplasm and under the cholinergic induction by Cch (A; $p < 0.05$, n = 3). In contrast, treatment by U could decrease Arc expression. Paraspeckle proteins are mostly found in the nucleus, and they did not show various regulation under treatment by Cch and U (B: P54nrb; C: PSPC1; D: PSF; E: SFPQ). Representative Western blots under each graph show the same results. *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126.

Based on these results, first, we show that Arc is expressed more in the cytoplasm than in the nucleus. Second, Arc protein expression was induced under the cholinergic induction by Cch both in the nucleus and cytoplasm. In comparison, treatment by U0126 inhibited Arc expression in both cytoplasmic and nuclear fractions. Third, we found paraspeckle proteins mainly in the nucleus, and they did not show various regulation under treatment by Cch and U0126 (B: P54nrb; C: PSPC1; D: PSF; E: SFPQ).

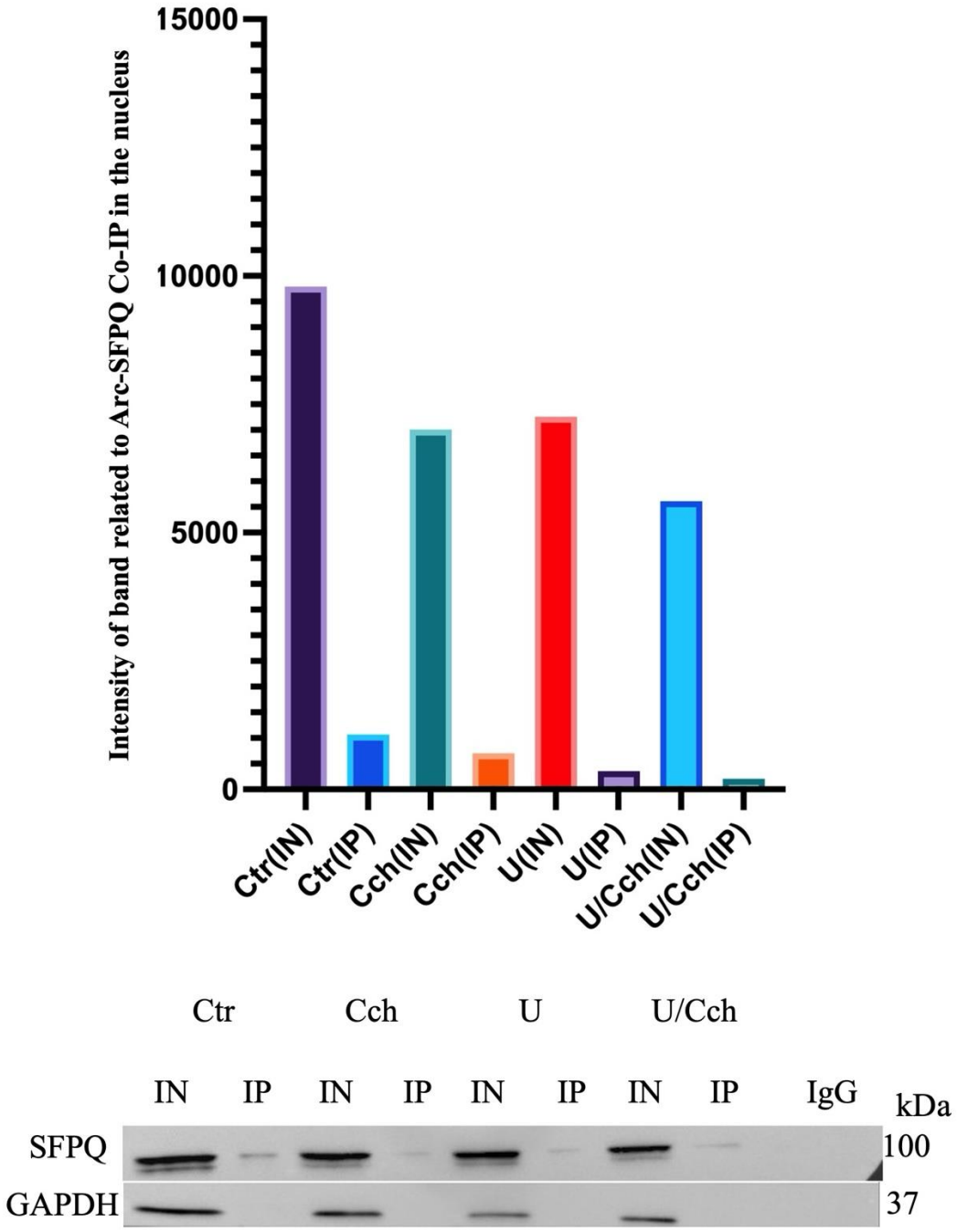
5.3.2 Arc coimmunoprecipitated with P54nrb, PSF/SFPQ in the nucleus, but not with PSPC1

