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Arc protein-protein interactions in long-term potentiation in the rat dentate gyrus in vivo

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Acknowledgements

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Bergen, 2 June 2014 Sudip Debnath

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Abstract

Synaptic plasticity is the ability of neuronal synapses to change in strength over time, and is considered to be a foundation for learning and memory. Long-term potentiation (LTP) is a widely studied form of synaptic plasticity in the mammalian brain. In LTP, brief high-frequency stimulation (HFS) of afferent fibers results in a long-lasting (hours to days) increase is excitatory synaptic transmission. HFS-evoked expression of the immediate early gene Arc, (*activity-regulated cytoskeleton-associated protein)* is required for stabilization LTP. However, the mechanisms by which Arc acts to generate and stabilize LTP are unknown. The identification of proteins that bind to interact with Arc during LTP is the key to understanding how Arc works. To this end, we evalutated possible interaction of Arc with two candidate interacting proteins, Dynamin 2 and Syntaxin 4 (Stx4). *In vivo* electrophysiological recording was done on anesthetized rats to determine LTP induction due to HFS and later compared with another group of rats, received baseline test stimuli but not any HFS. Differences between HFS-treated dentate gyrus and the contralateral control dentate gyrus in terms of Arc protein expression was measured by western blot method. HFS-treated and the contralateral control dentate gyrus were analyzed for Dynamin 2 and Stx4 co-immunoprecipitation (Co-IP) by Arc protein Immunoprecipitation (IP) at various time points. Our experimental proof indicated stable LTP induction after HFS and this percentage was higher to 40-50 % of increase to baseline. We also found that Arc is rapidly synthesized after tetanic stimulation. From our Co-IP experiments we found that Dynamin 2 and Stx4 are the prime binding partners of Arc protein during LTP. Arc-Dynamin 2 interaction may be highest during 60 minutes after HFS. Stx4 might be a candidate binding partner for Arc protein during LTP. Stx4 IP showed Dynamin 2 as a binding partner of Stx4. From this study we concluded that Arc is rapidly synthesized after HFS. During LTP Dynamin 2 and Stx4 are binding partners of Arc. Stx4 and Dynamin 2 are present in the sample protein complex.

List of abbreviations

LTP	Long-term potentiation
LTD	Long-term depression
Arc	*Activity-regulated cytoskeleton-associated protein*
Arg3.1	*Activity-regulated gene 3.1*
NIA	National Institute on Aging
DG	Dentate gyrus
LDG	Left dentate gyrus
RDG	Right dentate gyrus
CA	Cornu ammonis, Ammon's horn, hippocampus proper
EC	Entorhinal cortex
GABA	Gamma amino butyric acid
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor
Mg^{2+}	Magnesium ion
EPSP	Excitatory post-synaptic potential
fEPSP	Field excitatory post-synaptic potential
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response-element binding protein
mRNA	Messenger RNA
IEG	Immediate early genes
LEGs	Late effector genes
hArc	Human Arc
AS	Antisense oligodeoxynucleotides
KO	Knockout
E-LTP	Early phase of long-term potentiation
L-LTP	Late phase of long-term potentiation
PSD	Postsynaptic density
HFS	High frequency stimulation
PML-NBs	Promelocytic leukemia tumor suppressor protein nuclear bodies
Stx4	Syntaxin 4
PM	Plasma membrane
SNARE	Soluble NSF Attachment Protein
NSF	*N*-ethylmaleimide sensitive fusion proteins
REs	Recycling endosomes
Stx4	Syntaxin 4

1. Introduction

Our memory is our mind's recording framework. It holds all that we have learnt. We can store an astonishing measure of data. And thus at the end of the day, our memories contribute greatly to our personalities. However, learning and memory is a complex process, and because of this, there is an incredible drive towards neuroscience research aimed at breaking down and revealing the complexities of this system. From few decades scientist's came to know that memories are framed in the form of neuronal connections (synapse) and this process is called synaptic plasticity. Moreover they proved that hippocampus is requiring for memory formation. There are two major cellular mechanism of synaptic plasticity: long-term potentiation (LTP) and long-term depression (LTD). Increase in synaptic strength is known as LTP while Increase in synaptic strength is known as long-term depression (LTD). ***Activity-regulated cytoskeleton-associated protein*** **(which is also known as Arg3.1 or Arc Protein)** is an immediate early gene**,** is believed to be strongly involved in the process of synaptic plasticity.

The main goal of my thesis project was to evaluate potential Arc protein-protein interactions in long-term potentiation (LTP) in rats and to different time points. Most biological processes involve the action and regulation of multi protein complexes; we believe that stimulation dependent increase protein synthesis which will help us to elucidate the mechanism of Arc in synaptic plasticity. Activity-dependent changes in synaptic strength require more time for new protein synthesis.

1.1 Learning and memory

From nursery rhymes to mobile numbers, our brains grip an outwardly nonstop supply of information. Memory permits us to recollect facts and experiences. Memory comprises of encoding, putting away data, and recovery, making that data accessible for review. Learning, dissimilar to memory, is not about putting information. Rather, learning is a behavior. As it were, we could say that learning is a procedure, and memory is the record of that process.

Learning is closely connected to memory, in that it is completely dependent upon memory to capacity; however it goes past harboring realities into usage.

Regarding the matter of arrangement of memories, there are three fundamental classifications: sensory memory, short-term memory, and long-term memory.

Sensory memory is first in this arrangement of memories and is our capability to recollect certain parts of data for a brief moment (less than a second) after the stimulation has gone. Our tangible receptors can hold a huge measure of data yet everything held by these receptors keeps up for a small amount of a second (Carlson 2010).

Short-term memory is the capacity to hold a little amount of information. It is assessed that we can hold transient memories for up to 20 seconds. Long-term memory, it can hold an apparently boundless measure of data for a boundless time allotment. Long-term memories could additionally be deep rooted memories. One thing about long-term memory is the way that we could overlook data in the event that it is not reviewed at normal interims.

Hippocampus is the most critical brain region for these procedures. Both LTP and LTD have been seen at the excitatory synapses in the hippocampus (Neves, Cooke et al. 2008).

1.2 The Hippocampus

The hippocampus is found in the medial temporal lobe of the brain. It is known to assume a critical part in the combining of data from short-term memory to long-term memory. More specifically, studies using human subjects identified hippocampus to be a main player in episodic memory, the type of memory that links experiences with time and space (Kemp and Manahan-Vaughan 2007).

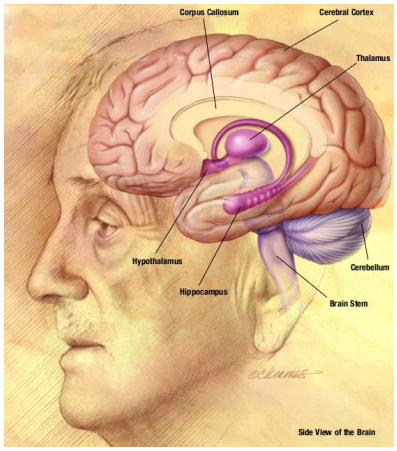


Figure 1.1: Human Brain (Adapted from NIA Human brain drawing)

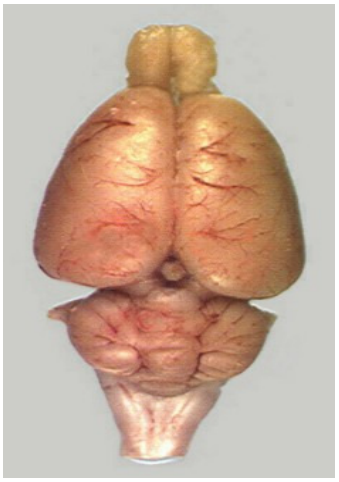


Figure 1.2: Rat Brain (Modified from Synapse *Web, Kristen M. Harris, PI, http://synapses.clm.utexas.edu/*)

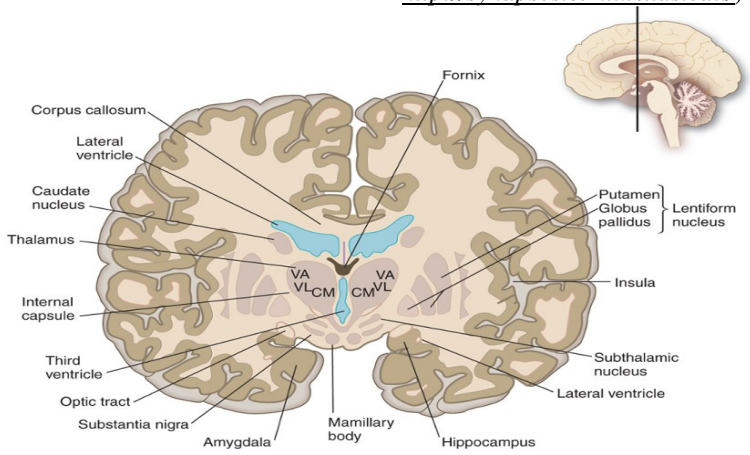


Figure 1.3: Coronal section of the brain. The rat brain and human brain outlining the confinement of the hippocampal creation. The volume of the hippocampus is something like 100 times bigger in humans than in rats, however the rodent hippocampus includes a noticeably vast volume contrasted with whatever is left of the brain, providing for it favorable element in the matter of mulling over the hippocampus. Therefore, a significant part of the accessible information has been determined from studies completed in the rat. There are however striking species differences, e.g. in the rat DG there is a monstrous commissural framework that provides nearly one---sixth of the excitatory data to the DG, although in people, commissural associations in the DG are very nearly completely absent (Amaral and Price 1984).

LTP is induced in dentate gyrus (DG) in the hippocampus and been a target for analysis molecular basis of learning and memory from decades. Likewise, interruptions of LTP and LTD in the hippocampus both interfere with spatial learning of laboratory animals, showing that long-term synaptic plasticity is fundamental for hippocampal memory (Kemp and Manahan-Vaughan 2007). It has a comparative capacity in human and rodents. On account of

ethical issues and the larger size of a rat brain compared to mice, a rat animal model is more suited and proper to lead such considers. Thus this study used a rodent model.

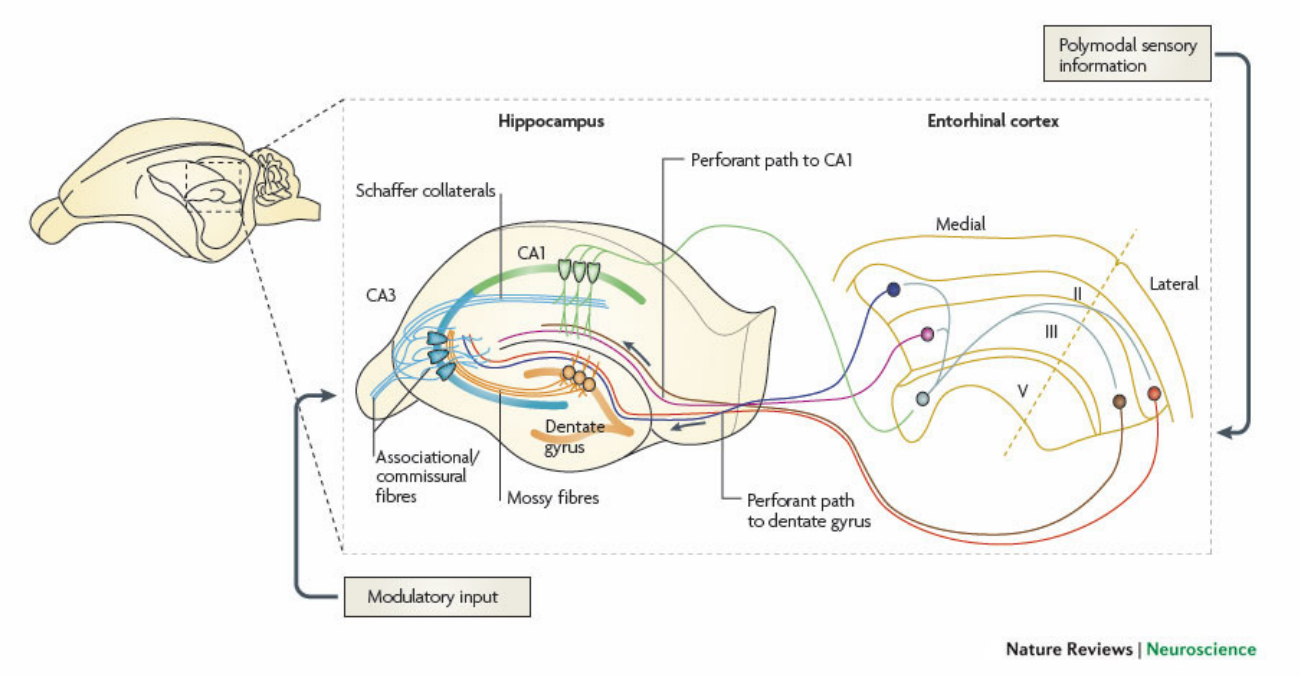


Figure 1.4: Hippocampal tri-synaptic loop. Here indicated in the rat brain. The primary indicating pathway of the hippocampus structures a circle. The entorhinal cortex passes on data to the granule cells of the dentate gyrus through the perforant way. Overgrown strands unite the granule cells to the pyramidal cells in the CA3 region, shaping a second synapse. At that point, Schaffer collaterals innervate CA1 pyramidal cells. Strands from this locale and the subiculum extend over to the entorhinal cortex (Neves, Cooke et al. 2008)

Synaptic transmission in the hippocampus structures a tri-synaptic loop. A functional tri-synaptic loop is created from the entorhinal cortex (EC) also known as perforant path to the dentate gyrus (DG) to the CA3 to the CA1 (Lavenex, Amaral et al. 2006). Formation of LTP can be achieved by provoking pharmacologically with drugs or with High frequency stimulation of brain by electrical stimulation.

Data enters this one-way circle through the axons of the EC. These axons make the loop's first association, with the granule cells of the dentate gyrus. From these cells, mossy fibers thus spread to make the loop's second association, with the dendrites of the pyramidal cells in region CA3.The axons of these cells are divided into two limbs. One extension structures the commissural fibers that extend to the contralateral hippocampus through the corpus

callosum. The other extension structures the Schaffer collateral pathways that make the third connection in the loop, with the cells in zone CA1 (Neves, Cooke et al. 2008).

1.3 Dentate gyrus

The hippocampus intervenes a few higher brain capacities, for example, taking in, memory, and spatial coding. The information locale of the hippocampus, the dentate gyrus, assumes a discriminating part in these procedures. Dentate gyrus granule cells accept excitatory neuron info from the entorhinal cortex and send excitatory yield to the hippocampal CA3 district by means of the mossy fibers. Besides, a few sorts of GABAergic interneurons are available in this area, giving inhibitory control over granule cell movement through input and feed forward hindrance. At last, hilar mossy cells intervene an alternate excitatory circle in the circuit. All around the brain, the dentate gyrus is one of a kind, in light of the fact that grown-up neurogenesis happens in this district. Consequently, new neurons are created and practically incorporated all around life.

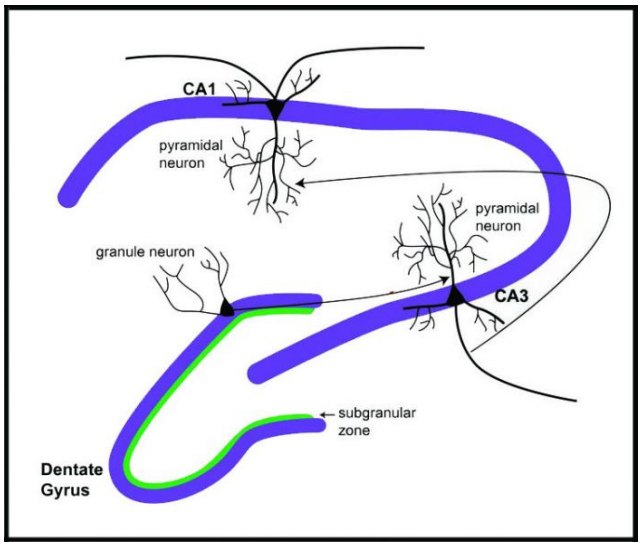


Figure 1.5: Neurons of the hippocampus and subgranular zone of the dentate gyrus (McCaffery, Zhang et al. 2006). The sub-atomic layer of dentate gyrus granule neurons get the hippocampal shaping's significant excitatory data from the cortex. This data is basically inferred from layer II of the entorhinal cortex and empowers the dentate gyrus to entryway data stream into the hippocampus trisynaptic circle.

