"The highest education is that which does not merely give us information but makes our life in harmony with all existence"

Rabindranath Tagore

University of Alberta

NOREPINEPHRINE-MEDIATED SYNAPTIC PLASTICITY: A NOVEL SIGNALING DIALOGUE BETWEEN RECEPTORS AND NUCLEUS

by

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Abstract

Norepinephrine (NE) helps in the consolidation and retention of memories of emotionally charged events. However, the pathway from membrane receptors to nucleus in noradrenergic signaling in the brain is not completely understood. The cyclic AMP-PKA pathway is one of the most studied signaling pathways recruited by norepinephrine to induce and maintain LTP, a cellular correlate of long term memory. In the present study, I describe a novel signaling mechanism of NE-mediated induction and expression of LTP when paired with a specific stimulus protocol, through cAMP receptors (Epac) instead of PKA. I demonstrate that this pathway involves transcriptional and epigenetic mechanisms other than local protein synthesis. Hence, this unique pathway may be recruited upon novel experience to form a stable memory. Considering that many memory-related cognitive impairments are due to altered pathophysiology of the noradrenergic system, these results both increase our understanding and move us closer to a possible solution for neurological diseases involving neuromodulators like NE.

Highlights

- Norepinephrine engages Epac to facilitate LTP
- Translation and transcription are recruited by norepinephrine
- Epigenetic mechanisms are recruited by noradrenergic stimulation
- Epigenetic regulation by NE offers a novel mechanism for maintaining LTP

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List of Abbreviations

4E-BP	4E-binding protein
α-AR	alpha-adrenergic receptor
AC	adenylyl cyclase
ACSF	artificial cerebrospinal fluid
AMPAR	L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance between groups
β-AR	beta-adrenergic receptor
bdnf	brain-derived neurotrophic factor
Ca^{2+}	calcium
CA	cornu ammonis
cAMP	adenosine 3', 5'-cyclic monophosphate
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CBP	CREB binding protein
CCAC	Canadian Council on Animal Care guidelines
CREB	cyclic AMP response element binding protein
DG	dentate gyrus
DMSO	dimethylsulfoxide
eIF4E	eukaryotic initiation factor 4E
EME	emetine
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase

fEPSP	field excitatory postsynaptic potential
G-protein	guanine nucleotide-binding regulatory protein
GluR	glutamate receptor
HAT	histone acetyl transferase
HDAC	histone deacetyl transferase
HFS	high-frequency stimulation
IEG	immediate early gene
LFS	low-frequency stimulation
LTP	long-term potentiation
LTD	long-term depression
LTM	long-term memory
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
Mg^{2+}	magnesium
mGluR	metabotropic receptor
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MSDS	Material Safety Data Sheets
MTL	medial temporal lobe
MWM	Morris water maze
NMDAR	N-methyl-D-aspartic acid receptor
PP1	protein phosphatase 1
PKA	cAMP-dependent protein kinase

PKC	protein kinase C
PSD	postsynaptic density
RAP	rapamycin
RNA	ribonucleic acid
S	stimulating electrode
SEM	standard error of the mean
STM	short-term memory
WT	wild type

Chapter 1: Introduction

1.1 Learning and memory: A historical perspective

The human brain, over decades of evolution, acquires properties which make us respond in accordance to our environment. The brain has specialized structures and components within it to perform higher cognitive functions. Learning and memory is one such higher nervous function where we learn about specific tasks, places, faces, etc., and store this information within the memory storage system of brain for future use.

Today's knowledge about synaptic plasticity and memory is rooted in psychological studies of the late 18th and 19th centuries. The German psychologist Hermann Ebbinghaus (1850-1909), performing many experiments on himself, such as memorizing lists of nonsense syllables and testing, revealed some of the basic properties of memory in terms of duration and influence of repetition on retention of that memory. Sergei Korsakoff (1887) published a classic paper on alcoholism and established a memory disorder as a measure to study mnemonic processes. William James in 1890 wrote *Principles of Psychology*, which introduced the concept of short- (primary) and long- (secondary) term memory with their distinctive features. Edward Thorndike then in 1898 published his work using animals to study memory, and introduced the concept of operant conditioning.

The 19th century brought the idea of behavioural neuroscience to the study of learning and memory. In 1904, Ivan Pavlov worked on classical conditioning, where he demonstrated that a conditioned reflex (i.e., salivation) could be altered with learning. In the 1930s and '40s, behavioural psychologists such as John Watson, B.F. Skinner, and Clark Hull introduced different theories of learning to explain complex behaviour. Tolman (1948) published a paper on cognitive maps in rats and men and argued for the cognitive mechanisms in learning involving knowledge about the world. Before the origin of behavioral psychology to explain memory mechanism, the great neuroanatomist Ramon y Cajal (1890) suggested that structural changes of synapses in the brain might be responsible for the memory engram, and Charles Sherrington (1897) supported this by naming these synaptic changes as a mechanism of learning. Karl Lashley in the early 1920s performed experiments by removing a mass of cortical tissue and measuring its effect on maze learning. He concluded that different memories were diffusely distributed throughout the cortical region. In 1938 Wilder Penfield demonstrated that electrical stimulation of the brain could elicit memories, perceptions, and hallucinations including voices, images, and music. In 1949, the Canadian neuroscientist Donald Hebb published his book the *Organization of Behavior* in which he argued for a neural network system to be responsible for memory storage in the brain. In the 1960s and '70s Scoville and Milner conducted tremendous work on the patient H.M. Their publications described H.M.'s severe loss of memory without loss of intellectual or cognitive abilities that resulted from partial removal of bilateral medial temporal cortex. This suggested a clear

functional heterogeneity within the brain, in contrast to the earlier theory of Lashley. Brenda Milner (1968) again demonstrated that procedural memory in H.M. was unaffected, suggesting different types of memory could be maintained by different brain regions.