After extraction of nuclear Arc and paraspeckle proteins from SH-SY5Y cells and detecting target proteins through Western blot, treated samples were used for Co-IP to determine whether there are interactions between Arc and paraspeckle proteins in the nucleus. The whole process was the same as Co-IP done for total lysate. We demonstrated that Arc could interact with P54nrb, PSF/SFPQ in the nucleus but not with PSPC1 (Figure 5-6).

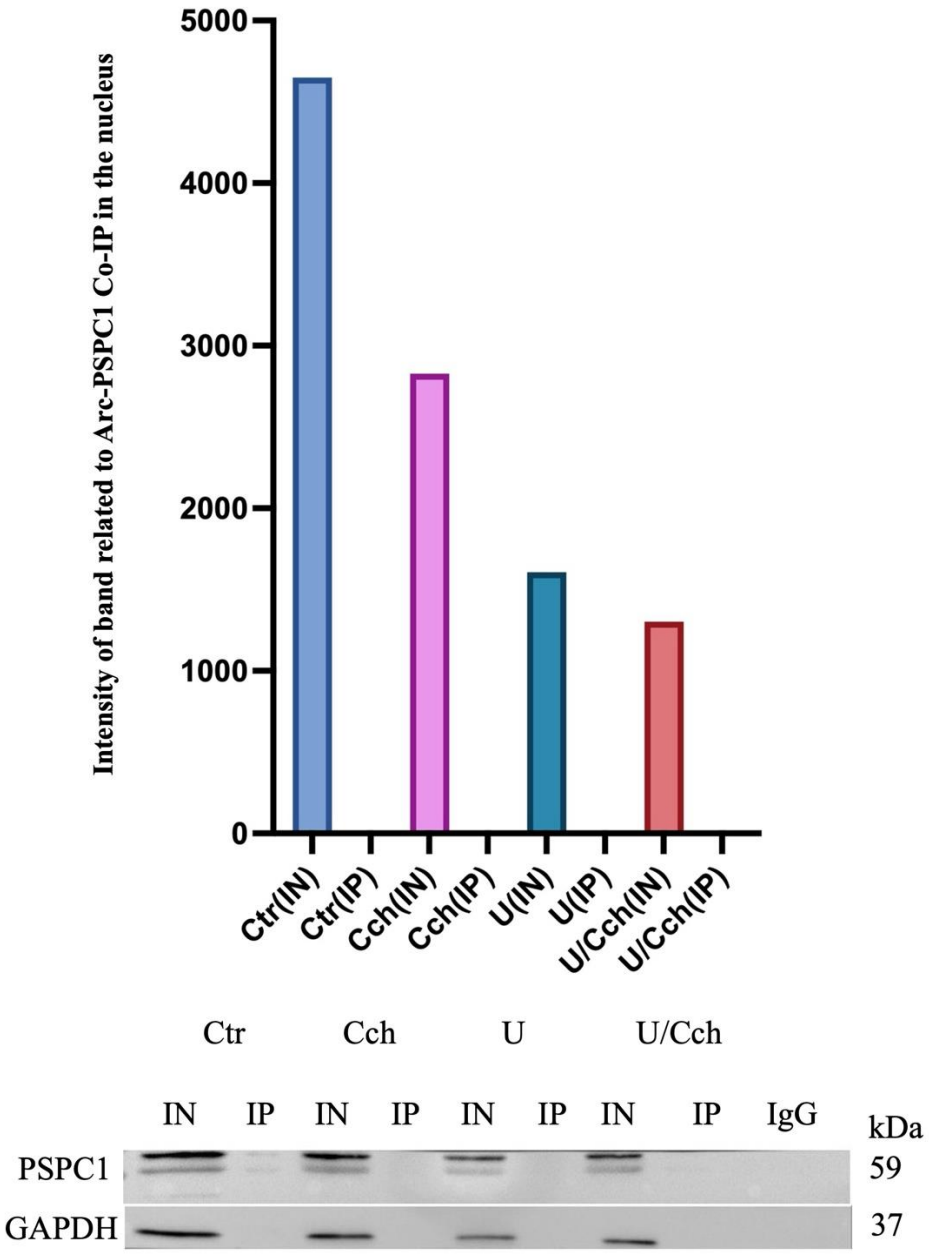
Following IP results for total lysates, first, we detected the interactions between Arc with P54nrb and SFPQ in nuclear fractions. Since paraspeckle proteins mainly exist and are expressed in the nucleus, these results are another confirmation for showing the specification of the interaction of Arc with P54nrb and SFPQ. Second, we did not find that Arc can coimmunoprecipitate with PSPC1 in the nucleus, similar to IP for total lysates.