1.4 Long-term potentiation (LTP)

Long-term potentiation (LTP) is a major cellular mechanism of synaptic plasticity. By definition synaptic plasticity is the capacity of the synapse to change in strength. This change might be attained by progressions in the amount of neurotransmitters discharged into a synapse, which is a structural intersection between two neuronal cells that transmits a signal to an alternate neuron. The degree of synaptic changes is also dependent on how adequately cells react to those neurotransmitters.

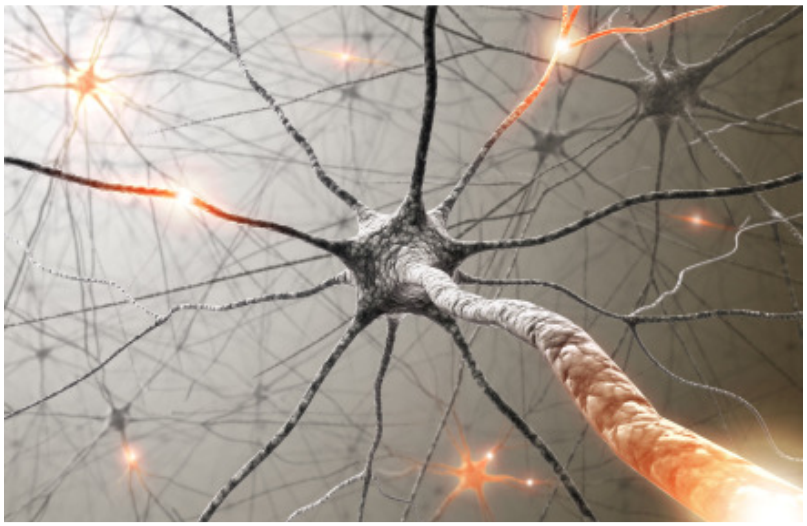


Figure 1.6: Neurons firing. A neuron is the working cell unit in the sensory system that is otherwise called a nerve cell. Neurons are answerable for sending motivation messages to other neural cells. Drive messages in a neuron are sent by means of the arrival of neurotransmitters. The neuron's phone body is known as the soma. The neuron cell comprises of 3 principle segments a soma, axon, dendrite.

From previous studies, we have come to know that alterations in the synaptic strength are bidirectional. Reinforcement of the synapses is known as LTP while declining of the synapses is called long-term depression (LTD).

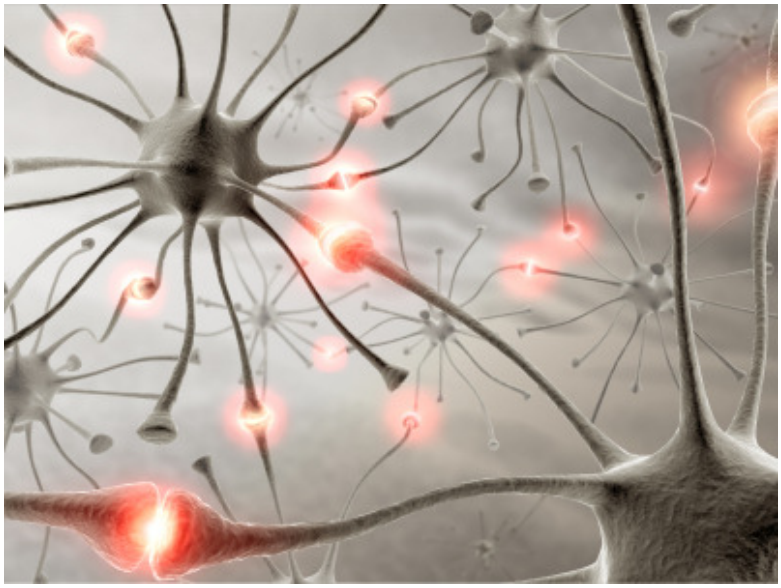


Figure 1.7: Neural synapses passing chemical messages. Synapse - Is the structural space between neurons in the sensory system that is the channel for a neuron to send a synthetic message indicator to the focused on neural cell. A synapse is otherwise called the terminal catch. When a message is accepted at the postsynaptic cell an electrical message is discharged and passes through the bordering neuron to the following presynaptic cell that discharges an alternate synthetic message. The procedure will rehash itself until the message achieves it fancied target. The expression synapse is Greek in source and means purpose of contact.

1.4.1 Mechanism of LTP

Two kinds of receptors are involved in synaptic transmission during LTP: N-methyl-D-aspartate receptor (also known as the NMDA receptor or NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor or AMPAR). The NMDA receptor is a glutamate receptor and the channel is penetrable to calcium ions but at normal synaptic transmission the channel is blocked at resting potential by physiological concentrations of Mg^{2+} ions.

The AMPA receptor is a non-NMDA-type ionotropic transmembrane receptor which opens to create fast excitatory postsynaptic potential (EPSP).

High-frequency stimulation of a synapse induces LTP. This reinforces the effectiveness of the synapse. Depolarization of the receptor in addition to glutamate tying discharges Mg^{2+} ions from NMDA receptor. This permits Ca^{2+} ions to enter the NMDA receptor channel and act as a second messenger in the post synaptic cell. Increased post synaptic Ca^{2+} ion concentration permits activation of various Ca^{2+} dependent protein kinases for instance Protein Kinase C, Calcium calmodulin kinases, etc. These kinases may act post synaptically to increase the sensitivity to glutamate release. On the other hand a retrograde sign may be discharged that follows up on the presynaptic terminal to improve neurotransmitters (Purves D 2001).

Influx of calcium ions in to the post synaptic cells permits a neurochemical course at the post synaptic terminal. Activated kinases bind to cAMP response element-binding protein (CREB). This leads to production of the transcript (mRNA) of immediate early genes (IEG) (Miyashita, Kubik et al. 2009). IEGs encode many particular late effector genes (LEGs). One effector protein encoding gene, that has received much consideration in connection to synaptic plasticity (i.e., LTP and LTD), is the gene that encodes Arc protein (Bramham, Alme et al. 2010).

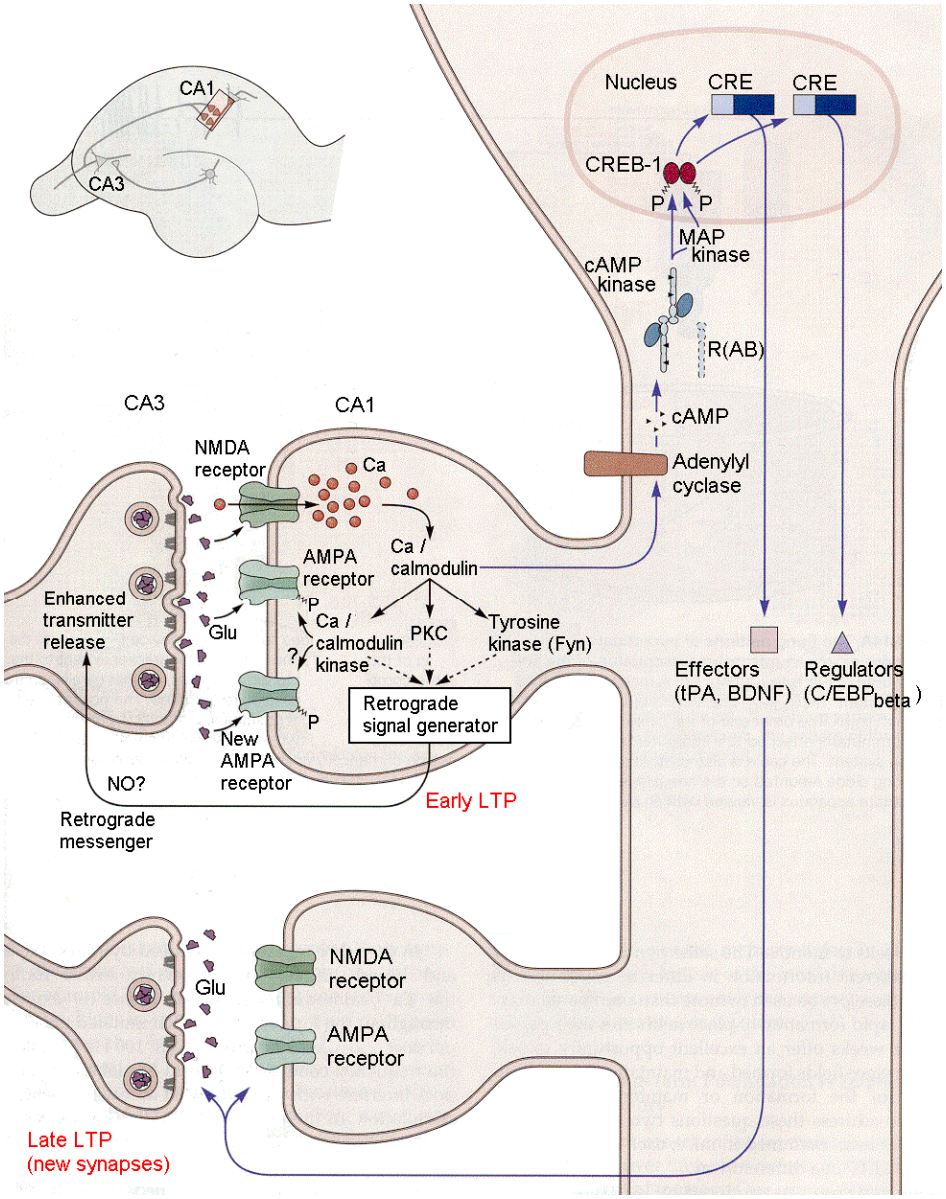


Figure 1.8: Mechanism in the LTP. (Kandel, ER, JH Schwartz and TM Jessell (2000) *Principles of Neural Science*. New York: McGraw-Hill)

1.5 Activity-regulated cytoskeleton-associated protein (Arc/Arg 3.1)

First characterized in 1995, the activity-regulated cytoskeletal protein (Arc/Arg 3.1) is an immediate early gene which is widely concerned with hippocampal dependent learning and memory. It is thought to play a central role in synaptic specific plasticity. Being an immediate early gene its expression is brief and fast in response to stimuli. It doesn't require any de novo protein synthesis. Arc is extraordinary among IEGs in light of the fact that its mRNA is quickly transported to distal dendrites and specifically confined at activated synapses, where it has the possibility to be locally translated (Steward and Worley 2001). Arc assumes a fundamental part in the late-phase of LTP and is needed for the consolidation of long-term memory. Induction of LTP in the hippocampus increases Arc protein expression, while blocking Arc outflow by antisense oligonucleotide infusion or gene knockout results in defects in both late-phase LTP and memory tasks (Guzowski, Lyford et al. 2000). Arc expression is vigorously prompted by activity, and the Arc protein is restricted to both active synapse and the nucleus. A previous study on the Arc protein found it to be highly localized to the nucleus that at the active synapse (Bloomer, VanDongen et al. 2007).

A recent study described human Arc (hArc) as a protein holding two globular domains on either side of a central disordered (Myrum et al 2013). High alpha helical content found in the same study and the authors showed that multiple independent structural domains are present in hArc protein which unfolds at an exclusive temperature. They have also showed that the primary cleavage site between the border of the two hArc domains as at residue 173 while the secondary cleavage site is at residue 35.

1.5.1 Role of Arc protein

In vivo studies in dentate gyrus have revealed that infusion of Arc antisense oligodeoxynucleotides (AS) in the hippocampus inhibits Arc mRNA translation. This however, was found to impair the maintenance of LTP and consolidation of Long term

memory (Guzowski, Lyford et al. 2000). Thus, in this case, even though the short-term memory was unaffected, long-term memory was significantly reduced.

In-vivo studies on Arc knockout (KO) mice recently revealed arc protein function in early-LTP (E-LTP) and late-LTP (L-LTP). Previously, it was known that E-LTP expression is facilitated by the addition of glutamate receptors into the postsynaptic membrane and the activation of various protein kinases. This process was known last from 30 minutes to a few hours. E-LTP is known to function in receptor trafficking, Postsynaptic density (PSD) development and F-actin remodeling. On the other hand, L-LTP continues from few hours to a few weeks. It is believed to be dependent on genetic variations. In Arc KO mice it is found that E-LTP was boosted while the L-LTP was absent in the CA1 region as well as the dentate gyrus (Messaoudi, Kanhema et al. 2007). Genetic deletion of Arc also showed reduced LTP and LTD in hippocampal slice preparations (Plath, Ohana et al. 2006).

Dysfunction of Arc results in to various neurodegenerative diseases like Angelman Syndrome (Greer, Hanayama et al. 2010) and Alzheimer's disease (Wu, Petralia et al. 2011). Generally, the Arc protein plays a major role in synaptic plasticity, memory and neurogenesis.

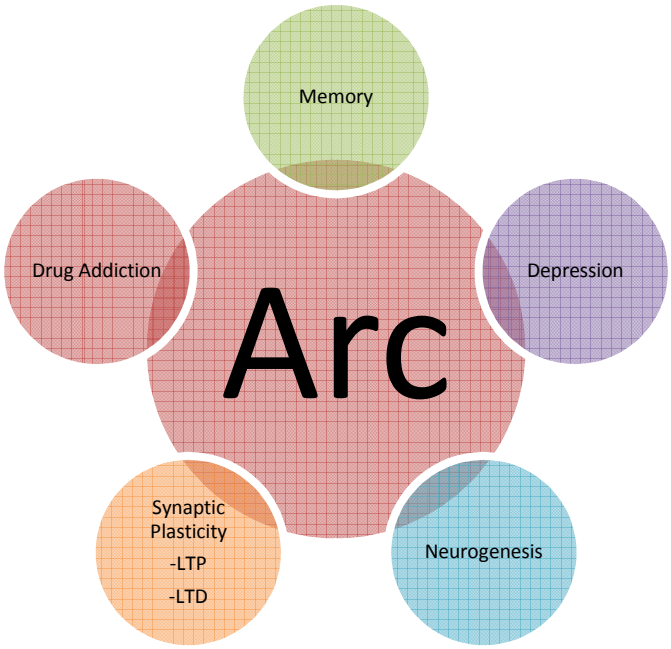


Figure 1.9: Arc protein function. Arc assumes discriminating parts in synaptic plasticity and memory stockpiling. Works in neurogenesis, drug fixation, and flexibility to stress have been proposed yet causal parts have not been characterized (Bramham, Alme et al. 2010).

1.5.2 Arc protein binding partners

Containing 3,948 base pairs and located on the long arm of chromosome 8 (8q24.3), Arc gene transcription can be induced by electrical stimulation by applying high frequency stimulation (HFS) (Messaoudi, Kanhema et al. 2007).

A schematic representation of Arc protein and its binding motifs is shown in Figure 10 below. In this figure, the binding sites for Endophilin 3 and Dynamin 2 proteins involved in AMPAR endocytosis in synaptic transmission are indicated. The PEST sequence between 351 to 392 bp which is rich in the amino acids Proline (P), Glutamate (E), Serine (S) or Threonine (T) could target Arc for proteasomal degradation (Peebles and Finkbeiner 2007, Bramham, Alme et al. 2010).