1.2 Theories of learning and memory

1.2.1 Multiple memory systems

The idea of memory not being a single faculty of mind appeared in the writing of famous psychologists and philosophers more than a century ago. The philosopher Maine de Biran (1804/1929) proposed three forms of memory: mechanical, sensitive, and representative, each of which has unique properties and mechanisms (Schacter and Tulving, 1994). Although the idea of distributed memory systems in the brain was promising at the beginning, those ideas were ignored until the 1960s and '70s, when combined evidence from cognitive neuropsychology, psychology, and neurobiology supported the view of *multiple memory systems.* The research was directed then toward the efforts to experimentally dissociate different memory systems. Work with experimental animals and amnesic patients then renamed these systems into procedural, declarative, and emotional memory, respectively (Scoville and Milner, 1957; Schacter, 1990; Squire, 1992). Declarative memory refers to the conscious recollection of facts and events that is impaired in amnesia and dependent on structures in the medial temporal lobe. On the other hand, procedural memory is learned by gradual acquisition of skills and expressed through performance or

motor functions. Emotional memories are modified by emotionally charged events **(Fig. 1.1).**

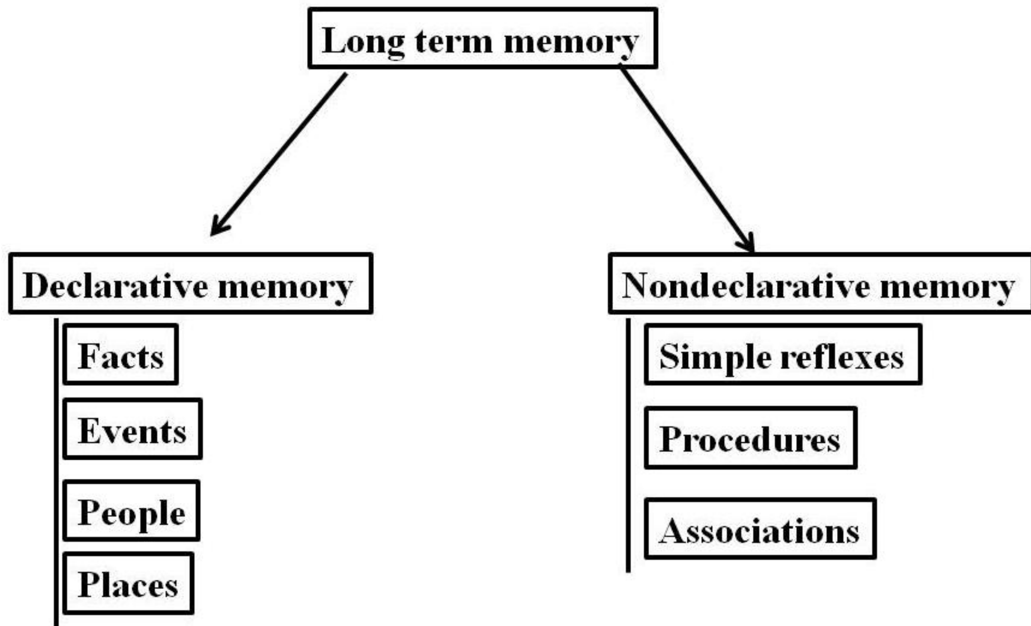


Fig. 1.1: **Various forms of long term memory**

The evidence supporting the notion of multiple memory systems came from the various psychological experiments conducted on the amnesic patient H.M., who had a brain surgery in which a portion of medial temporal lobe (MTL) was removed to cure the epileptic attacks which made H.M.'s life unimaginable. Though the surgery was successful, H.M. developed a severe anterograde amnesia in which he was unable to form any new memories of people, places, facts and events, while maintaining a perfect intellectual capability (Corkin, 1984; Milner et al., 1968). However, H.M did retain specific types of memory (Corkin, 2002), although he performed poorly on tests designed to assess retention of information such as pictures, stories, etc. For example, H.M. could learn new sensory motor skills such as the task of mirror-drawing, and improve over trials despite an inability to remember the event of performing the task before. He did perform normally in other tests such as repetition priming, classical conditioning, and habit learning. In summary, H.M. had a specific deficit in declarative memory whereas his non-declarative memory was intact . H.M.'s study, along with studies from other amnesic patients, strongly supported the notion of different memory systems being localized at different part of the brain (Cohen and Squire, 1980; Squire and McKee, 1993; Tulving and Schacter, 1990; Warrington and Weiskrantz, 1968). With the advancement in pharmacological and surgical manipulations of the brain using different animal models, an in-depth knowledge of neural pathways responsible for memory can be assessed. To this end, several lines of amnesic animal models were developed and a rigorous study on rats and mice provided solid information about multiple memory systems in the brain and their

interactions. Based on several key observations from human studies and animal experiments regarding distinctive memory systems in the brain, Squire coined the term "medial temporal lobe system", which consists of the hippocampal formation, perirhinal cortex, and parahippocampal cortex, and suggested that it is responsible for encoding of declarative memories distinct from other types of memories. The striatum is responsible for habitual memory, neocortex for percepts and priming, amygdala for emotional memory, and cerebellum for motor learning or procedural memory (Squire and Zola-Morgan, 1988; Squire, 1992; Squire and Zola, 1997; Squire and Zola-Morgan, 1991).