A) Graph and Western blot Arc-P54nrb Co-IP in the nucleus (explanation comes in following)



B) Graph and Western blot Arc-SFPQ Co-IP in the nucleus (explanation comes in following)



C) Graph and Western blot Arc-SFPQ Co-IP in the nucleus (explanation comes in following)

Figure 5-6: Arc interaction with P54nrb, SFPQ, and PSPC1 in nuclear fractions. The results (densitometric analysis of Western blots) show that Arc could coimmunoprecipitate with P54nrb and SFPQ in nuclear fractions (A and B). Mean + S.E.M for each protein is displayed. While no interaction between Arc and PSPC1 was detected (C). The graphs represent band intensities for each protein treated with Cch, U, or U/Cch (n = 1). Western Blots results are presented under each graph. *Ctr*: untreated cells/ control; *Cch*: treated by Carbachol; *U*: treated by U0126; *U/Cch*: treated by both Carbachol and U0126.

5.1 Arc co-localized with p54nrb in the nucleus of SH-SY5Y cells

The identified interaction between nuclear and cytoplasmic Arc with paraspeckle proteins through the Co-IP method was novel and promising, making us do more complementary investigations. For this, we applied the immunocytochemistry (ICC) approach to explore possible co-localization of Arc with p54nrb protein. SH-SY5Y cells treated with Cch, U0126, and U/Cch were prepared for doing ICC based on the previously mentioned method (4.6). Different immunostainings (Arc anti-mouse and P54nrb anti-rabbit) and Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies were applied to visualize target proteins. DAPI (405) and Phalloidin (647) were used to visualize the nucleus and actin cytoskeleton, respectively.

By using a simple Red-Green-Blue (RGB) merge method, we were able to detect possible co-localization. So, in the case of co-localization of target proteins, red and green pixels from two separate images will overlap and change to yellow. Red colors were relevant to Arc protein, while green colors showed the P54nrb.