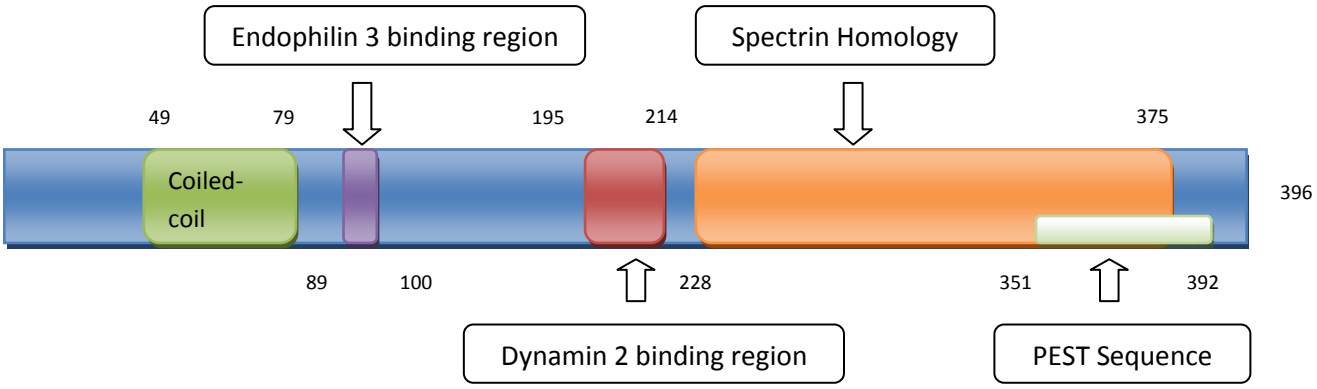


Figure 1.10: Schematic representation of Arc Protein and binding motifs. The endophilin and dynamin tying locales intercede AMPAR endocytosis in synaptic depression. The PEST arrangement, a district rich in the amino acids proline (P), glutamate (E), serine (S), or threonine (T), could target Arc for proteasomal degradation. (Bramham, Alme et al. 2010)

Chowdhury S et al., (2006) proposed a model for Arc protein regulation of AMPAR trafficking. Arc protein occurs in the synapse-particularly through local protein translation. The formed Arc protein then initiates Endophilin 3 and Dynamin 2 binding. The Arc-endophilin-dynamin complex in turn causes adjustment of endosome framing and and enrollement of the AMPA receptor. Ultimately, the fate of the Arc-endophilin-dynamin complex may be subject to 3 destinies. First, it may be reused once again at the synaptic membrane. Secondly, it may move to the lysosomal compartment, where it is degraded or sent to the proteasome, or move to the nucleus where it may act as a signaling endosome (Chowdhury, Shepherd et al. 2006). However the regulation of Arc function over the time course of its production remains poorly understood.

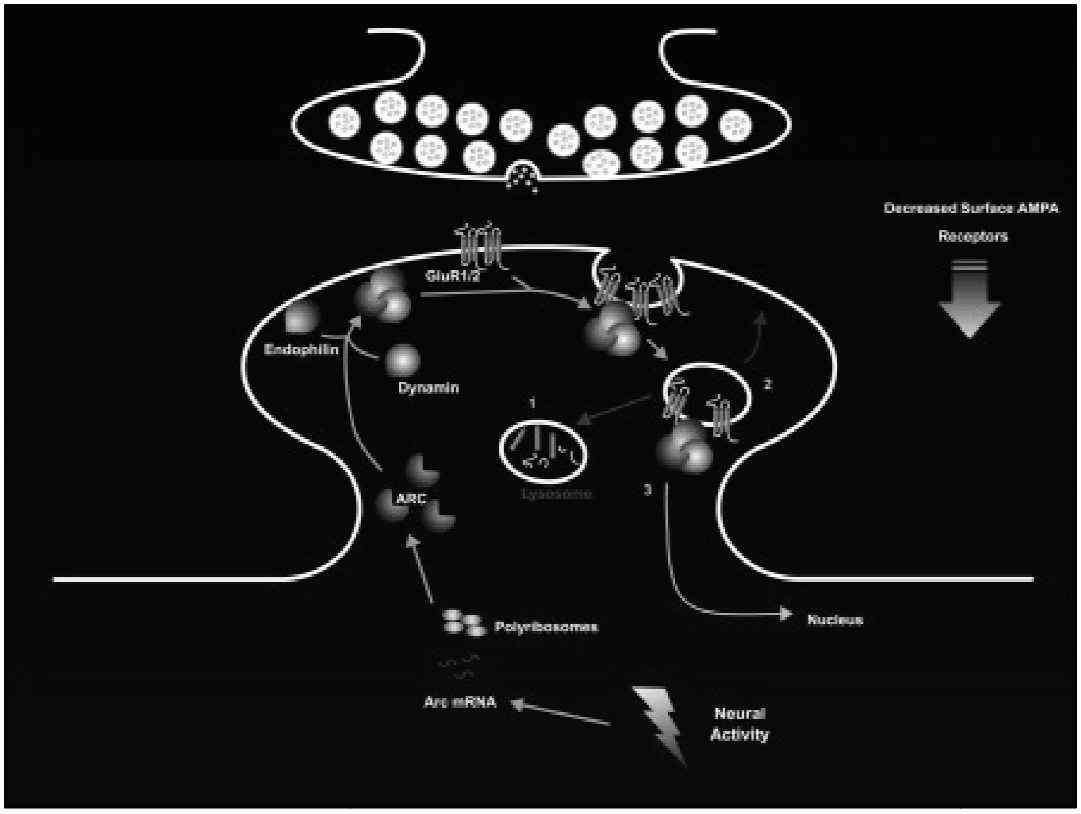


Figure 1.11: Proposed model for Arc modulation of AMPAR trafficking. The intricate of Arc/Arg3.1, endophilin, and dynamin adjusts endosome establishment and specifically enlists AMPA receptors. These endosomes might then (1) reuse again to the membrane, (2) movement to the lysosomal compartment where protein is degraded or sent to the proteosome, (3) activity to the core as an indicating endosome convey other load. It is fascinating to note that Arc/Arg3.1 protein is available in the nucleus of select populations of brain neurons where it is coincidently instigated with Arc/Arg3.1 protein in dendrites. Changes in the energy of these vesicular pathways are expected to underlie changes in unfaltering state levels of AMPAR at synapses (Chowdhury, Shepherd et al. 2006).

It has been found that Arc induces the formation of Promelocytic leukemia tumor suppressor protein nuclear bodies (PML-NBs). PML-Nbs have function in the nucleus, including role in transcription (Bernardi and Pandolfi 2007). Most recent study indicated that Arc localization to the nucleus, which diminishes Glua1 transcription and synaptic strength (Korb, Wilkinson et al. 2013). Glua1 transcription could be controlled through CRE sites inside its promoter (Borges and Dingledine 2001) and PML-Nbs manage CRE-dependent transcription in other cell types (St-Germain, Chen et al. 2008). Yet how this regulation influences about whether course throughout Arc processing is remains

inadequately caught on. The role of Arc in the nucleus and its binding partners are little understood.

1.5.3 Candidate binding partners for Arc

Recent studies from Kennedy et al. (2010) establish specific role of Syntaxin 4 (Stx4), a plasma membrane (PM) soluble NSF-attachment protein receptor (SNARE) protein in activity-dependent exocytosis in hippocampal neurons. Stx4 is localized in the postsynaptic membrane and it defines an exocytic domain in activity-dependent exocytosis of AMPA receptor (Kennedy, Davison et al. 2010). Figure 1.12 below illustrates that Stx4-mediates postsynaptic exocytosis of AMPA receptors.

According to their model recycling endosomes (REs), a subunit of AMPA receptor are shaped in a dendritic spine. These are shaped by endocytosis and comprise various PM components. For example, AMPA receptor, NMDA receptor, N-cadherin. REs moved to the adjacent post-synaptic density (PSD) by Bic/Gly stimulation (Mg^{2+}-free solution containing 200 μM glycine, 30 μM bicuculline) of the neuron by bathing for 5 min to increase total network activity which also induces clustering of Stx4. At the last stage of the proposed model, Stx4 facilitates fusion of REs attached with the PM and at the end mediates diffusion of recycling endosome contents into the plasma membrane. Endosomal contents are available at synapse after fusion (Kennedy, Davison et al. 2010).

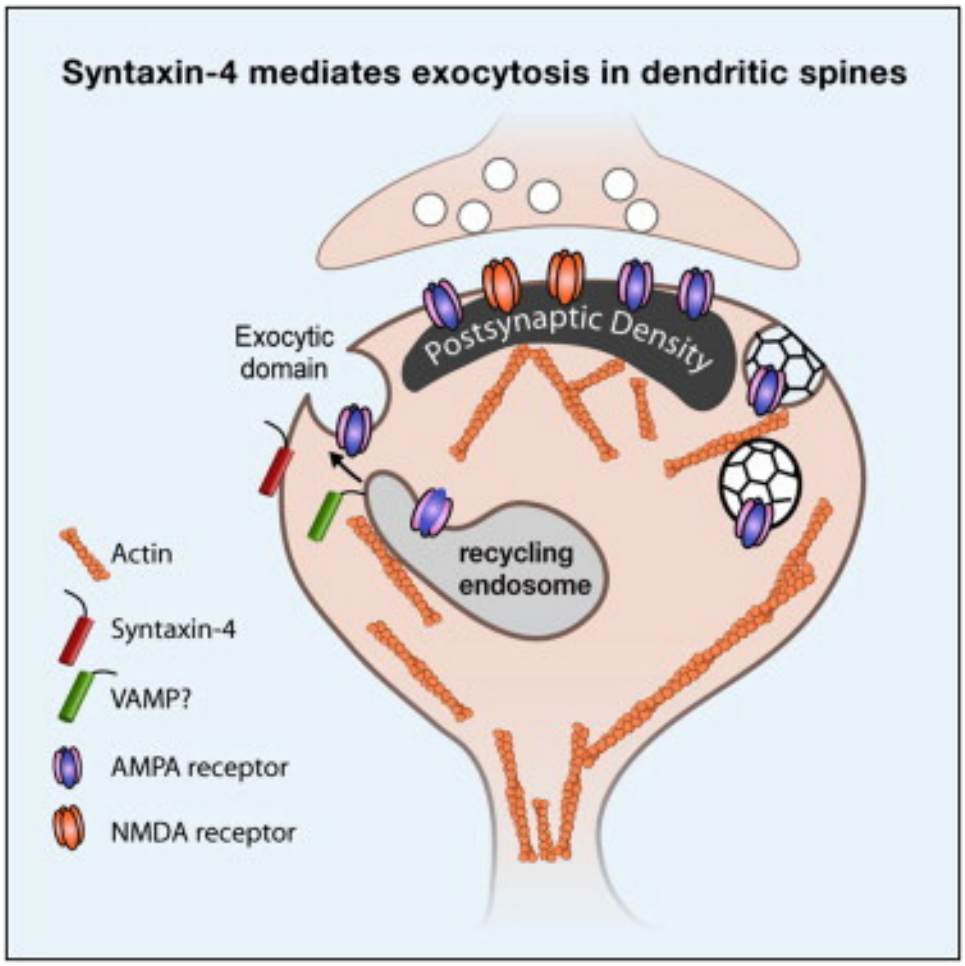


Figure 1.12: Stx4 mediates exocytosis in dendritic spines. Activity triggers exocytosis of AMPAR-holding endosomes in dendritic spines. Recycling endosomes wire with the spine layer lateral to the postsynaptic density. The plasma membrane t-SNARE Stx4 intercedes spine exocytosis. Disrupting Stx4 blocks spine exocytosis and synaptic potentiation. (Kennedy, Davison et al. 2010)

From a neurological point of view, AMPA receptor trafficking is very important in the sense that supplement or elimination of postsynaptic AMPA receptors regulates the scale and foundation of LTP (Kennedy et al 2010). Additionally, altered AMPA trafficking in laboratory animal models shows various neurological dysfunctions like Alzheimer.s disease and schizophrenia (Kessels and Malinow 2009). Stx4 plays a vital role in this regards. So from the above review there might be possibilities that Stx4 can bind with Arc to facilitate AMPA trafficking and thus can be a novel binding partner for Arc.

1.6 Aims

The overriding goal is to understand and how Arc works in LTP through the biochemical identification of its protein interaction partners.

We know that Arc protein interacts with Dynamin 2 during clathrin- mediated endocytosis. Dynamin 2 is a known binding partner for Arc protein (Chowdhury, Shepherd et al. 2006). Stx4 is a plasma membrane soluble SNARE protein in activity-dependent exocytosis in post synaptic neurons (Kennedy, Davison et al. 2010). So, it can be a candidate binding partner for Arc protein.

The specific aims of the study were as follows:

1. To establish methodology for performing Arc IP in DG tissue and to optimize lysis buffer salt (NaCl) concentration for Co-IP.
2. To examine possible interaction of Arc with Dynamin 2 and Stx4.
3. To examine interaction of Arc with Dynamin 2 and Stx4 follows LTP induction in the dentate gyrus.

2. Materials and Methods

In vivo electrophysiological study in the dentate gyrus was followed by analysis of biochemical changes in dentate gyrus with the coimmunoprecipitation method.

2.1 Materials

The following reagents and buffers have been used to perform the study.

2.1.1 Reagents & Buffers

- Lysis Buffer: 20 mM Hepes-HCl (pH 7.4), 100 mM NaCl, 0.5% (v/v) Nonidet P-40, 5 mM Ethylenediaminetetraacetic acid (EDTA), 0.5 mM (Sodium fluoride) NaF, 0.5 mM activated sodium vanadate (Na_3VO_4), 20 mM N-ethylmalemide: Freshly prepared lysis buffer used to lysis the tissue otherwise stored in a tube at 4^0C.
- Phosphate-buffered saline (PBS) (130 mM NaCl and 20 mM sodium phosphate, pH 7.5).
- Protease inhibitor cocktail (Roche Diagnostics GmbH). 1 tablet/10 ml was added into the final solution.
- BCA protein assay kit (Thermo scientific, # 23225) for Protein analysis.
- Washing Buffer: 1 x PBS (In some cases with lysis buffer), 1x TBST (mixture of Tris-Buffered Saline and Tween 20)
- Protein G-sepharose beads (GE Health Care Bioscience AB, 17061801)
- 2x Laemmli sample loading buffer (Bio-Rad #161-0737)
- Specific antibodies (Polyclonal, monoclonal) such as Arc (C-7) Mouse Monoclonal Antibody (Santa Cruz Biotech #sc-17839), Arc (H-300) Rabbit Polyclonal Antibody (Santa Cruz Biotech #sc-15325), Anti-Syntaxin 4 Rabbit Polyclonal Antibody (SYNAPTIC SYSTEMS GmbH, #110043), Anti-Dynamin, Hudy 1 Mouse Monoclonal Antibody (Upstate cell signaling of analysis).

2.2 Methods

2.2.1 *In vivo* electrophysiology

2.2.1.1 Animals

Adult male Sprague-Dawley rats weighing 250 g-350 g were housed in group of 4 rats per cage in pathogen free under 12 light/dark inverted cycle, 40-60 % humidity and standard rat food and water. Animal experiments were carried out in harmony with the European Community Council Directive of 24 November 1986 (86/609/EEC) and permitted by the Norwegian Committee for Animal Research. *In vivo* electrophysiological recording have been detailed previously (He, Huang et al. 2008). The protocol is described below:

2.2.1.2 *In vivo* electrophysiological recording

Rats were anesthetized with intraperitoneal (IP) injection using urethane (1.5 g/kg) dissolved in sterile water. After 15 min of anesthesia all rats were tested with hind limb pinch and according to given extra amount of anesthesia. Rats were placed in a stereotaxic apparatus. Temperature of rats was monitored with rectal probe and kept 37^0C by a heating pad (HSE-Harvard Germany). The skull bregma point was decided to be the stereotaxic reference zero point. A local craniotomy was performed by making a slit on the scalp. Carefully bregma point had been pointed.