1.2.2 Memory phases and consolidation

Though different classification systems exist to describe the phases of memory duration, for the purpose of simplicity it can be classified into three distinct phases: working memory, short-term memory (STM) and long-term memory (LTM). Working memory lasts for 10-30 seconds (Craik, 1979) and incorporates fragments of information such as a visuospatial sketchpad to hold and manipulate visual images, a phonological loop to retain speech, a fraction of episodic information of an episodic event, and an attentional component from the central executive to hold the information during the learning period (Baddeley, 1996; Baddeley, 2003; Repovs and Baddeley, 2006; Pickering, 2001). The prefrontal cortex plays an important role in the regulation of working memory (Fuster, 1998). Though patients with selective damage of MTL demonstrate severe anterograde amnesia, they have perfect working memory (Squire and Zola, 1997).

This proves that working memory is separate from other types of memory. Another example of this separation is H.M.'s normal working memory (in terms of recognition and recall without any distraction) (Corkin, 2002).

The STM can retain memory for longer than working memory but shorter than LTM (from minutes to hours). STM and LTM operate in parallel fashion (Izquierdo and McGaugh, 2000) instead of a temporal progression of memory storage as previously thought (James, 1890). Injecting specific kinase inhibitors of STM into the hippocampus inhibits STM without affecting LTM expression at later time points (Izquierdo et al., 1999). Therefore, STM and LTM may use separate mechanisms to progress from one phase to the other.

The formation of LTM requires a process called consolidation during which new or temporary memories are transferred from a labile to a more resilient form (Alvarez and Squire, 1994; McGaugh, 2000). Although the processing of information can exist in the hippocampus, the long term storage of this information is thought to be the neocortical region (Alvarez and Squire, 1994; Bontempi et al., 1999). Consolidation is a slow process during which cortical structures are reorganized and finally become independent of the memory processing systems. The slow process of consolidation can be understood from the studies of retrograde amnesia, in which patients experience an impairment of retention of more recent memories but old memories remain intact (Squire and Alvarez, 1995). For example, patient E.P. who had bilateral hippocampal damage

was able to recall the neighbourhood where he spent his childhood (Teng and Squire, 1999) and his performance was perfect in comparison to the aged-matched control who also grew up in the same place and later moved. However, E.P. had no memories of his current place, where he moved after he became amnesic (Teng and Squire, 1999). Therefore, the hippocampus as memory processing system reorganizes over time so that it is no longer needed for retention of memories.

1.2.3 Hippocampal memory system

The hippocampus receives neuronal projections from all different cortical areas to process specific information **(Fig. 1.2**) and act as a window through which the brain can see the outside world. In doing so, it acts as a gateway to associate information from different inputs over time to encode explicit memory (Mishkin et al., 1998). For example, cortical association areas from frontal, parietal and temporal lobes converge on the hippocampus through parahippocampal and entorhinal cortices (Suzuki and Amaral, 1994a; Suzuki and Amaral, 1994b) and information from the hippocampus also flows back to the same cortical association areas (Deacon et al., 1983; Van Hoesen et al., 1972; Amaral and Witter, 1989).

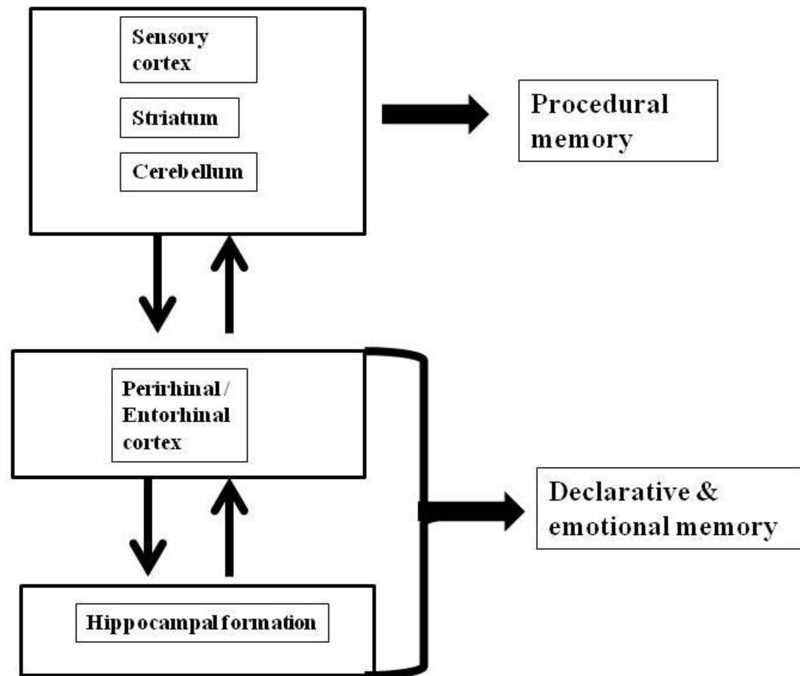


Fig. 1.2: Brain regions and associated Memory systems (adapted and modified from Squire and Zola-Morgan, 1988).