Based on the staining results, first, we detected higher red color pixels related to Arc protein in the treated group with carbachol. This means Arc expression was induced after Cch treatment. In contrast, there were almost zero red pixels related to the inhibitory effect of U0126 on Arc expression. We did not see any differences in green pixels related to P54nrb after being treated with Cch or U0126. Second, the RGB merge of Arc and P54nrb showed a co-localization of Arc and P54nrb in the nucleus, as there was a change of color from red (Arc) and green (P54nrb) to yellow (Figure 5-7).

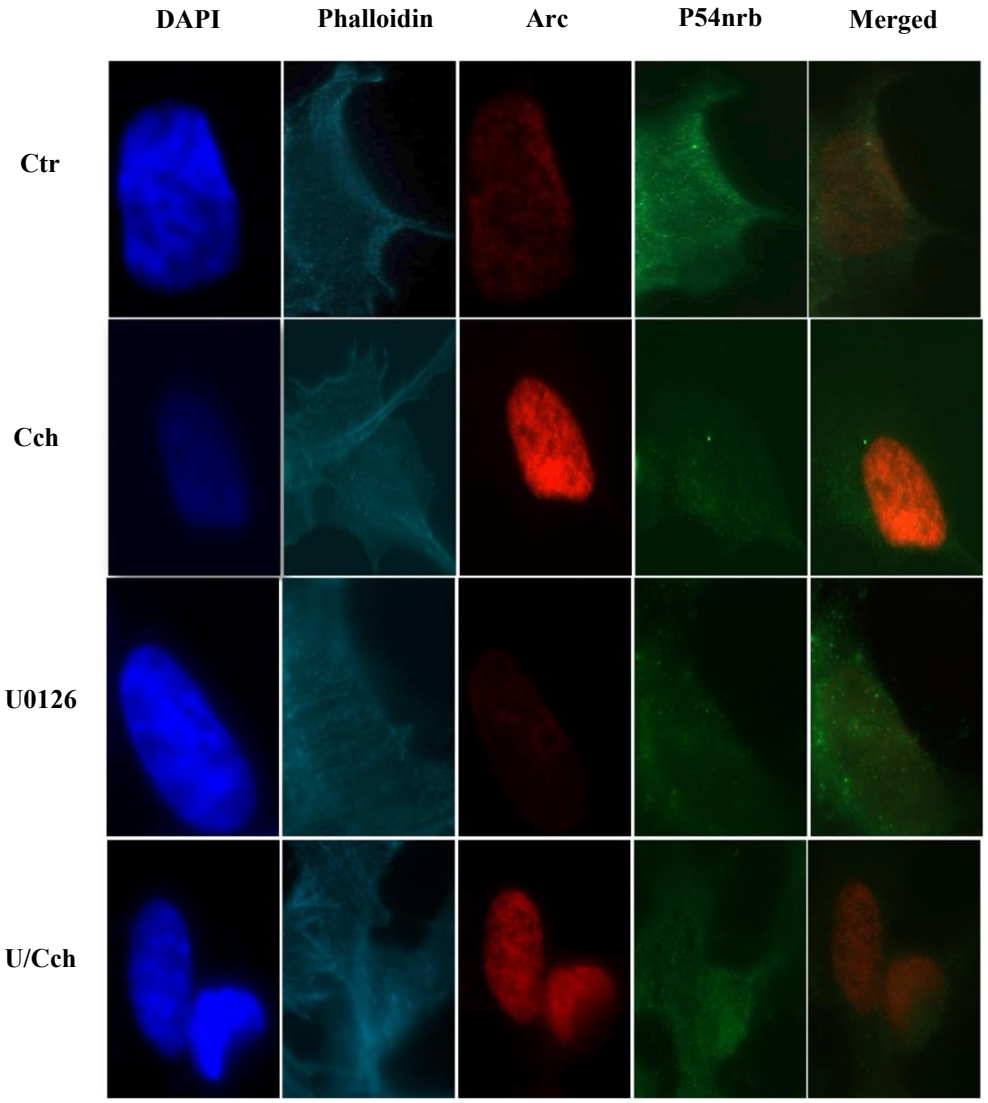


Figure 5-7: Co-localization of Arc and P54nrb. Differently treated SH-SY5Y cells (rows) were fixed and immunocytochemically stained for Arc (red) and P54nrb (green). Red-green-blue (RGB) merge was used to visualize co-localization (yellow). We found that Arc and P54nrb co-localize (merged group). Cell nuclei and actin filaments and the nucleus were visualized using DAPI (first column) and Phalloidin (second column), respectively.