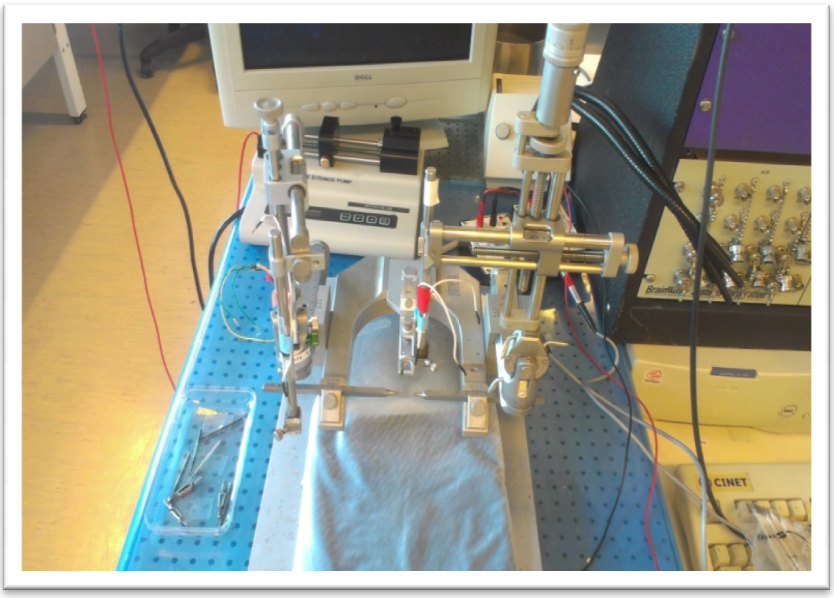


Figure 2.1: Stereotaxic Apparatus. It is mechanical assembly is a gadget that is utilized, alongside a cerebrum chart book of directions, to exactly embed a recording terminal into a particular brain locale. The device uses a set of three directions (x, y, and z) to check each one purpose of the cerebrum in three dimensional spaces**.**

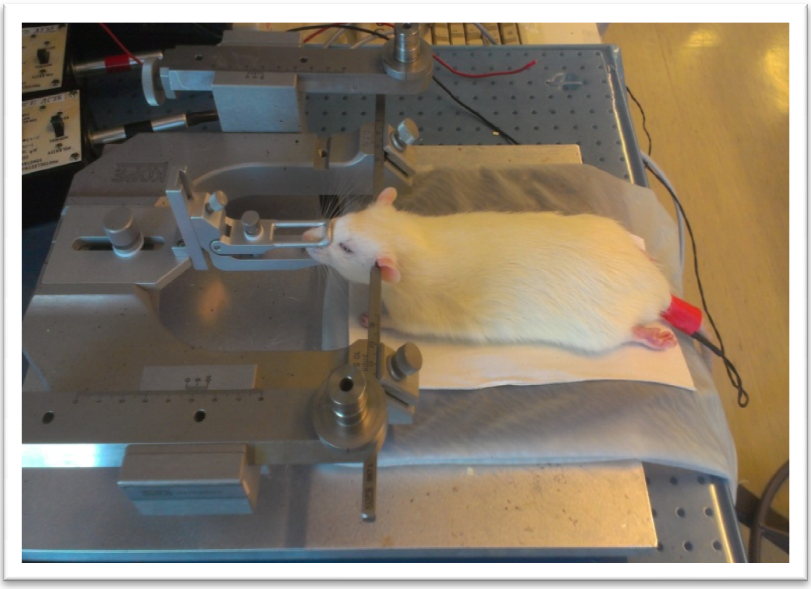


Figure 2.2: Anesthetized rat placed in a stereotaxic apparatus. The rat was put in a stereotaxic device (David Kopfs Instruments, USA). The instrument comprised of a tough "U" casing to which a corner clasp, that held the terminal arms, was joined at each one corner. The ear bars were set at the side of the head in the common jaw attachments of the skull front to the rat's ear canals and a nose-and-tooth bar upheld the upper jaw of the rat.

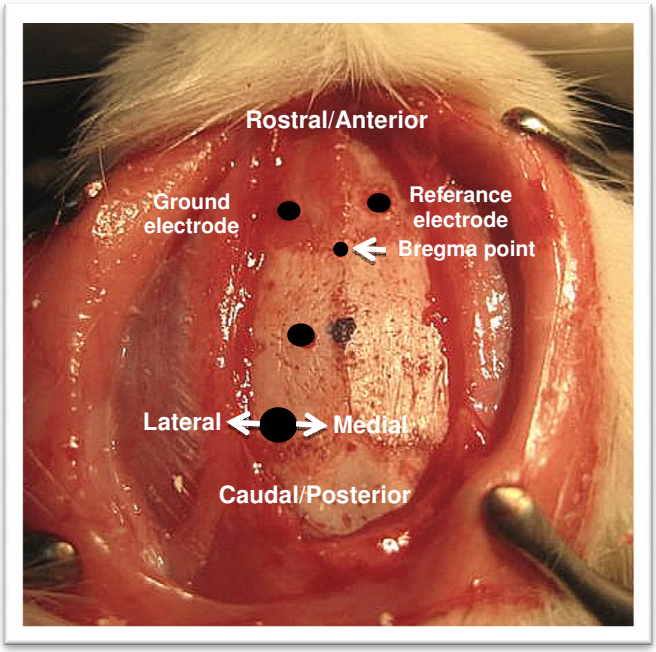


Figure 2.3: Local craniotomy of anesthetized rat. A surgical tool was utilized to make a 1.5 cm long intudinal on top of the creatures head from between the eyes and counter directionally.

Two holes bored with a width of 1-2 mm at the anterior side of the brain above the bregma point for ground/reference. Another two holes bored with a width of 1.5-2.0 mm on respective recording and stimulation positions such as one hole was made between 8.0 mm posterior to the bregma and 4.0 mm lateral to the midline for perforant path fibers stimulating. An alternate opening was made at 4.0 mm posterior to the bregma and 2.3 mm lateral to the midline for dentate region recording. The slit was made in dura while lowering the electrode and the surface was saturated with saline intermittently all around the test.

Bipolar stimulating electrodes (140 µm diameter with an inter electrode separation of 500 um from SNEX 100, Rhodes medical instrument, woodland hills CA) were put in the perforant path. A stainless steel needle monopolar tungsten recording electrode coated with Teflon was used to record the extracellular field potentials in the hippocampus.

The stereotaxic directions of stimulating was lowered in angular bundle from entorhinal cortex (Anterior to posterior: 7.9 mm, ML: 4.2 mm lateral to midline and 2.5-3.0 mm dorsal to ventral

from dura) while recording electrode (tungsten, 112 um in diameter) was lowered slowly for dentate gyrus (Anterior to posterior: 3.9 mm, 2.3 mm lateral to midline and 2.5–3.3 mm dorsal to ventral from dura). The coordinated were adjusted until large positive fields EPSPs (fEPSPs) with negative population spikes were evoked. The coordinate was advanced to maximize the field responses to a biphasic current pulse of 150 µs and 300-500 µA that was delivered by an isolated pulse stimulator (S11 stimulator, Grass technologies, USA). The recorded signals were amplified x1000 and filtered (1 Hz - 10 Hz bandpass) using a differential AC amplifier (Model 1700; A-M Systems) and digitized using with A/D converter. Acquisition and analysis of field potentials were accomplished using Data Wave Technologies (Longmont, CO).

Single-pulse test stimulation consisted of monophasic square wave pulse (150 us duration, 0.033Hz) at 30s-interval was delivered to perforant path by stimulator. By slightly varying the depth of the stimulating and recording electrode at the above-mentioned range below the cortical surface, the characteristic waveforms evoked by stimulation perforant path could be acquired.

2.2.1.3 Experimental protocol

Before start of recording in each experiment, the whole system was equilibrated until the field potentials keep in a steady condition for 20-40 min. The recorded traces shown in the figures were averages of 10 consecutive records. High-frequency stimulation (HFS) consisted of three sessions repeated in 5 min. Each session consist 4 trains each composed of 8 pulses at 400 Hz with 10s interval between each train.

The baseline fEPSP was measured for 20 min (at 0.033 Hz) applied every 30 second. After high-frequency stimulation, lasting 10.5 min, the fEPSP was measured for different time points (30, 60, 120 min post HFS) and the percentage of the ratio of absolute fEPSP to baseline value was used to represent the increased fEPSP level. It was defined as a successful induction of long-term potentiation if the amplitude of fEPSP change exceeded 20% (Messaoudi, Kanhema et al. 2007).

2.2.1.4 Data Analysis

The evoked fEPSP was measured by computing the steepness of the slope at five consecutive points (figure 2.5). The information records were changed over to ASCII arrangement and further investigated in Microsoft Office Excel 2003 (Microsoft Corporation, USA). Figure showing two points were EPSP was measured.

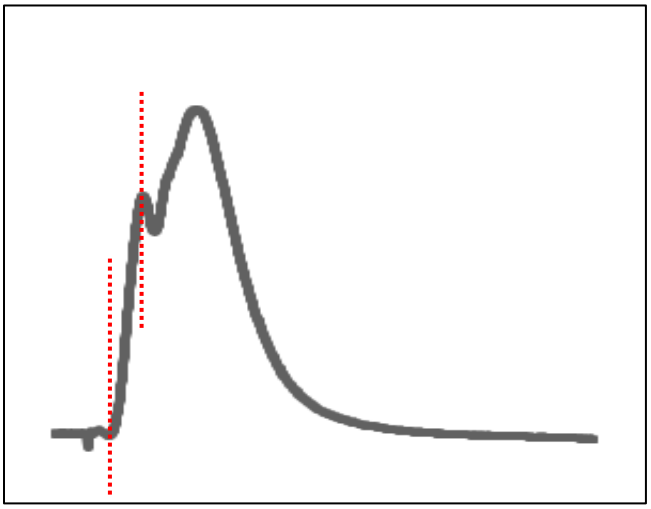


Figure 2.5: Field potential analysis. The evoked fEPSP was measured by computing the maximum slope along five consecutive points on the rising phase of the fEPSP.

2.2.1.5 Microdissection and collection of tissue

Microdissection of the dentate gyrus was completed within ~5 minutes to maintain a strategic distance from protein degradation. Temperature is another factor for protein degradation so the whole process was completed on ice. The cerebellum is uprooted from the cerebrum and the two cerebral sides of the equator are differentiated. The sagittal segment demonstrates the dorsal side down. Here we saw the right side of the equator surface up; this white part is corpus collosum. Then the remaining brainstem was pulled to get to the hippocampus which is arranged in the fleeting fold, it is "tilted" out. The dentate gyrus and CA regions were separated and stored in a micro tube with proper labelling immediately under -80^0C in a freezer for further analysis.

2.2.2 Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) was carried out to detect two proteins whether present in the same protein complex under physiological conditions. The Co-IP principle is as portrayed in the figure 2.6. Co-IP seeks to mimic the intracellular conditions for distinctive protein complexes in a cell solution. The protein-protein interactions are often transient and weak interaction between short amino acid sequences may occur (Alber, Dokudovskaya et al. 2007).

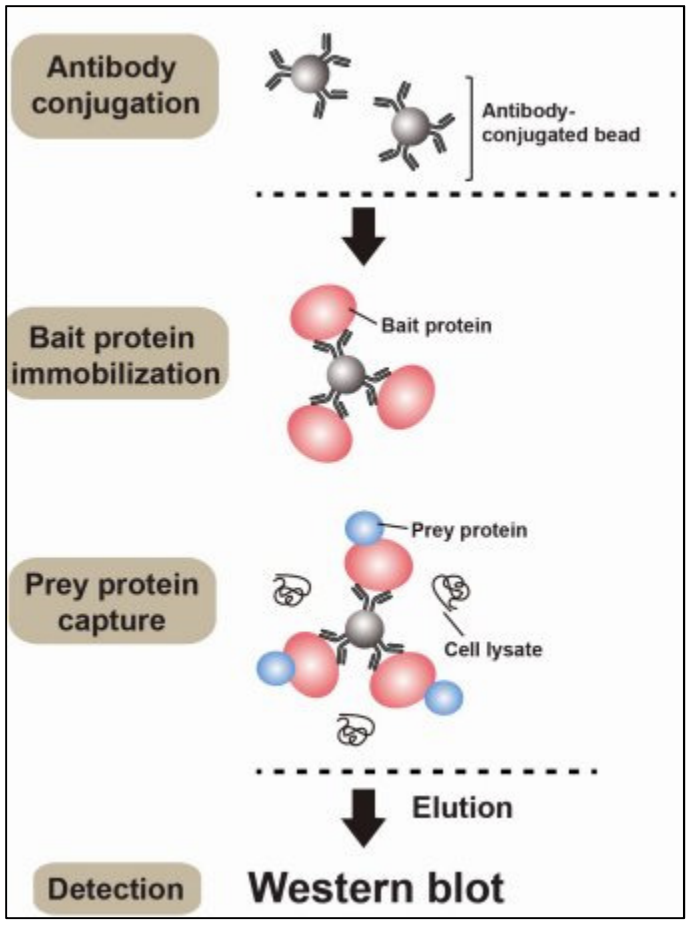
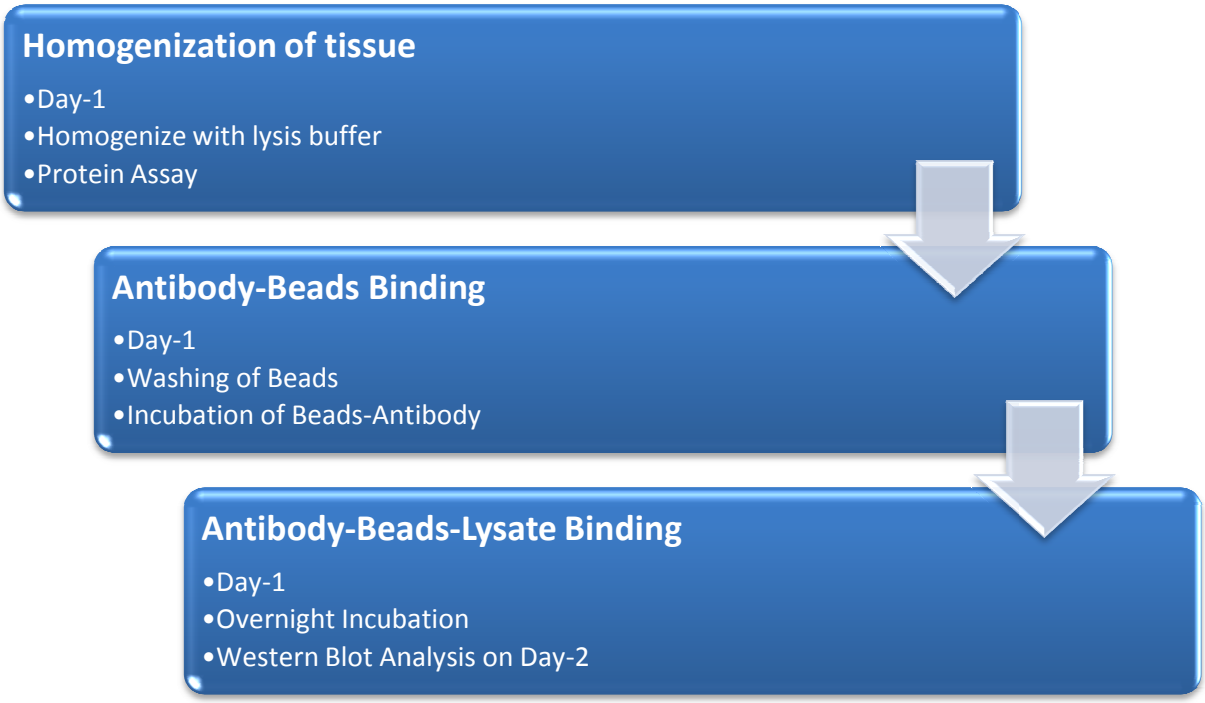


Figure 2.6: Co-Immunoprecipitation (Co-IP) assay. See below for the description of method.