Why does the hippocampus have those complicated innervations? Initially it was suggested that the hippocampus is involved in olfaction, sensation, perception and higher cognitive functions (reviewed in Anderson et al., 2007). However, after tremendous research on human case studies, the MTL theory was proposed by Larry Squire, where he suggested that the hippocampus, along with the parahippocampal and perirhinal cortices, plays a selective role in the formation of declarative memory (Cohen and Squire, 1980). Even before MTL theory, hippocampal single unit recording from awake rats suggested a cognitive map theory of hippocampal function (O'Keefe and Dostrovsky, 1971) in which an individual neuron has a spatial map of the environment and that information is coded by its firing patterns. Anatomical and behavioral studies across species indicate that the functional organization of the MTL system is similar (Squire, 1992), and the requirement of hippocampus for memory formation is common among humans, non-human primates and rodents.

In summary, the hippocampus covers a broad range of functions that include spatial, non-spatial, and contextual forms of learning and memory, which allow it to support the formation of relational representations of information in memory (Eichenbaum et al., 1992).

1.3 Hippocampal neuroanatomy and physiology

1.3.1 Hippocampal tri-synaptic circuitry

The mammalian hippocampus is a C-shaped structure in the temporal lobe extending from the caudoventral temporal lobe to the septal nuclei rostrodorsally (Amaral and Witter, 1989). Histologically, the hippocampus is separated into distinct subregions thought to be responsible for information processing and memory formation. These subregions are termed Ammon's horn, the dentate gyrus and the subiculum. Ammon's horn is further subdivided into four regions: cornu ammonis 1-4 (i.e., CA1-CA4). Several anatomical (Blackstad et al., 1970; Hjorth-Simonsen, 1973; Hjorth-Simonsen and Jeune, 1972) and electrophysiological (Krnjevic and Ropert, 1982) studies on hippocampus have described it as a layered, organized structure which is amenable to cellular electrophysiology experimentation. A typical hippocampal slice, commonly used for in vitro preparation, contains the tri-synaptic circuit consisting of the dentate gyrus, CA1 and CA3 connected sequentially **(Fig. 1.3)**. Though all subregions receive direct subcortical input, cortical output flows systematically through the hippocampal circuitry. The perforant pathway, originating from the entorhinal and perirhinal cortices, terminates in the molecular layer of the dentate gyrus. The granule cells of the dentate gyrus then send output through mossy fibres to the proximal dendrites of the CA3 pyramidal cells. The large collateral axons of CA3 cells then either terminate in CA1 region through the Schaffer collateral pathway or project to other CA3 cells within the same field. A connection from area CA1 to subiculum, and efferents from both areas back to the parahippocampal region

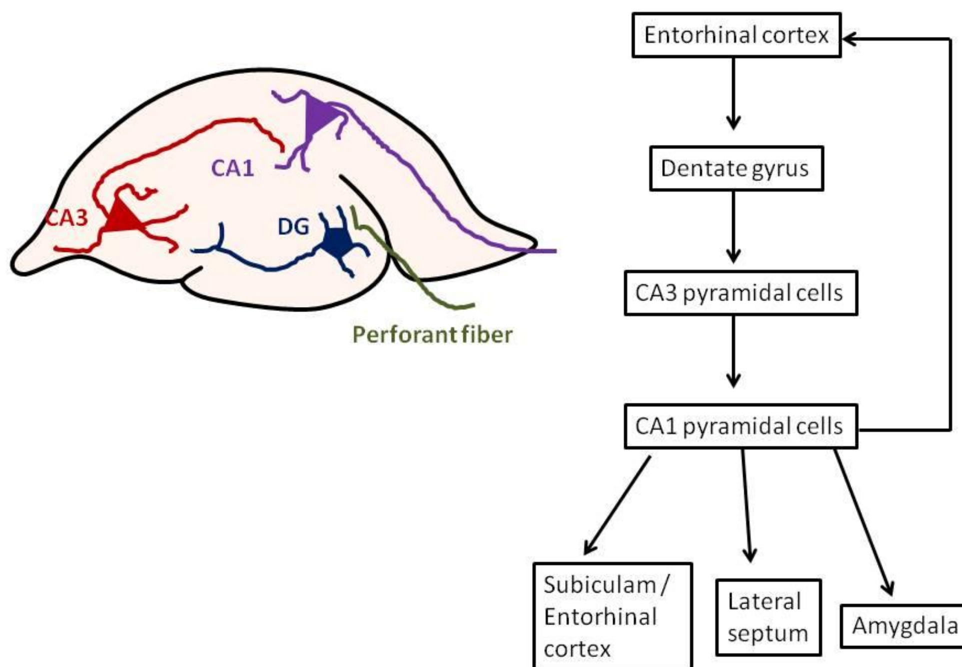


Fig. 1.3: Hippocampal circuitry and information flow

complete the circuit. The parahippocampal region can also project to the CA1 region directly without following through long pathways.

Evidence from neural network organization, lesion studies and computational modelling suggests a subregional specificity of function (Zola-Morgan et al., 1986; Gold and Squire, 2005; Suthana et al., 2009; Kim and Frank, 2009; Kartsounis et al., 1995). Furthermore, with the advancement of science, studies using genetic approaches have shown that synaptic plasticity in the CA1 region is required for certain forms of memory (Tsien et al., 1996; Nakazawa et al., 2002).