6. Discussion

6.1 Carbachol and U0126 regulate Arc, but not paraspeckle protein expression

To investigate the effect of carbachol and U0126 on the target proteins, first, we detect the expression of Arc and paraspeckle proteins in neuroblastoma (SH-SY5Y) cells through the Western blot technique. These cell lines were found as a cellular model through which it is possible to study the signaling pathways regulating Arc expression [81]. Second, we treated the SH-SY5Y cells with carbachol and U0126 and mixed carbachol and U0126 to see the regulation effects on Arc and paraspeckle expressions. We found endogenous Arc expression induced after using carbachol, while U0126 almost inhibited it. Previous studies determined that cholinergic stimulation by Cch increased Arc mRNA expression by activating M1/M3 mAchR subtypes [83]. It was also shown that Cch treatment can activate mAchr-coupled ERK and increase intracellular calcium release, thus reducing transient Arc mRNA expression in SH-SY5Y cells. ERK signaling is necessary for Arc protein translation [81].

U0126 (1,4-diamino-2,3-dicyano-, 4-bis[2-aminophenylthio]-butadiene) is a small molecule that inhibits a central intracellular pathway, MEK. It has been used in studies such as cancer, development, and neurobiology [84]. Studies proved that U0126 inhibits MEK and ERK activation [85-87]. U0126, the ERK inhibitor, can block ERK phosphorylation and inhibit Cch-induced Arc mRNA and protein expression [82]. Consistent with other studies, we found that pretreatment with U0126 can significantly reduce the carbachol-increased Arc [77, 88].

Additionally, we showed that paraspeckle expressions were not affected by Cch or U0126. It is possible to conclude that paraspeckle proteins are regulated through different pathways than Arc protein, which is unrelated to the MEK/ERK pathway. This can be a new finding since there is no studies to show similar or opposite results.

6.2 Arc coimmunoprecipitated with NONO/P54nrb, PSF/SFPQ, not PSPC1

Mostly, proteins function as team players in a dynamic network. It was determined that protein-protein interactions are vital in many biological processes. One biochemical method to identify this interaction in living cells is co-immunoprecipitation [89, 90]. We applied IP and Co-IP methods to evaluate the possible interaction between Arc and paraspeckle proteins. Within a total lysate, there are many different proteins. We first purified Arc protein using the IP method from a total lysate of SH-SY5Y cells. We used a specific antibody for Arc (mouse anti-Arc polyclonal) to enrich Arc protein from the protein mixture. We applied Western blot for IP samples to analyze proper purification and used positive and negative controls. We showed that Arc protein was effectively purified. Besides that, we evaluated the effect of Cch and U0126 on Arc expression and found more purified Arc proteins in the samples were treated with Cch or U/Cch. While there was much less purified Arc protein in the U0126 samples. These results are consistent with our previous results (5.1.1).

Following Arc purification, we applied the Co-IP method using different antibodies specified for paraspeckle protein on the same blots achieved from IP samples. We analyzed the samples for the presence of the binding partner (paraspeckle proteins) by immunoblotting. For this reason, the membrane was cut based on the different molecular weights of paraspeckle proteins. After blocking the membrane, specific primary and secondary antibodies were applied to the cut membranes, followed by using ECL to visualize possible interactions between target proteins. First, we found that Cch and U0126 do not have regulation effects on paraspeckle protein expressions, consistent with our previous results (5.2.2). Moreover, Arc co-immunoprecipitated with SFPQ/PSF and NONO/P54nrb in SH-SY5Y neuroblastoma cells but not PSPC1. The other study was done in the same research group, showing the Arc-PSF/SFPQ interaction in SH-SY5Y cells (Kanhema, not published).