Steps

Day-1

- Protein G-sepharose beads was washed 3 times with 1x PBS buffer at 3000 rpm, for 3 minute at 4^0C
- A final volume of 20 µL compact beads was taken in each 1.5 ml Eppendorf tube.
- Beads were incubated with 3 µg of specific antibody (primary) for 2 hours at 4^0C
- Tissue was homogenize in lysis buffer
- Homogenized solution was centrifuged at 4^0C, 14,000 rpm for 10 min
- Supernatant was collected in a properly labeled tube. The solution was stored in -20^0C
- BCA protein assay was then done to determine the protein concentration (Wiechelman, Braun et al. 1988).
- 250-500 µg of protein lysate incubated with antibody bound beads at 4^0C, 25 rpm for overnight**.**



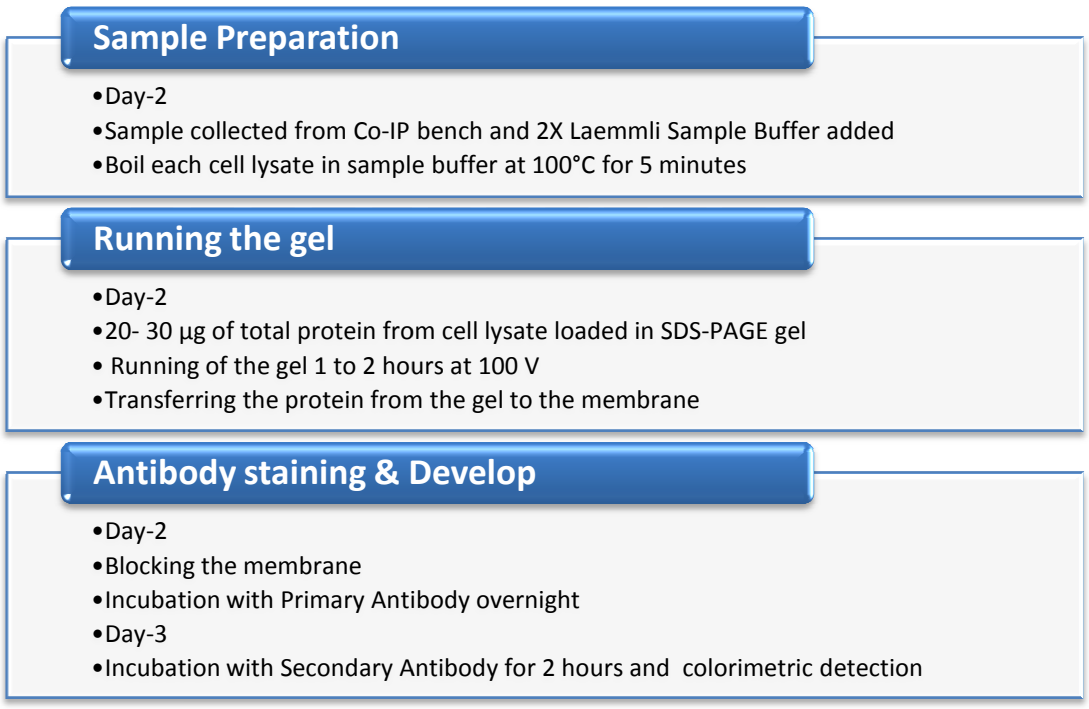
Flow Chart 2.1: Co-Immunoprecipitation work course

Day-2

- Overnight incubated samples were centrifuged at 3000 rpm for 3 min at 4^0C with washing buffer 1x PBS for 3 times.
- Laemili sample buffer with DDT was used 1:1 ratio to elute protein complexes.
- Equal amounts of protein were loaded onto SDS-PAGE gels (10%) and run 80 V for the first 20 min. the run was completed at constant 120 V later on.
- Separated proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Little Chalfont, UK) at a constant voltage of 100 V for 70 min.
- After transferring the gel in to a membrane, it was stained for 5 minutes on shaker in Ponceau reagent (0.25% Ponceau S in 40% methanol and 15% acetic acid).
- After ponceau staining the membrane was blocked with 5% non-fat milk (NFM), for 30-60 minutes at room temperature.
- Primary antibody was added after blocking for overnight at 4°C. Antibody concentration was selected according to working concentration.

Day-3

- After overnight incubation with the primary antibody the membrane was washed with TBST for 5 minutes 3 times.
- Secondary Antibody was added at a concentration of 1:5000.
- Proteins were visualized using enhanced chemiluminescence (ECL Western Blotting Analysis System; GE Healthcare)
- Blots were stripped with 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, at 60°C for 1 h and reprobed with another antibody detecting the protein of interest.
- Protein bands were quantified using ImageJ Software. Statistical analyses were based on unpaired or paired *t* tests as appropriate.



Flow Chart 2.2: Western Blot Work Flow

2.2.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most extensively used procedure to separate proteins from complex samples of mixture. Being available an electricity, proteins migrate towards the negative anode inside the poly-acrylamide gel under denaturing conditions. In SDS-PAGE, the detergent SDS and a heating step confirm that the electrophoretic portability of a solitary sort of protein is just separated by its molecular weight in the permeable acrylamide gel.

2.2.4 Western-blot

The capacity to exchange proteins from SDS-PAGE gels to nitrocellulose or Polyvinylidene fluoride, or polyvinylidene difluoride (PVDF) membrane has gotten standard in most labs. The western blot is a broadly utilized scientific system used to catch particular proteins in an example of tissue homogenate or extract. In principle, it uses gel electrophoresis to isolate local proteins by 3-D structure. It also separate by denatured proteins by the length of the polypeptide. The proteins are then exchanged to a membrane nitrocellulose or PVDF). At the end they are stained with antibodies particular to the target protein and chemiluminescent detection is performed to visualized the protein complexes as band (Towbin, Staehelin et al. 1979).

3. Results

3.1 Depth profile through the CA1 and down to dentate gyrus: Groundwork of the electrophysiological system

The recording electrode is positioned for optimal recording of evoked field potentials in the dentate gyrus by performing a depth profile. After the stimulating electrode is placed in the angular bundle (fiber tract of the perforant path), the recording electrode is incrementally lowered through the hippocampus and into the dentate gyrus. Charactertistic changes in the shape and polarity of the response during the depth profile confirm correct placement of the electrodes and are used to determine the final depth of the recording electrode in hilar region of the dentate gyrus.

In our study the recording electrode was lowered in the dentate gyrus to record the maximum medial perforant path evoked fEPSP in the hilar region. The stimulating electrode was positioned in the angular bundle 2.8 mm beneath dura matter, as illustrated in the methods. The stimulation electrode increases stimulated fibers from layer II neurons in enthorinal cortex. Test pulses at 300-500 μA were given at 30 second intervals and the recording began at 1.5 mm beneath dura matter.

Figure 3.1 shows the depth profile and outlines how the recording electrode was brought down to the structural surface of the neocortex through the CA1 pyramidal cell layer, stratum radiatum, stratum lacunosum-moleculare, and the consecutively the molecular layer, granule cell body layer, and hilar region of the dentate gyrus.

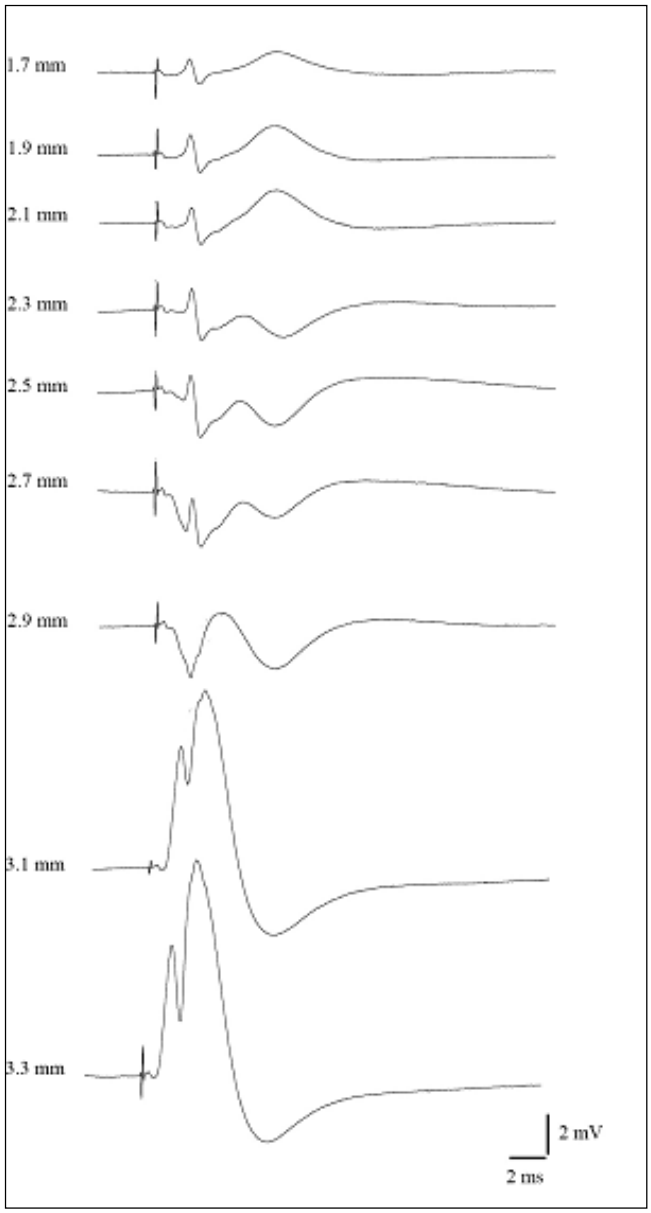


Figure 3.1: Depth profile of the medial perforant path-evoked field potential in urethane anesthetized rat. Depths are measured in mm from the dura matter on brain surface. Increasing negative reaction was observed when the recording electrode was in between 1.7 to 2.7 mm beneath dura matter. Maximum negativity was observed when the recording electrode was in 2.9 mm down to dentate gyrus but from that point the extremity turns around and gets positive as the terminal enters the cell body area. The last depth position at 3.3 mm exhibits a superimposed positive-going spike which is discovered when greatest fEPSP incline is attained.

3.2 High-Frequency Stimulation (HFS) induces LTP

LTP was induced by applying brief HFS to the medial perforant path. HFS consisted of eight pulses at 400 Hz repeated four times at 10 seconds intervals. Three sessions of HFS were given at intervals of 5 minutes at different time point (test group). Three time points such as 30 minutes, 60 minutes and 120 minutes has been chosen to record the fEPSP changes. Unless otherwise specified HFS had been applied into the left dentate gyrus (LDG), after which it collected for further biochemical analysis. Untreated tissue from the right dentate gyrus was collected as an internal control. In addition, another group of rats received baseline test stimuli, but not HFS.

In the figure 3.2 (A-F) percentages of fEPSP changes has been plotted into graph to see the change after applying HFS significant increases in fEPSP slope had been observed in post-HFS test group compared to baseline. The HFS induced approximately 40% increase in the fEPSP from baseline in the test group. No change in control group was observed in this study.

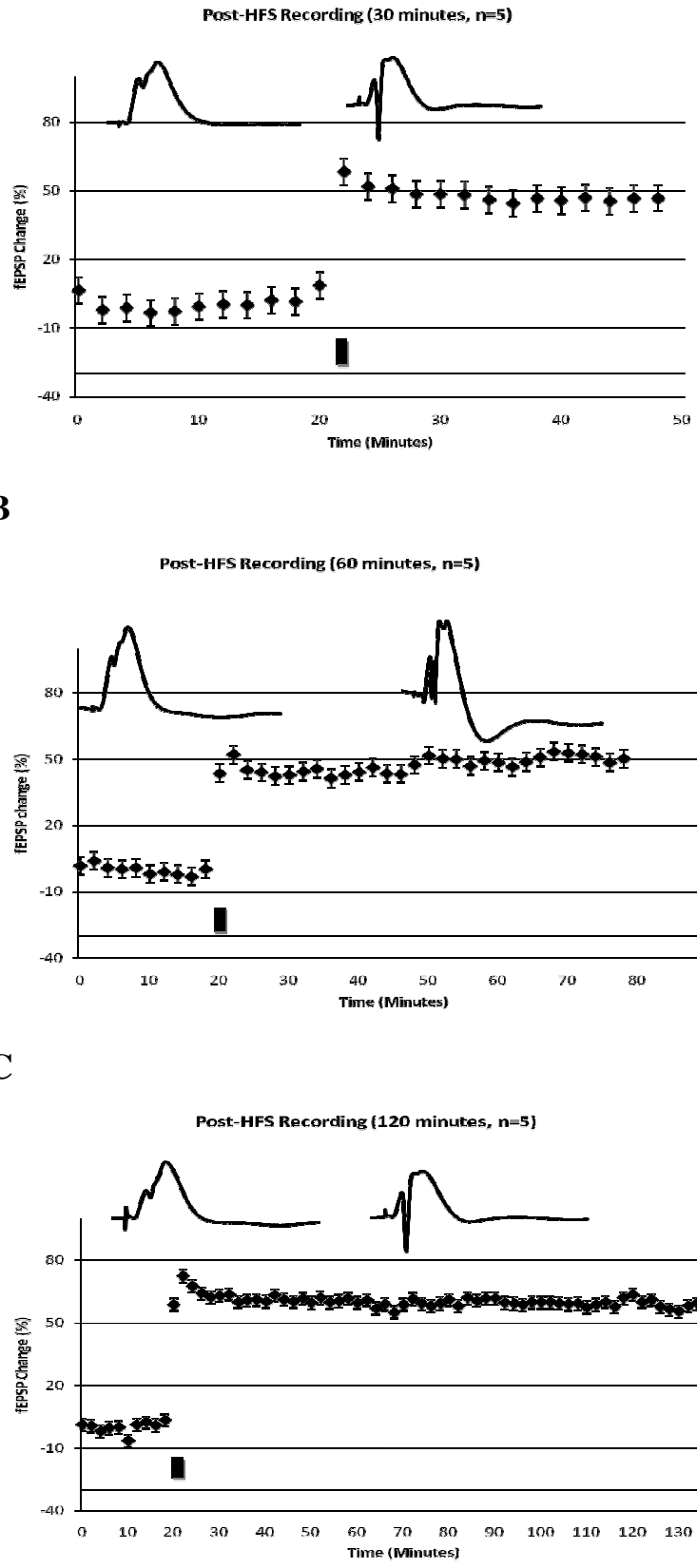


Figure 3.3: High-Frequency Stimulation (HFS) induces LTP. Time course plots show changes in the medial perforant path-evoked fEPSP slope showed in percentage of baseline. Values are means ± Standard Error Mean (SEM). HFS is indicated by the thick black box. Distinct increase of fEPSP at 30 minutes, 60 minutes and 120 minutes observed at 400 Hz that is represented in A,B and C (n=5). The peaks shows pattern of sweeps at the time points indicated in baseline recording and post-HFS recording plots.

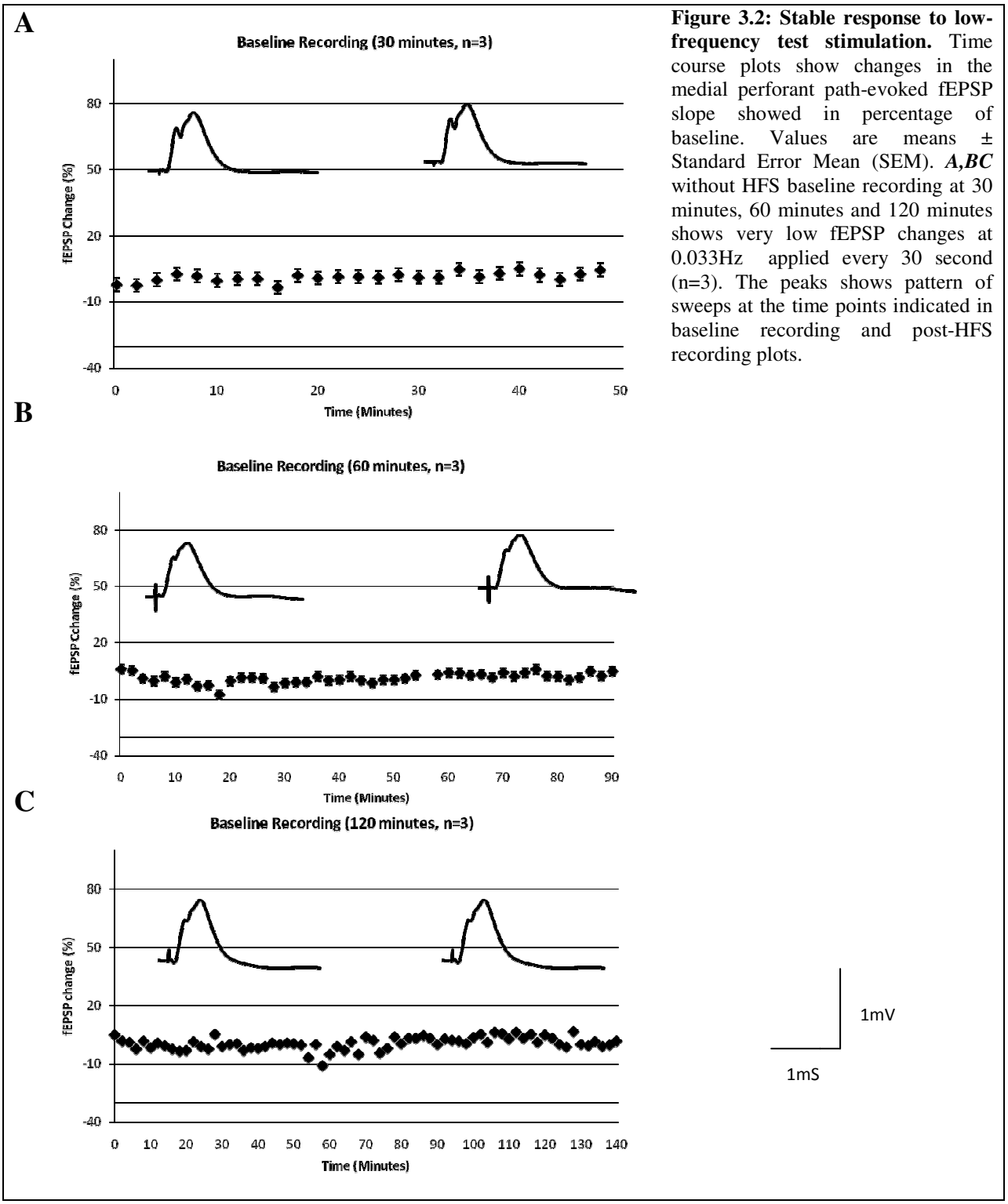
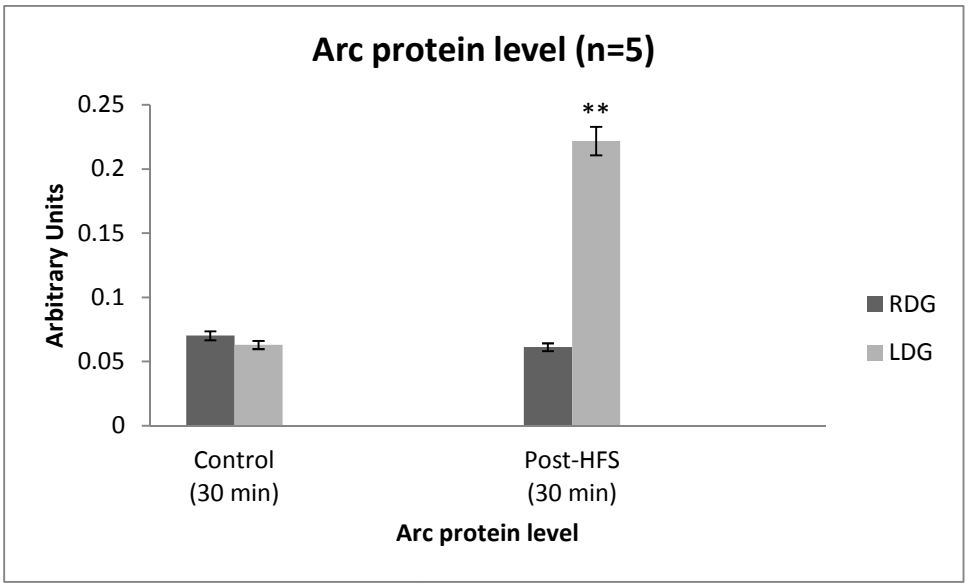