1.3.2 Neuronal communication and glutamatergic transmission in the hippocampal circuit

Neurons communicate through a specialised structure called a synapse, termed by Sir Charles Scott Sherrington and colleagues. Synapses are junctions where presynaptic and post synaptic neurons communicate by chemical or electrical signals. In the brain, most neurons use chemical synaptic transmission to communicate and this underlies numerous cognitive processes. Though the final outcome of complex neuronal information processing is visible through a behavioral or cognitive phenomenon, individual neurons act as a unit for this purpose. Thus, an understanding of basic properties of individual neurons would be helpful to elucidate the cellular basis of behaviour (Kandel, 1976).

Upon depolarisation of the presynaptic terminal by an invading action potential, the Ca^{2+} channels open up, causing fusion and release of neurotransmitter-

containing vesicles from presynaptic terminals onto the synaptic cleft. The neurotransmitter then binds to its specific receptor on the post synaptic membrane close to the synaptic cleft. This binding of neurotransmitter leads to changes of the ion channels and of the membrane conductance, which leads to initiation of a graded membrane potential. This electrotonic membrane potential is known as an excitatory or inhibitory post synaptic potential (EPSP or IPSP, respectively). A special property of the synapse is its plastic nature, which means the strength of a synapse often can be enhanced or depressed depending upon the type of input it receives from other sources of influence. Such modifications of synaptic strength are key players in many important cognitive and behavioural phenomena such as sensory adaptation and alteration of receptive fields (O'Shea and Rowell, 1976) and habituation of escape responses to repeated stimuli (Auerbach and Bennett, 1969; Zucker, 1972; Zilber-Gachelin and Chartier, 1973). The plasticity of synapses in learning and memory is further described in the next section.

The majority of synapses in the hippocampal circuit are glutamatergic, which means these synapses release glutamate as a neurotransmitter at their nerve terminal. Most of the action of glutamate is upon the binding of two separate classes of glutamate receptors: the ionotropic receptors (or ligand-gated ion channels) and the metabotropic receptors (G-protein coupled receptors) (**Fig. 1.4**). This discussion will focus on ionotropic receptors as they have been shown to play an active role in synaptic plasticity**.** On the basis of reactivity to specific

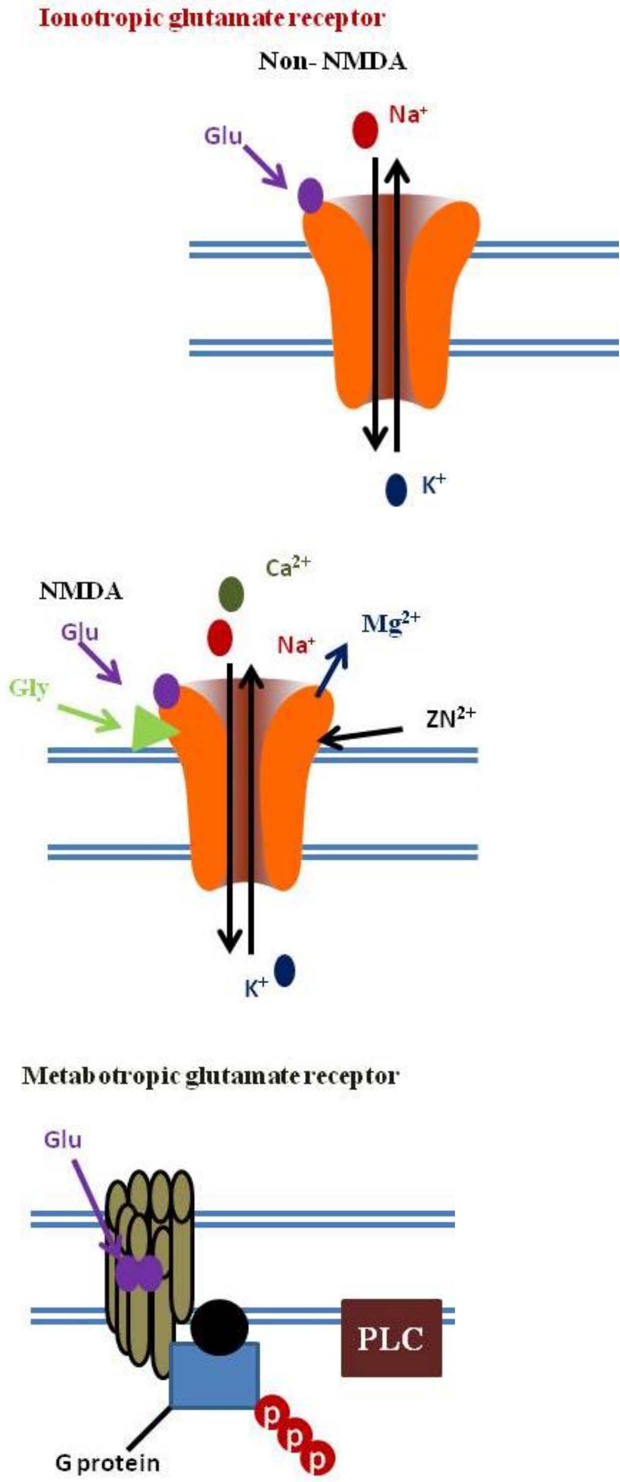


Fig. 1.4: Three types of glutamate receptors.

agonists and antagonists, the ionotropic receptors were categorised into two distinct types of glutamate receptors (Watkins and Evans, 1981): N-methyl-D-aspartate (NMDA) and non-NMDA types. The post synaptic potential/current (EPSP/EPSC) of CA1 pyramidal cells has been characterized by the electrophysiological whole-cell recording technique (Blanton et al., 1989). The excitatory synaptic responses of CA1 pyramidal cells have two kinetically distinct components: a fast, rapidly decaying non-NMDA mediated response, and a late, slow-rising NMDA-mediated response (Hablitz and Langmoen, 1982; Herron et al., 1985; Collingridge et al., 1988; Collingridge et al., 1992; Hestrin et al., 1990).