Although the Co-IP is used widely for studying protein-protein interactions, there are some issues to keep in mind. For example, the ability to detect this type of interaction depends on the strength of the interaction and the expression levels of the target proteins [91]. In order to overcome this problem, we extracted nuclear and cytoplasmic proteins from the lysate of SH-SY5Y cells, as paraspeckle proteins mainly exist in the nucleus.

6.3 Arc interacted with NONO/P54nrb and PSF/SFPQ, not PSPC1 in the nucleus

The main focus of this study was on the nuclear Arc and its binding partner in the nucleus. We extracted the nuclear Arc and paraspeckle proteins based on the identified interaction between Arc and PSF/SFPQ and P54nrb in total lysate. We applied subcellular fractionation using the specific kit (Figure 4.5). This kit was used for many different cell types and showed that it efficiently extracts concentrated nuclear protein (www.Thermofisher.com/NE-PER^{TM}). To optimize the protocol, we checked the presence of Arc and paraspeckle proteins in the nuclear and cytoplasmic fractions using Western blot and immunoblotting. In this project, we always used different groups of samples treated with Cch, U0126, and U/Cch as well as the control group. We found that using the fresh cells for subcellular fractions yielded better results. Final data showed Arc protein was more expressed in the cytoplasmic fractions and under the effect of Cch stimulation. Under normal circumstances, there are low levels of Arc expression, and only a few cells express Arc [92]. However, upon Arc expression stimulation, it primarily localizes in the cytoplasm of hippocampal neurons [53]. After several hours following stimulation, Arc accumulates within the nucleus [57, 93]. In this study, cells were collected 1h after stimulation by Cch. However, this period is insufficient for the effective accumulation of Arc in the nucleus, which may explain the higher concentration of identified Arc in the cytoplasm.

Additionally, we discovered a higher concentration of paraspeckle proteins within the nuclear fractions compared to the cytoplasmic ones. Paraspeckles are nuclear structures that form by binding specific proteins to NEAT1 in the cell nucleus [94]. However, paraspeckles can localize in the cytoplasm, contributing to gene regulation through RNA nuclear retention [70, 72].

Lastly, we used nuclear fractions for detecting Arc-paraspeckle protein interactions in the nucleus. We identified that Arc coimmunoprecipitates with NONO/P54nrb and PSF/SFPQ, not PSPC1, in the nucleus, the same as the previous results (5.3.2). Although there is no similar study, these results are aligned with studies showing the roles of Arc and paraspeckle in the nucleus. Additionally, there are a few similar identified binding partners for Arc and paraspeckle proteins in the nucleus, to which they bind and function in different cellular processing.

Research has indicated that paraspeckle proteins contain lncRNAs and have a role in chromatin remodeling [95]. There are multiple binding sites for NEAT1 at various genomic loci. Studies have demonstrated that NEAT1 contributes to chromatin's regulation and spatial organization [96, 97]. Additionally, paraspeckles have been found to play a role in maintaining chromatin organization [98]. The study concluded that nuclear Arc is a brake on gene regulation and chromatin remodeling in response to Cocaine-induced Arc accumulation in the nucleus. This can also be related to the homeostatic role of Arc in neuron [99].

The other example concerns the association between Arc and paraspeckle proteins with PML bodies. It was identified that a lack of paraspeckles causes a lack of PML bodies, another type of nuclear body [63]. PML bodies are sites of transcriptional regulation [100] and have various regulation roles in several cellular processes, including transcription, apoptosis, and response to DNA damage [101]. The association between Arc and PML bodies was identified via Arc interaction with nuclear spectrin isoform (βSplVΣ5). This interaction increases the number of PML bodies and demonstrates Arc's regulatory effect on PML function [7].