Figure 3.2: Stable response to low-frequency test stimulation. Time course plots show changes in the medial perforant path-evoked fEPSP slope showed in percentage of baseline. Values are means ± Standard Error Mean (SEM). ***A,BC*** without HFS baseline recording at 30 minutes, 60 minutes and 120 minutes shows very low fEPSP changes at 0.033Hz applied every 30 second (n=3). The peaks shows pattern of sweeps at the time points indicated in baseline recording and post-HFS recording plots.

.3.3 Arc protein expression after HFS

Differences were observed between HFS-treated dentate gyrus and the contralateral control dentate gyrus in terms of Arc protein expression (Figure 3.3-3.5).

A



B

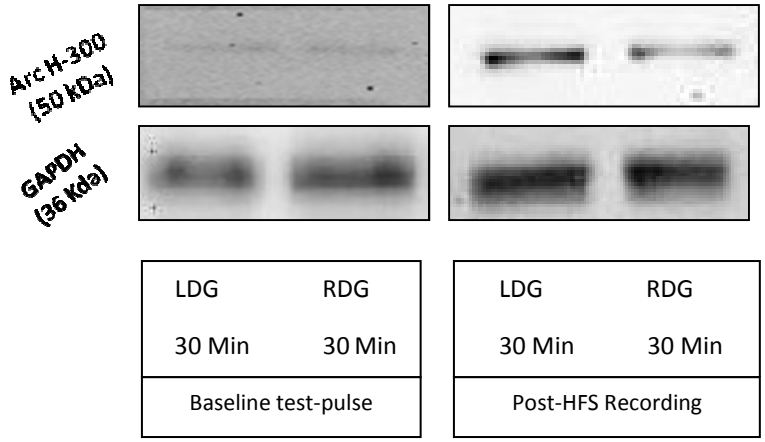
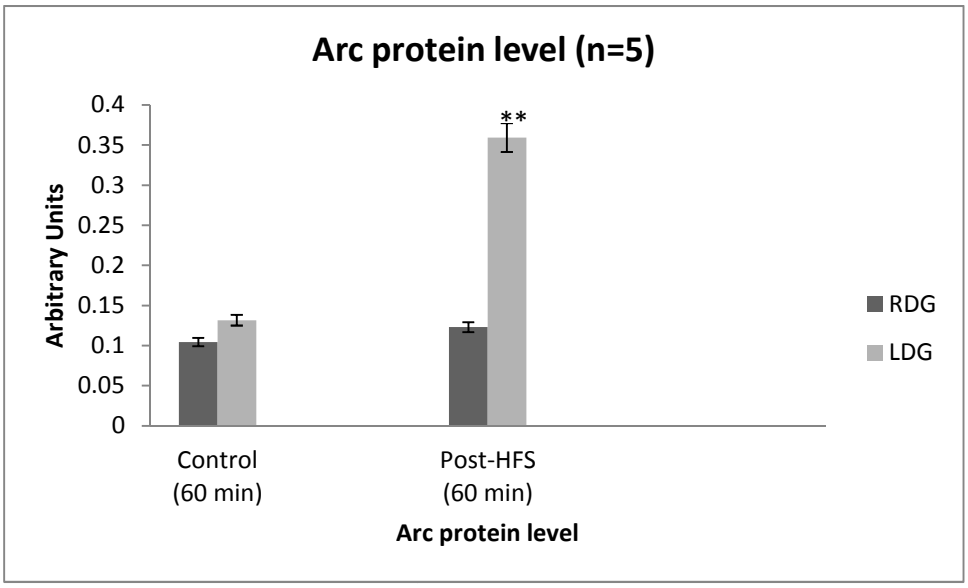


Figure 3.3: Arc protein level during baseline and Post-HFS recording for 30 min (n=5). Results of five independent experiments (error bar = SEM). ***A,*** total area of Arc protein level in HFS-treated dentate gyrus (LDG) and the contralateral control dentate gyrus (RDG) expressed where differences had been observed in terms of 30 min Post-HFS recording. Sample immunoblots for results in A (*-P<0.05, **-P<0.001,***-P<0.001). Normalized to GAPDH. ***B,*** high enrichment of Arc protein was observed in the HFS-treated dentate gyrus (LDG) compare to the contralateral control dentate gyrus (RDG) in 30 min Post-HFS recording. GAPDH was used a loading control.

A



B

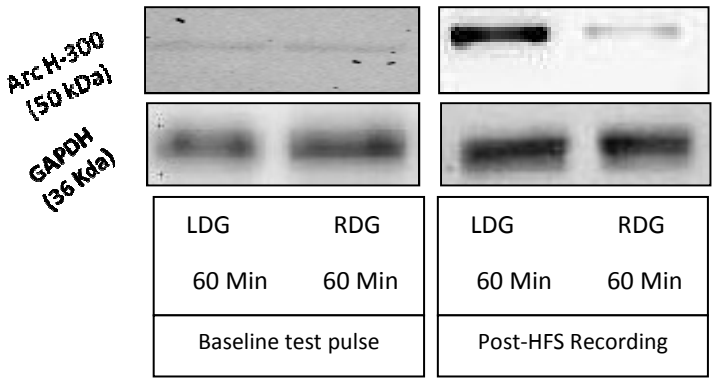
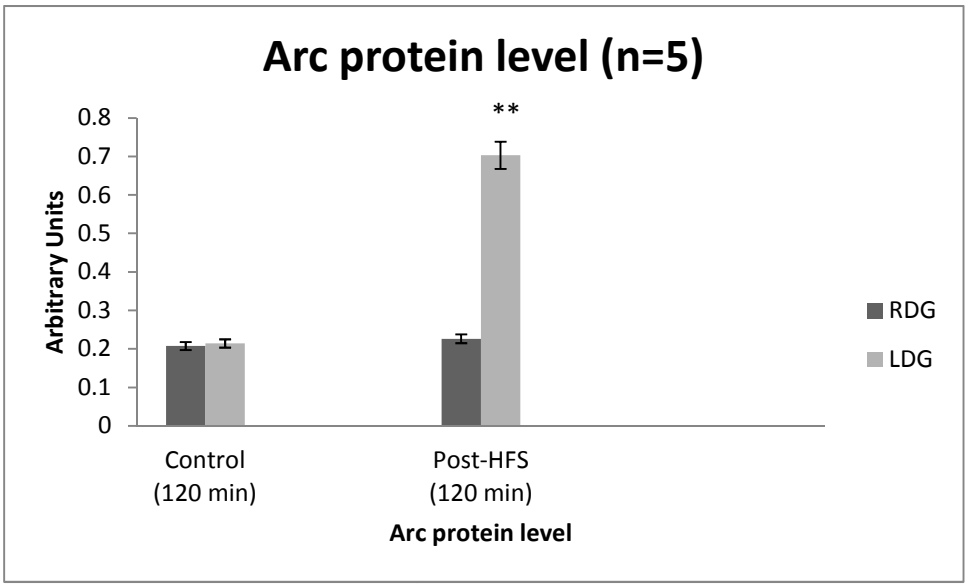


Figure 3.4: Arc protein level during baseline and Post-HFS recording for 60 min (n=5). The figure shows the results of five independent experiments (error bar = SEM). ***A,*** total area of Arc protein level in HFS-treated dentate gyrus (LDG) and the contralateral control dentate gyrus (RDG) expressed where differences had been observed in terms of 60 min Post-HFS recording. Sample immunoblots for results in A (*-P<0.05, **-P<0.001,***-P<0.001). Normalized to GAPDH. ***B,*** high enrichment of Arc protein was observed in the HFS-treated dentate gyrus (LDG) compare to contralateral control dentate gyrus (RDG) in both 60 min Post-HFS recording. GAPDH was used a loading control.

A



B

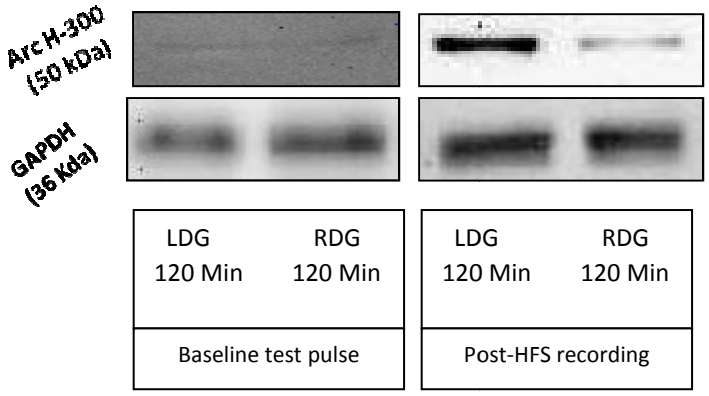


Figure 3.5: Arc protein level during baseline and Post HFS recording for 120 min (n=5). The figure shows the results of five independent experiments (error bar = SEM). ***A,*** total area of Arc protein level in HFS-treated dentate gyrus (LDG) and the contralateral control dentate gyrus (RDG) expressed where differences had been observed in HFS-treated dentate gyrus (LDG) and the contralateral control dentate gyrus (RDG) in terms of 120 min Post-HFS recording. Sample immunoblots for results in A (*-P<0.05, **-P<0.001,***-P<0.001). Normalized to GAPDH. ***B,*** high enrichment of Arc protein was observed in the HFS-treated dentate gyrus (LDG) compare to the contralateral control dentate gyrus (RDG) in 120 min Post-HFS recording. GAPDH was used a loading control.

3.4 Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation allows the isolation of native protein interaction complexes associated with a specific antigen (protein) in tissue samples. Here we sought to detect Arc binding in dentate gyrus tissue after LTP induction. As it was shown that Arc protein is maximally expressed at 120 min post-HFS (Messaoudi, Kanhema et al. 2007), this time point was used to verify our protocol.

Arc (c-7) mouse monoclonal antibody was incubated with Protein G-sepharose beads and after that this forms an immune complex with HFS treated sample when incubated overnight with the cell lysate. After performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the membrane was probed with Arc (H-300) rabbit polyclonal antibody. Enrichment of Arc was observed in the HFS-treated dentate gyrus compare to the contralateral, untreated dentate gyrus (Figure 3.6).

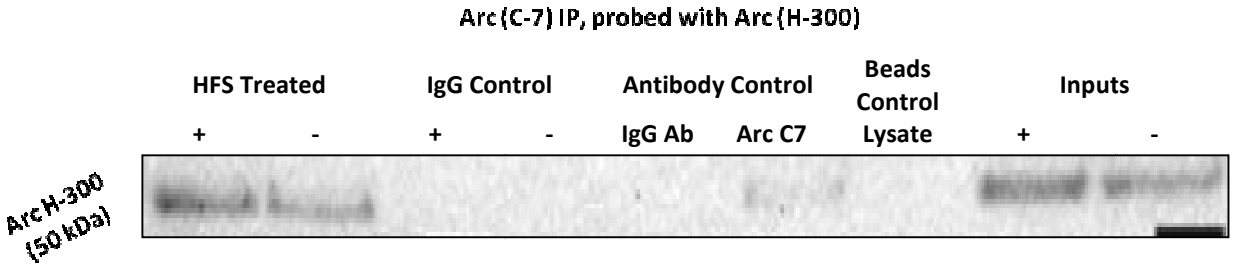


Figure 3.6: Arc Immunoprecipitation and detection. Arc C-7 IP showed detection of Arc protein. Here, minus sign (-) indicates untreated dentate gyrus while plus sign (+) indicates HFS treated dentate gyrus. After performing SDS-PAGE the membrane was probed with Arc (H-300) rabbit polyclonal antibody. Enrichment observed in the HFS-treated dentate gyrus compare to the contralateral control dentate gyrus. IgG antibody was used as a negative control. Lysate (Lys) was used as a beads control.

To test the binding interaction between Arc-Dynamin 2 and Arc-Stx4 we performed Arc protein IP and checked for binding partners, Dynamin 2 and Stx4. In both cases high enrichment of Arc protein was observed in the HFS-treated dentate gyrus compared with the untreated dentate gyrus.

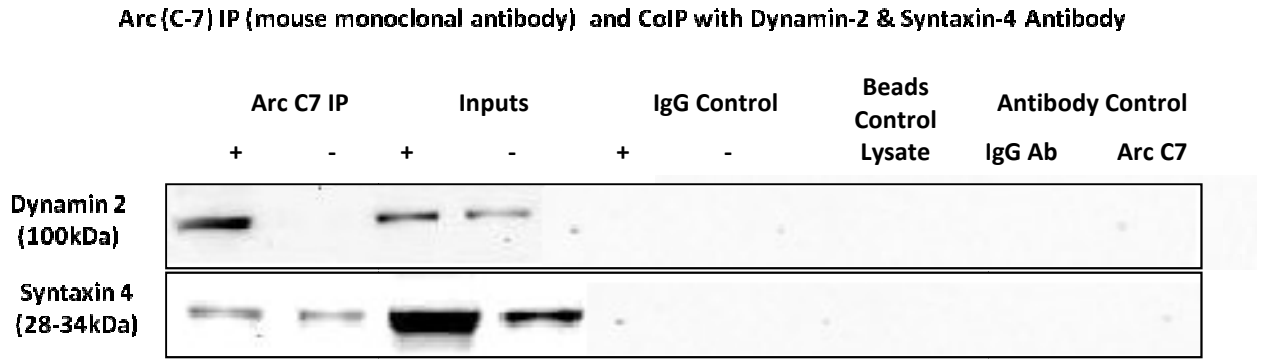


Figure 3.7: Dynamin 2 and Stx4 co-immunoprecipitated with Arc. Arc protein IPed with Arc (c-7) mouse monoclonal antibody and looked for Dynamin 2 and Stx4. Here, minus sign (-) indicates untreated dentate gyrus while plus sign (+) indicates HFS-treated dentate gyrus.