The NMDA receptors are composed of two subunits: NMDAR1 and NMDAR2, with NMDAR2s having four isoforms: NMDAR2A-D (Hollmann and Heinemann, 1994; Dingledine et al., 1999; Bochet and Rossier, 1993). The benefit of having different subunits is to alter receptor function according to subunit combination; NMDAR1 serves as a fundamental unit and NMDAR2 subunits as modulatory. The NMDARs in CA1 pyramidal cells are Ca^{2+}-conducting glutamate receptor ion channels (MacDermott et al., 1986; Mayer and Westbrook, 1987; Iino et al., 1990; Ogita et al., 1998) and act as coincidence detectors of simultaneous presynaptic firing with postsynaptic depolarization. This coincidence detection is needed to expel Mg^{2+} ions from the NMDAR channel pores and to conduct Ca^{+2} influx subsequently (Mayer et al., 1984; Nowak et al., 1984; Kumamoto, 1996; Mayer and Westbrook, 1987).

Another type of fast excitatory glutamatergic synaptic transmission in hippocampal cells is mediated through a type of glutamate receptor activated by α-amino-3-hydroxy-5-methyl-4-isoxazol-proprionate (AMPA), known as AMPA receptors (AMPAR). The AMPARs are composed of four subunits, GluR1-4 or GluRA-D (Hollmann and Heinemann, 1994; Dingledine et al., 1999). The kinetics of AMPA receptors are dictated by the subunit composition, which can be expressed as either homomeric or heteromeric oligomers. For example, AMPARs containing GluR2 subunits are predominantly Ca^{2+} -impermeable and outward-rectifying (Jonas et al., 1994; Liu and Cull-Candy, 2002; Tanaka et al., 2000).

An additional, kainate-sensitive glutamate receptor subtype has been also identified (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking et al., 1998), and these kainate receptors are encoded by the GluR5-7 and KA-1/2 family of genes with structural homology to AMPA receptors. The final class of glutamate receptors are G-protein coupled receptors, known as metabotropic glutamate receptors. Based on their homology, these receptors are divided in three broad categories: mGluR 1-3. Activation of mGluRs causes physiological functions such as inhibition of calcium and potassium channels (Anwyl, 1999).

1.4 Synaptic plasticity

Since the discovery of neurons as signal-conducting elements of the brain, it was postulated that they may also have the ability to store and retrieve information in terms of various electrochemical mechanisms. Ramon y Cajal (1893) suggested a

correlation between mental activity and synaptic strength. Interestingly, Donald Hebb (1949) in his landmark study postulated a theory of cellular memory formation where synaptic activity could generate long-lasting changes in excitability of the neurons and these changes comprise a cellular memory. He wrote, "When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased," (p.62). Since then, it was hypothesized that neurons retain previous information and mediate learning and memory.

1.4.1 Synaptic plasticity: Study from **Aplysia**

Although hypotheses regarding cellular mechanisms of memory formation existed in the 19th century, it was difficult to design and perform experimental investigation on the neuronal level considering the complex architecture of mammalian nervous system. To this end, in the 1970s Eric Kandel and groups used the marine invertebrate *Aplysia californica,* which has a relatively simple nervous system consisting of fewer and larger easily identifiable neurons, to address and investigate the cellular mechanisms of memory formation (Kandel et al., 1976). One of the observable and modifiable behavioural responses of *Aplysia* is the gill-withdrawal reflex, which allows it to protect its gill and siphon from an external noxious stimulus. In the laboratory, the strength of this behavioral response to a graded stimulus to the siphon is determined by its previous experience and considered indicative of learning (Pinsker et al., 1973). Generally,

a tactile stimulus activates siphon sensory neurons which eventually excite the motor neurons innervating the gill and initiate withdrawal of the gill from the stimulus environment. These gill-withdrawal responses are sensitized in such a way that a noxious stimulus at the tail induces a withdrawal response which is increased in both strength and duration upon subsequent stimulation with a neutral stimulus. Mechanistically, sensory input from the siphon skin modulates responses of excitatory and inhibitory interneurons to alter postsynaptic motor neuron responses. A tail shock activates excitatory serotonergic interneurons which release serotonin (5-HT) onto presynaptic sensory neuronal terminals originating from the siphon and generates a stronger postsynaptic response to the motor neuron and consequently a larger withdrawal response of gill (Mackey et al., 1989; Glanzman et al., 1989). The relative simplicity and accessibility of the nervous system in *Aplysia* has permitted an in-depth investigation of the neuronal mechanisms of short- and long-term sensitization in *Aplysia* (Byrne et al., 1974; Hawkins, 1981). In brief, transient local kinase activation and protein phosphorylation constitutes presynaptic facilitation in short-term sensitization (Castellucci et al., 1980; Klein and Kandel, 1980), whereas persistent protein kinase activation and macromolecular synthesis constitute long-lasting sensitization of the gill-withdrawal response (Sweatt and Kandel, 1989; Sossin et al., 1994; Castellucci et al., 1986) **(Fig. 1.5)**.