Regarding gene regulation, paraspeckle and Arc proteins showed that they are involved in transcriptional gene regulation [64, 102]. There are several RNA binding proteins (RBPs) to which Arc binds and regulates protein expression. Moreover, Arc can recruit RNAs through binding to RNAs and facilitates intercellular RNA transport [102]. It was also shown that Arc could broadly inhibit RNA polymerase II [99]. Additionally, paraspeckles contain over 40 RNA binding proteins assembled onto the substantial scaffolding of long noncoding RNA, NEAT1, and their essential roles in RNA processing, splicing, and transcription [63, 103]. The studies showed the roles of PSF and P54nrb at several steps in RNA processing [104]. P54nrb is a bridge between RNA-Poll II and the coactivator complex, mediating the expression of cAMP-response genes [105]. RNA Polymerase II is a crucial component of the transcription process, and its function is influenced by the chromatin organization [106]. Moreover, SFPQ and NONO bind to RNA-Poll II and mediate co-transcriptional processing [107].

These similar activities for Arc and paraspeckle proteins and the identified interactions between Arc and paraspeckle proteins in this study may reveal new binding partners for nuclear Arc. It is possible to conclude that nuclear Arc interacts with paraspeckle proteins and plays several roles in

the nucleus. However, we need to determine whether these interactions occur specifically between target proteins through IP and Co-IP methods. Accordingly, we used the ICC method to evaluate possible colocalization between nuclear Arc and paraspeckle proteins. The results showed that Arc and p54nrb co-localize in the nucleus of SH-SY5Y cells. Based on these results, there is additional evidence for a specific interaction between Arc and P54nrb. We could not apply the ICC method for Arc and SFPQ/PSF protein because of time limitations.

7. Conclusion and Future Perspective

In this master study, we found that Arc interacts with NONO/P54nrb and PSF/SFPQ in the nucleus of the neuroblastoma (SH-SY5Y) cells. These two proteins are members of the human DHBS family and the two mains of paraspeckle proteins. Paraspeckle proteins are linked to several neurodegenerative diseases, such as Parkinson, Huntington and Alzheimer's. However, more studies will be needed to understand the exact and important mechanisms through which paraspeckle proteins are involved in different molecular mechanisms in the brain [64]. We also found that Arc colocalizes with NONO/P54nrb in the nucleus. According to these findings, it is possible to conclude that NONO/P54nrb and PSF/SFPQ proteins are two new binding partners for Arc protein. Additionally, based on the roles of Arc and these two paraspeckle proteins in the nucleus of neurons, Arc contributes to different molecular mechanisms like chromatin remodeling and RNA processing regulation possibly through interacting with paraspeckle proteins. Although the methods used in this study cannot confidently prove our findings, these findings are promising and can guide future studies related to the Arc function in the nucleus.

The Co-IP assay is a common method for detecting protein-protein interactions, but it has some limitations. One of these limitations is that some protein-protein interactions are week and timescale-dependent meaning they can only be detected during a special period. Therefore, it is recommended to perform Co-IP experiments for different amounts of time. [91]. In this study, localization and accumulation of Arc in the nucleus require enough time. For the next similar study, it would be helpful to use a different time scale, for example, 2 hours instead of 1 hour, for treating Arc protein with carbachol. Additionally, it is possible that the interactions will not be identified because of the overlapping of the protein-binding site with the binding site of the first antibody used for purifying the target protein. Using multiple antibodies against the target protein and applying reverse IP can optimize this protocol to yield better results [91].

The other problem is related to SDS detergent and low concentrations of nonionic detergents of lysis buffers, which can interfere with Co-IP [91]. Thus, for future studies, using different methods, including RNA immunoprecipitation (RIP), and GST pulldown assay are highly recommended.

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