3.5 Optimizing lysis buffer salt (NaCl) concentration

Co-Immunoprecipitation (CoIP) is the most powerful method to study protein-protein interactions in vivo. It follows the basic principle of the specific antigen-antibody reaction. The strength of protein-protein interactions is commonly temporary and weak. It is hence vital to optimize the binding conditions regarding using of salts (NaCl) in lysis buffer solution. NaCl acts as buffering agent and prevents non specific protein aggregation.

In the initials experiments tissues were homogenized with three different NaCl concentrations i.e., 75 mM, 100 mM, and 150 mM into a lysis buffer. Arc (c-7) mouse monoclonal antibody was incubated for 2 hours with Protein G-sepharose beads and then incubated overnight with cell lysate obtained post-HFS. After performing SDS-PAGE the membrane was probed with Arc (H-300) rabbit polyclonal antibody. To confirm high enrichment in the HFS treated site i.e, in LDG

compared to untreated site RDG. However, no difference was seen between 100 mM NaCl and 150 mM NaCl containing lysis buffer while doing Arc C-7 IP. Low binding affinity of Arc protein was seen at 75 mM compared to 100 mM and 150 mM of NaCl concentration (Figure 3.8a). However, throughout Arc C-7 CoIP with Anti- Syntaxin 4 rabbit polyclonal antibody showed high enrichment of with Arc protein when 100 mM of NaCl was used (41.12%). This is in contrast to a concentration of 75 mM and 150 mM of NaCl containing lysis buffer which gave a representation of 21.74 % and 37.14 % respectively when used separately (Figure 3.8b). On the basis of above result we decided to use 100 mM salt concentration for further studies.

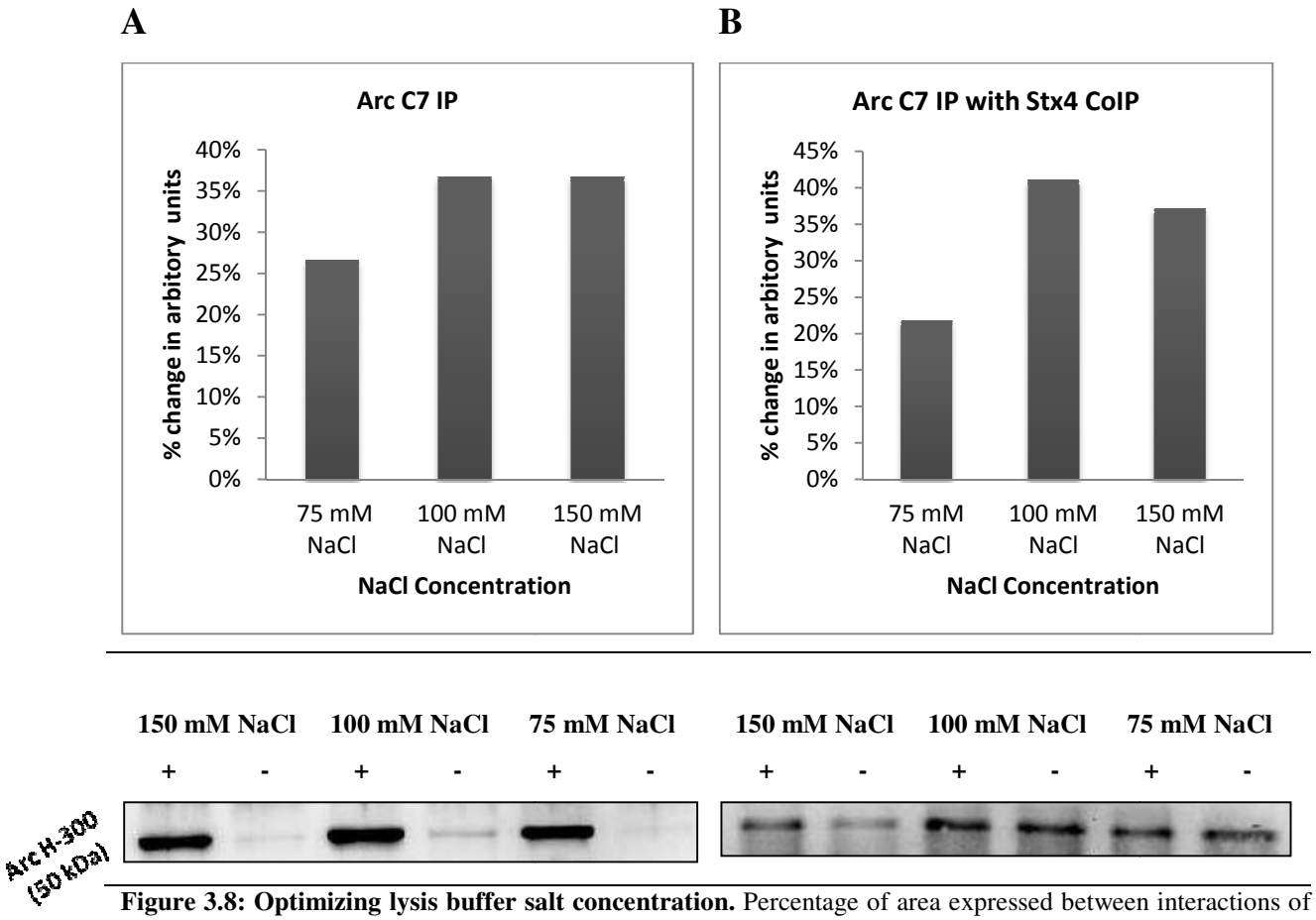


Figure 3.8: Optimizing lysis buffer salt concentration. Percentage of area expressed between interactions of two proteins using three different salt concentrations (75 mM, 100 mM, and 150mM) in a lysis buffer. Here, minus sign (-) indicates untreated DG while plus sign (+) indicates HFS treated DG. ***A,*** No differences had been observed between 100mM NaCl and 150 mM NaCl containing lysis buffer in Arc C-7 IP, probed with Arc (H-300) antibody. Very low expression (26.67 %) found when 75 mM of NaCl containing lysis buffer was used in the same experiment. ***B,*** However, during Arc C7 CoIP with Stx4 antibody showed increased interaction when 100 mM was used (41.12%) compare to 75 mM and 150 mM of NaCl containing lysis buffer.

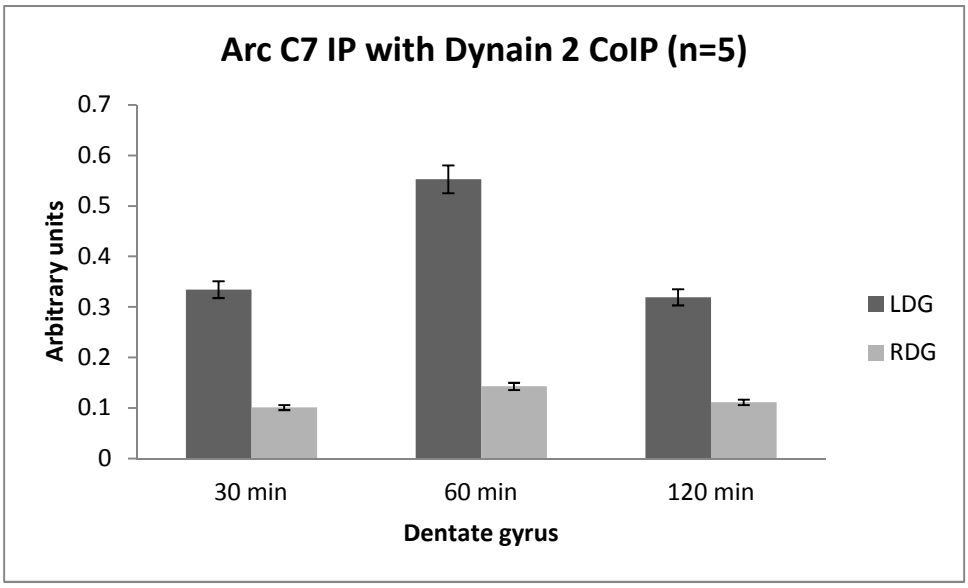
3.6 Arc-Dynamin Interaction may be highest during 60 minutes after HFS

It has as of recently been indicated in a study that Arc interfaces with Dynamin (Chowdhury, Shepherd et al. 2006). Here we have showed that Arc interact with Dynamin 2 in vivo. Dynamin protein is essential for clathrin-mediated synaptic vesicle recycling and receptor-mediated endocytosis.

When Arc protein IP was carried out to look time dependent interaction between Arc protein and Dynamin 2 where the highest interaction had been observed in 60 minutes after HFS recording and a high level of enrichment observed in the LDS of 60 minutes after HFS recording compared to 30 and 120 minutes.

Figure 3.9 shows the Arc-Dynamin protein interaction level in LDG and RDG expressed at different time points.

A



B

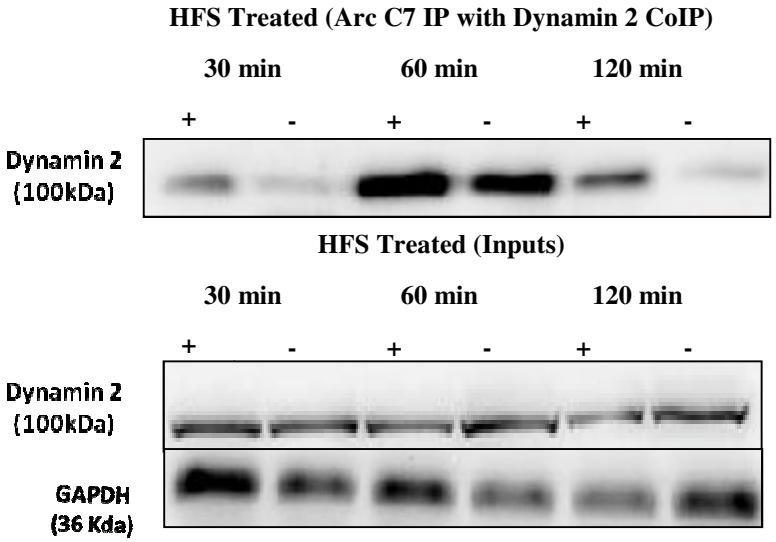


Figure 3.9: Arc-Dynamin protein interaction level during Post-HFS recording. The figure shows the results of five independent experiments (error bar = SEM). ***A,*** total arbitory units of Dynamin 2 protein interaction level in HFS-treated dentate gyrus (LDG) and the contralateral control dentate gyrus (RDG) expressed at different time point where highest interaction had been observed in 1 hr after HFS recording. Normalized to GAPDH. ***B***, high level of Dynamin 2 enrichment was observed in the HFS-treated dentate gyrus (+) of 1 hr after HFS recording compare to 30 and 120 min. Here, minus sign (-) means the contralateral control dentate gyrus while plus sign (+) means HFS-treated dentate gyrus.

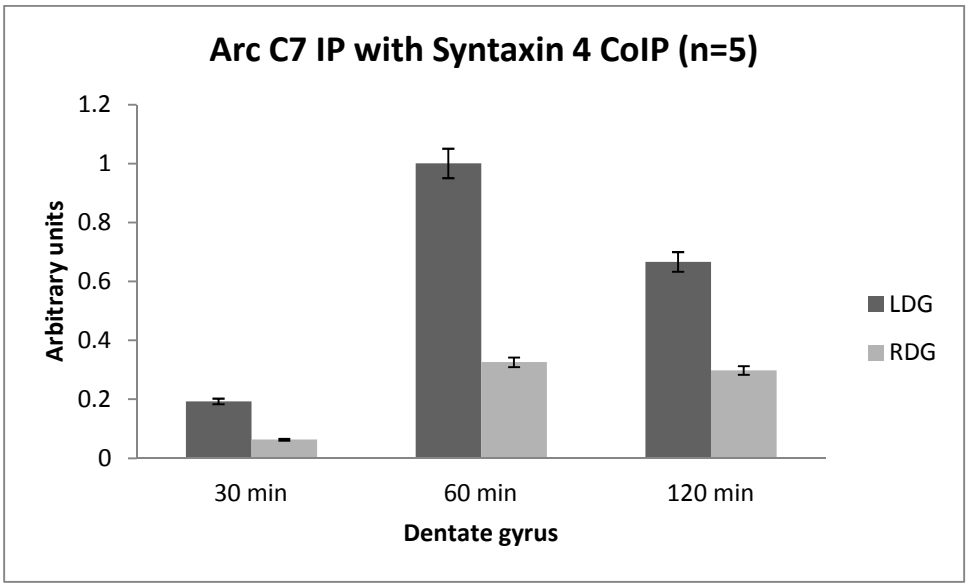
3.7 Stx4 might be a candidate binding partner for Arc protein during LTP

Whether Stx4 is a partner with Arc or not we performed Arc C7 IP with Stx4 co-IP. We found Stx4 enrichment in the HFS-treated site in the co-IP (Figure 3.7). This led us for further investigation of Arc-Stx4 interactions during LTP.

To determine Arc-Stx4 interaction during LTP, Arc protein IP was carried out to look for time-dependent interaction between Arc protein and Stx4 where the highest interaction had been observed in 60 minutes after HFS recording and a high level of Stx4 enrichment observed in the LDS of 60 minutes after HFS recording compared to 30 and 120 minutes.

Figure 3.10 shows the Arc-Stx4 protein interaction level in LDG and RDG expressed at different time points.

A



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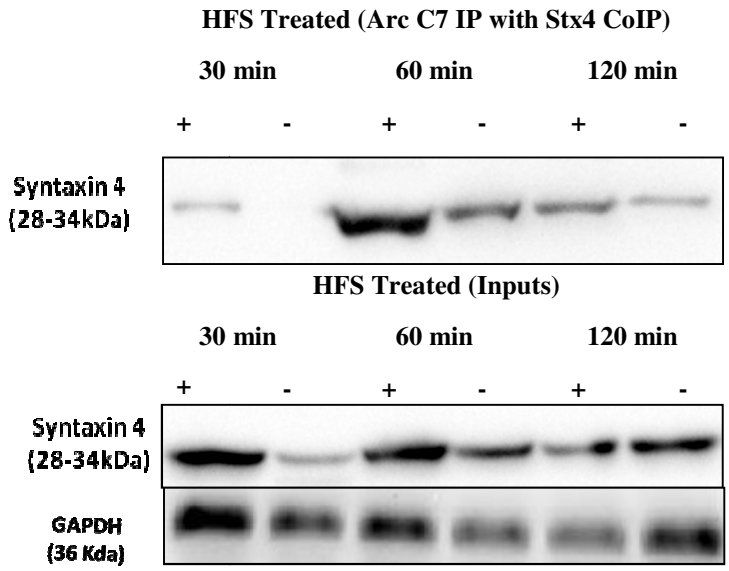

Figure 3.10: Arc-Stx4 protein interaction level during Post-HFS recording. Results of five independent experiments (error bar = SEM). ***A,*** total area of Arc-Syntaxin 4 protein interaction level in HFS treated and untreated expressed at different time point where highest interaction had been observed in 60 minutes after HFS recording. Normalized to GAPDH. ***B,*** high level of Stx4 enrichment was observed in the HFS treated site of 60 minutes after HFS recording compare to 30 and 120 minutes.

3.8 Stx4 CoIP showed interaction with Dynamin 2

From our above results we found that Stx4 is a candidate binding partner for Arc protein during LTP. To confirm our finding we performed reverse IP, Stx4 IP followed by immoblotting for Arc as well as Dynamin 2. Though we were unable to detect any enrichment of Arc in Stx4-Arc, we saw enrichment of Dynamin 2 in Stx4-Dynamin 2 CoIP (Figure 3.11).

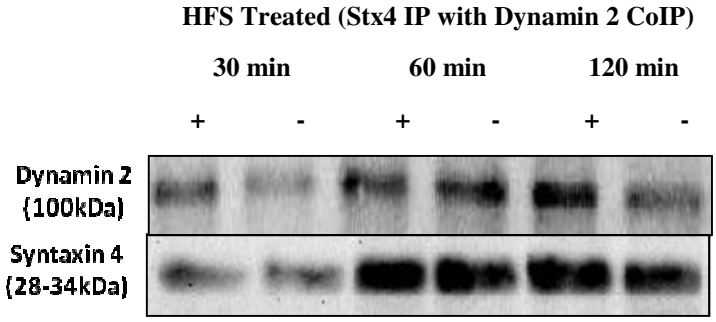

Figure 3.11: Stx4-Dynamin 2 protein interaction levels during Post-HFS recording at 30 min, 60 min and 120 min. Enrichment of Dynamin 2 was in the HFS-treated dentate gyrus.