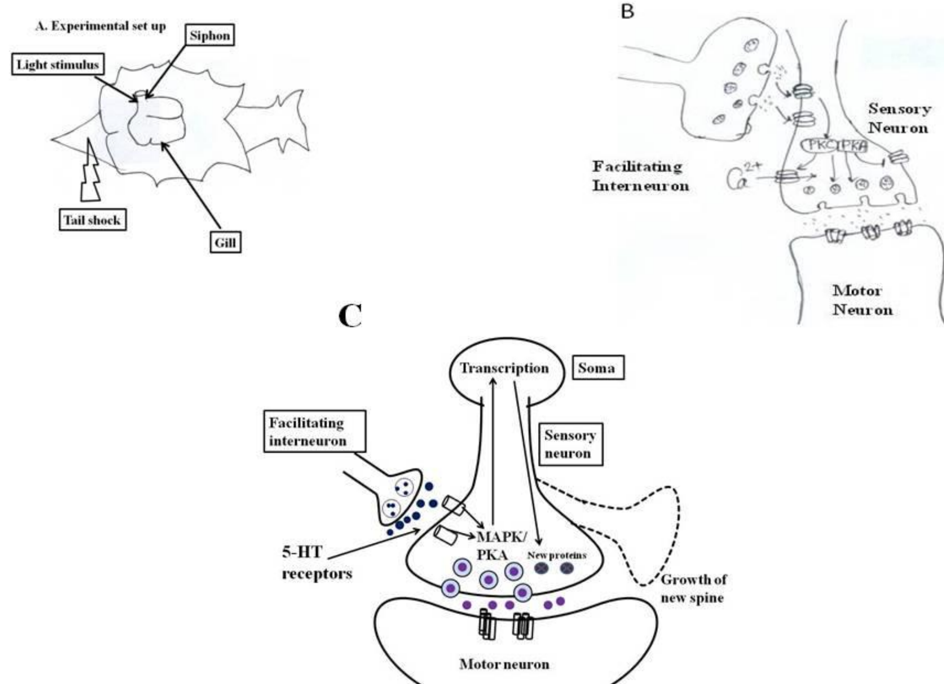


Fig. 1.5: Gill withdrawal response in *Aplysia* (adapted and modified from Kandel et al., 2001)

A: Aplysia Anatomy and experimental set up.

B: Cell-signaling in short-term sensitization response

C: Cellular-signaling in long-term sensitization response

Thus, studies on *Aplysia* demonstrate a direct link between the neuronal mechanism of action to the behavioral response and establish a framework for investigation of synaptic plasticity and learning and memory that is also observed in the more complex mammalian system.

1.4.2 Synaptic plasticity in the mammalian brain: Long-term potentiation and long-term depression

Though studies from the simple *Aplysia* model postulated that alterations of strength of synapses could mediate information storage in the mammalian brain, the experimental evidence of such phenomena was not available until the 1970s, when Bliss and Lømo conducted a series of experiments on rabbit hippocampus and discovered long-term potentiation (LTP) of synaptic strength in dentate gyrus, similar to the long-term sensitization of *Aplysia* (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). In short, they observed a persistent enhancement of synaptic strength when a high frequency electrical stimulation was applied at synapses in the hippocampus of the rabbit, and thought it to be responsible for encoding of new information. Accumulating evidence supported the idea that LTP is responsible for memory formation and consolidation (Bliss and Collingridge, 1993; Martin and Morris, 2002; Moser et al., 1998). Initial observations on the characteristics of LTP have made it a crucial candidate for the information storage system. Like long-term memory, the duration of LTP may persist from days up to a year (Bliss and Gardner-Medwin, 1973; Abraham et al., 2002; Staubli and Lynch, 1987).

There are several other properties of LTP which make it a unique candidate for memory formation and storage. Importantly, LTP is pathway-specific, which means only stimulated synapses are affected, not nearby inactive synapses (Andersen et al., 1977). The purpose of this pathway specificity is to process information precisely from individual synapses through a complex computational mechanism. LTP also shows the properties of cooperativity and associativity. Cooperativity means numerous presynaptic fibres should fire together to bring sufficient depolarization of the post-synaptic neuron to induce LTP (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973; Malenka, 1991; McNaughton et al., 1978). Associativity implies that LTP could be elicited if a weak stimulus at one input is paired temporally with a strong stimulus at an independent input (Gustafsson et al., 1987; Levy and Steward, 1979). Using these properties, neurons can perform information processing and generate LTP.

Unlike long-term potentiation, synaptic strength can also be persistently weakened, termed long-term depression (LTD), which is thought to balance the enhancement of synaptic strength during LTP and prevent the saturation of synapses. A low frequency stimulation of presynaptic neurons or an inconsistent presynaptic firing which eventually fails to activate postsynaptic cells would decrease the synaptic strength between these cells and cause LTD (Stent, 1973; Dudek and Bear, 1992; Heynen et al., 1996; Milner et al., 2004). Some forms of

LTD are thought to be necessary for information storage (Etkin et al., 2006; Manahan-Vaughan and Braunewell, 1999; Nakao et al., 2002).