4. Discussion

4.1 Methodological deliberations

Although in vivo electrophysiology and co-IP are all well established methods in the laboratory, there are sources of in all steps:

1. Experimental use of animals
2. *In vivo* recording
3. Microdissection
4. Homogenization of tissue and protein determination
5. Co-immunoprecipitation
6. Western blot

These issues are described below.

4.1.1 Experimental use of laboratory animals

The utilization of laboratory rats permits control of numerous variables to guarantee clear examinations that inevitably can generate measurably solid information. Body weight and brain size are important variables. The brain size of rats is important because of the stereotaxic coordinated framework created for exact electrode positioning.

In this study it is very important that the rats used for electrophysiology tests were within a similar weight and age range, as brain size increases with body weight. In our study body rats weighed 250–350 g and were of 2-4 months old. Sex was another important variable for this study. Male rats were picked as they don't have an oestrus cycle that is acknowledged to influence numerous physiological parameters in the hippocampus. All animal were housed and maintained in compliance with Norwegian animal regulations.

Use of a proper anesthetic agent in order to maintain a stable level of anesthesia was important as the evoked response can change can be affected. For decades urethane is the standard anesthetic used for in vivo recordings in the hippocampus and found have no effect on evoked excitatory responses or LTP (Pearce, Stringer, & Lothman, 1989). In this current study a specific unique dose of urethane (1.5mg/kg) was used to anesthetize animals.

4.1.2 *In vivo* recording and evading tissue injury

Electrophysiology profoundly relies on an electrode with reproducible electrical properties. Resistance of the recording and stimulation electrode was checked before each experiment. After every test the electrodes were ready cleaned to be used again. This was carried out to evade coagulated blood leftovers that could compromise the stimulation and recording.

At the point when stimulating the medial perforant path of the hippocampus, the placement of the recording electrode in the dentate gyrus must be precise. The dendrites from the granule cells structure a dipole upon stimulation and the ionic flows between these shafts are recorded. The depth profile suggests to these characteristics changes as the recording electrode is deliberately brought down through the brain and into the dentate gyrus. The depth profile is performed with an exact and precise methodology; enrollments from all tests ought to have the same basis. Otherwise the response won't be comparable to the signal needed for consistent data set.

The positioning of the recording and stimulating electrodes causes tissue disturbance and injury. To minimize tissue damage and pressure build-up the recording electrode (112 µm) is gently lowered 200 µm every 30 seconds.

The methods by which the situating of the electrodes and the changes of stimulus intensities set to one third of greatest population spike were standardized between rats. Then again, the threshold for stimulation needed to evade a field potential varies between rats. The perforant path evoked responses were standardized by a depth profile identification of a placement that gives the largest fEPSP slope response. This is also standardized by the stimulus intensities set to one third of the most intense population spike.

4.1.3 Microdissection

When the recording was completed all rats were killed followed by brain removal and hippocampal dissections on an ice-cooled stage. Hippocampal sections were cut using a tissue chopper and transferred to a petri dish containing ice-cold PBS for DG microdissection. Microdissections was completed rapidly to avoid tissue degradation therefore all microdissections were done and DG tissue immediately stored in dry-ice within less than 5 min.

4.1.4 Homogenization of tissue and protein determination

Lysis buffer was used to homogenize the dentate gyrus after microdissection. The composition of the lysis buffer used for tissue homogenization is important. Hepes-HCl was used as a buffering agent to prevent protein denaturation. NaCl used as a buffering agent to prevents non-specific protein aggregation. A mild non-ionic detergent was used to extract protein. EDTA was used which is sequester free Ca^{2+} (ca-chelator) that breaks the cell membrane as Ca^{2+} functions as a stabilizer. The lysis buffer also contains DTT which is a reducing agent of S-S bonds.

A protease inhibitor mixed cocktail was added to avoid protein degradation. In this way, throughout homogenization, the non-homogenized specimens were kept in the cooler as far as might be feasible. Absolute protein concentration in lysate was determined using the BCA protein assay. Accordingly, aliquots were made to avoid degradation of protein because of sample freezing and thawing. Protein determination was done as fast as possible under the circumstances after homogenization to avoid protein degradation.

4.1.5 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) is a well known method for distinguishing physiologically pertinent protein-protein interactions. Specific antibodies (Polyclonal, monoclonal) beads were used and sufficient incubation time of the specific protein and the antibody-binding is vital part of experiment. The ideal incubation time for our experiments, giving the best yield of protein-protein interaction distinguished by Western blotting analysis, was overnight. Co-IP is a procedure that allows isolation of whole protein complexes, and not only a solitary protein. Protein-protein interactions are reserved, and the protein composition of the complex might be described. Antibody-bound beads with no sample were used as an antibody control while bead with lysate was used as a bead control as a part of the co-IP. This antibody control will allow the detection of false positive bands of both heavy and light chains of IgG. Consequently, two groups were available on the membrane, relating to the heavy (50 kda) and light (25 kda) chains of the precipitated primary antibody. Moreover, the bead control allows detection of false positives bands due to proteins in the lysates binding non-specifically to the beads. These control experiments are essential in evaluating the specificity of a coIP experiment. For Arc coIP with Dyn2 and Stx4, no non-specific bands were detected following optimization of the procedures.

4.1.6 Western-blot

Proteins were separated by SDS-PAGE in a ten percent polyacrylamide gel in light of the fact that the molecular masses of the potential binding partners of Arc protein were in the reach of ~20-150 kda. Higher percentage of gel was used as the molecular weights of Arc binding partners are small. The SDS-PAGE was run for approximately 2 hours until the 20 kda proteins approached the base of the gel, for maximal partition of the proteins.

Regardless of the fact that the fold change in LTP-affected specimens contrasted with controls is ascertained, the contrast in densitometric qualities from blot to blot can result in little varieties in

the results. Equivalent measures of lysate protein were stacked in each single well. GAPDH is used as a loading control to determine whether samples have been loaded equally across all wells or not. It also helps to see the effective transfer of protein during western blotting.

Western blotting is a semi-quantitative technique satisfactory for looking at the measure of protein between samples, yet does not give an outright estimate of protein amount. A sufficient amount of protein lysate was used for Co-IP, to increase the likelihood to capture Arc binding partners.

The choice of using antibodies for Western blotting is critical for an improved discovery of the proteins of interest. Monoclonal antibodies are frequently favored because they can detect only one epitope on the antigen. Because of their specificity they are very good as the primary antibody in an assay. They give very less background signal as they detecting one target epitope On the other hand polyclonals can identify multiple epitopes on the antigen. But it produces large amount of non-specific antibodies which can give background signal. In our study we used monoclonal antibody to bring specificity in results. Secondary antibodies are the fundamental driver of background signal, yet careful washing of the membranes after incubating with antibodies diminishes background signal. Moreover

4.2 Results

4.2.1 Large increase in Arc protein expression after HFS

A study by Messaoudi and colleagues exhibits a dynamic, stringent prerequisite for activity-induced Arc synthesis in LTP upkeep in the dentate gyrus in vivo. They uncovered that Arc synthesis was vital for LTP support. They inferred that early synthesis of Arc may characterize for LTP expression, while sustained Arc synthesis is needed for LTP maintenance (Messaoudi et al., 2007).

Current data suggest that from 30 min to 2 hour post HFS, Arc protein expression at maximum plateau. Moreover our results comply with long term presence of Arc in the dentate gyrus. Moreover it has been shown that Arc mRNA/protein is degraded rapidly (Bramham et al., 2010; Greer et al., 2010), it means Arc mRNA may be translated only once. Prolong availability of Arc in dentate gyrus recommend us to stress on, with whom Arc protein is engaged with different protein at different time point and does it show the perspective on mechanism involved?

4.2.2 Arc-Dynamin interaction in dentate gyrus LTP

Dynamin is belongs to a family of large GTPase and is essential for intracellular membrane trafficking and actin cytoskeleton dynamics. This enzymatic activity is strikingly increased through binding with microtubules, acidic phospholipids and certain regulatory proteins that contain Src homology 3 (SH3) domains (Urrutia, Henley, Cook, & McNiven, 1997). The Dynamin is included in squeezing off and fissioning of membrane, which discharges endosomes from the plasma membrane surface, although its enzymatic activity on lipids, dynamin likewise enlists numerous different proteins to control endosome arrangement (Chowdhury et al., 2006). Mutations in Dynamin , *Shibire*, which causes paralysis due to a severe defect in synaptic vesicle recycling at the neurotransmitter junction (Chen et al., 1991).

Postsynaptic endocytosis of receptors is believed to be mediated by similar kinds of proteins, even though possibly through specific protein isoforms (Chowdhury et al., 2006). It has been show that Dynamin 2 is post synaptic and is localized to the PSD through interaction with its post-synaptic scaffolding protein, Shank (Okamoto, Gamby, Wells, Fallon, & Vallee, 2001). During receptor endocytosis Arc protein recruits Dynamin which is anchored to the PSD through its interaction with Shank (Okamoto et al., 2001). Arc-Dynamin interaction is known to be required for endocytosis of AMPA-type glutamate receptors in LTD. Interestingly; the present study suggests Arc interaction with Dynamin 2 in the context of dentate gyrus LTP. From the present study it has been found that the Arc protein interacts with Dynamin 2 during LTP. Their interaction is found higher during 60 min of post-HFS compare to 30 min and 120 min post-HFS recording. This all evidence shows from the literature survey and our results shows that dynamin and arc interaction also play a vital role is LTP and However further studies are required to better understand the proper mechanisms of Arc-Dynamin 2 interaction during LTP. This all evidence showed

4.2.3 Identification of Stx4 as a candidate binding partner for Arc in LTP

An earlier study from Kennedy et al. (2010) showed that Stx4, a plasma layer soluble NSF-attachment protein receptor (SNARE) protein in activity-dependent exocytosis in hippocampal neurons. Stx4 is limited to the postsynaptic layer and it characterizes an exocytic space in activity-dependent exocytosis of AMPA receptor.

Stx4 is localized in the postsynaptic membrane and it defines an exocytic domain in activity-dependent exocytosis of AMPA receptor (Kennedy, Davison, Robinson, & Ehlers, 2010). From our findings we believe that Arc-Stx4 may be have role in recycling of endosome to active membrane or introducing new protein. However while doing the reverse CoIP with Stx4 with Arc didn't show any enrichment may indicate the interaction between Arc- Stx4 is indirect over time. Moreover, binding of Stx4 with Arc has shown increase at 60 min post-HFS as dynamin,

which is an indicator of possible unknown mechanism. However we proposed a model for Arc-Dynamin2-Stx4 interactions during LTP in figure 4.1.

Stx4 IP showed Dynamin 2 as a binding partner of Stx4, which lead us to think on putative possible direct or indirect role of arc protein in exocytosis. Although number on mechanism has been described on role of Arc protein endocytosis whereas limited data available on role of Arc in exocytosis. We need to design further possible experiments on these proteins to define possible mechanism.

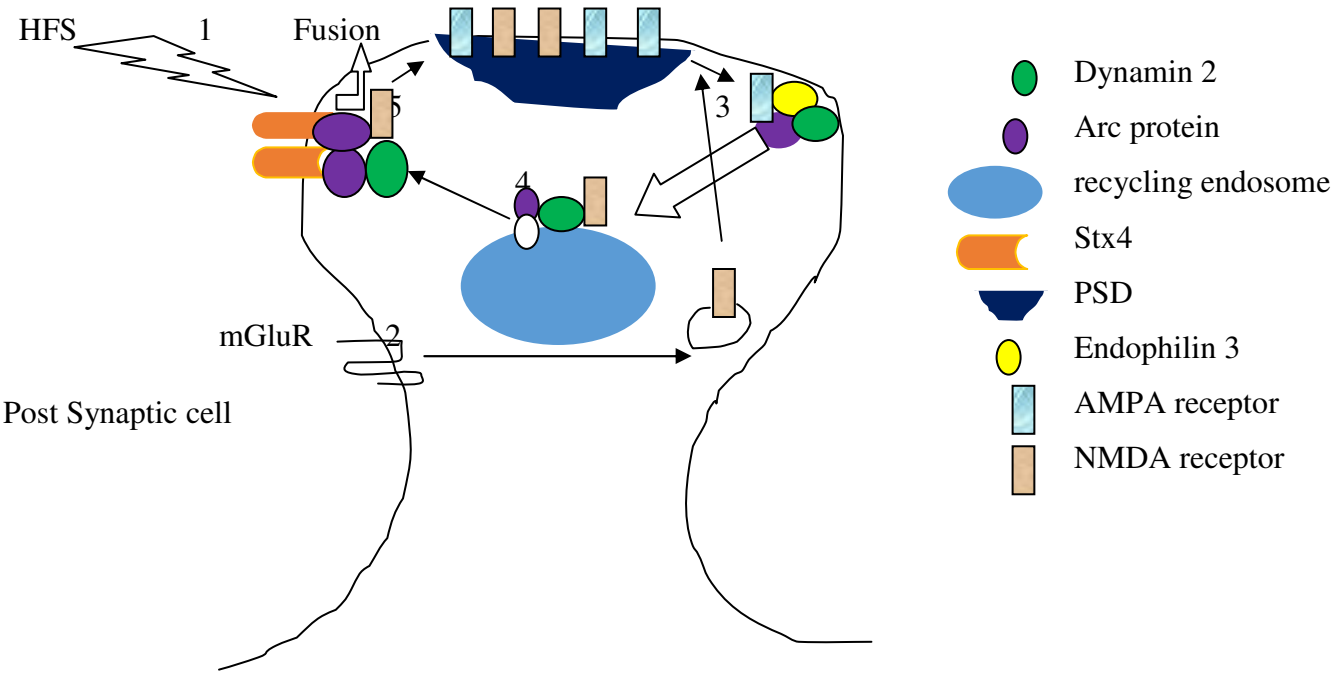

Figure 4.1: Proposed model for Arc-Dynamin2-Stx4 interactions during LTP. In the dentate gyrus due to HFS activation mGluR induced LTP thus increases NMDA fEPSP. This result in over expression of Arc protein near PSD. Arc protein recruits Dynamin 2 and Endophilin 3 for AMPA receptor endocytosis (3). We found that arc-Dynamin 2 and Arc-Stx4 interaction is highest in 60 min of post-HFS and findings from Stx4-Dynamin 2 co-IP this is evident that during LTP Arc protein interacts with Dynamin 2 to recycling endosomes where they bind with Stx4 that mediates exocytosis in dendritic spines. At the same time the receptor is internalized (endocytosed) and then recycled to be exocytosed (inserted) in the membrane to induce LTP.

4.3 Conclusions

Our preliminary results from this study support the following conclusions

1. Arc is rapidly synthesized after HFS.
2. Dynamin 2 and Stx4 are binding partners of Arc during LTP.
3. Stx4 and Dynamin 2 are present in the sample protein complex.

4.3.1 Future perspectives

These are preliminary data in many respects. You should outline what experiments are needed to consolidate and confirm the conclusions.

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Many possible questions arise from the present studies where further investigations are necessary. Such as,

1. How Arc protein regulating exocytosis in co-operation with Stx4?

2. CoIP of Dynamin 2 with Stx4 lead us to think on, does Arc-Dynamin binding require Stx4?

3. Why 60 min post HFS showed increased binding of Dynamin and Stx4 to the Arc protein?

We believe that Arc- Stx4 may be having role in recycling of endosome to active membrane during LTP while to define possible mechanism can be the future perspectives.

5. Appendix

A. User Manuals

1. Pierce® BCA Protein Assay Kit
   https://www.piercenet.com/instructions/2161296.pdf
2. Pierce® ECL Western Blotting Substrate
   https://www.piercenet.com/instructions/2161743.pdf

B. Polyacrylamide gel recipes

Solution components	Resolving gel (10 %) for 10mL preparation	Stacking gel (5%) for 4 mL preparation
	mL	mL
dH_2O	4.0	2.7
30 % acrylamide mix	3.3	0.67
1.5 M Tris base (pH 8.8)	2.5	0.5
10 % SDS	0.1	0.04
10 % ammonium persulfate	0.15	0.04
TEMED	0.004	0.004

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