1.4.3 Hippocampal LTP phases and mechanisms

Traditional teaching divides hippocampal LTP into three phases: immediate, early and late LTP. Immediate LTP is thought to be protein-kinase independent and lasts about 30 minutes. Early LTP lasts for 2-3 hours and is due to persistent activation of various protein kinases. The late phase of LTP lasts many hours to days, and is hypothesized to be due to translation and transcription mechanisms.

LTP typically goes through three stages: induction, expression and maintenance. Commonly, a brief, 100Hz, high frequency stimulation (HFS) is used to elicit LTP. Several other protocols have also been employed to induce LTP, such as the application of electrical stimulation in the range of 3-12 Hz (also known as theta frequency) (Staubli and Lynch, 1987). This type of stimulation is believed to be more physiological as oscillations in this range are observed in the hippocampus and thought to be mediated by release of acetylcholine, GABA and glutamate from neurons of medial septum and entorhinal cortex (Alonso and Llinas, 1989). For example, short high-frequency bursts of stimulation with an interburst interval of 200 ms, which resemble and phase-lock with the complex spike activity of pyramidal neurons, facilitates the induction of LTP (Buzsaki, 1986; Otto et al., 1991). Electrophysiological recordings of LTP make use of these stimulation protocols to induce LTP in hippocampal slices. Mechanistically, induction of LTP is due to increased intracellular calcium concentration through NMDA receptors

(Collingridge et al., 1983; Lynch et al., 1983; Malenka et al., 1988). In this respect, NMDA receptors play a crucial role as coincidence detectors to ensure that LTP is induced properly only when there is a simultaneous activation of pre- and post-synaptic neurons (to remove the magnesium blockade from NMDARs). For example, weak tetanic stimulation might not be able to depolarise the post-synaptic neuron to reach the threshold for NMDAR activation, even though there is enough glutamate present at the synaptic cleft for AMPAR activation. However, a strong synchronous tetanic stimulation of presynaptic neurons (within a specific spatial and temporal window) activates immediate or nearby postsynaptic neurons to reach to their threshold depolarization for NMDAR activation and calcium influx, leading to LTP induction.

LTP, after its induction through NMDAR activation, follows through the expression (E-LTP) and maintenance (L-LTP) phases. LTP expression is a consequence of the interaction of multiple intracellular signaling cascades (Sanes and Lichtman, 1999; Soderling and Derkach, 2000) leading to enhancement of synaptic transmission through increasing post-synaptic receptor conduction or insertion of extrasynaptic receptor subunits on post-synaptic sites. In this respect, signaling molecules such as CaMKII, PKA and PKC have been described to phosphorylate subunits of AMPA and NMDA receptors (Malenka and Bear, 2004; Roche et al., 1996; Lee et al., 2000) to alter the kinetics of function (Benke et al., 1998; Derkach et al., 1999). Many studies have reported a deficiency in LTP expression using pharmacological blockers or genetic approaches to block

signaling molecules such as CAMKII or PKC, confirming the previous notion (Malinow et al., 1989; Hu et al., 1987; Silva et al., 1992). PKA, however, behaves in such a way that its inhibition does not disrupt E-LTP generation by 1x100Hz stimulation (Abel et al., 1997; Duffy and Nguyen, 2003; Huang and Kandel, 1994). Further investigation revealed that PKA phosphorylates I-1, which suppresses the inhibitory effect of PP1 on synaptic potentiation (Blitzer et al., 1998). In this way, PKA can gate LTP expression through phosphatase regulation (Blitzer et al., 1998; Woo et al., 2002). Insertion of extrasynaptic AMPARs to the "silent synapses" (synapses with no AMPARs) or AMPAR trafficking due to enhanced CAMKII and PKA activity are proposed to be other candidate mechanisms of LTP expression (Kullmann, 2003; Poncer, 2003; Esteban et al., 2003; Hayashi et al., 2000). Thus, both E-LTP (LTP expression) in hippocampus and short-term facilitation in *Aplysia* are decremental (lasting only hours) and rely on covalent modification of pre-existing proteins. The subsequent phase of LTP maintenance (L-LTP) requires additional synaptic modifications, such as macromolecular synthesis.

L-LTP or LTP maintenance phase lasts for several hours and is generally initiated by applying multiple (>2) trains of HFS (Abraham et al., 2002). The multiple trains of HFS activate intracellular signaling that engages translation and transcriptional machinery to produce new mRNAs or proteins in support of structural and functional plasticity (Deadwyler et al., 1987; Nguyen et al., 1994; Stanton and Sarvey, 1984). In this context, it should be noted that studies using

inhibitors of translation and transcription mechanisms suggest a temporal window for these mechanisms to support LTP maintenance. Translation is required for persistence of LTP at the beginning but transcription is generally recruited at a later time point (Frey and Morris, 1997; Frey et al., 1996; Nguyen et al., 1994). The requirement of translation and transcription for LTP maintenance is a complex mechanism needing further clarification.

The initial idea of a translational requirement for LTP was revealed by a series of studies showing the presence of polyribosomal complexes in dendrites (Steward and Schuman, 2001; Steward and Schuman, 2003). Meanwhile, several groups also showed the presence of translational factors in dendrites. These translational factors could be recruited at synaptic sites upon electrical stimulation (Kanhema et al., 2006; Tang and Schuman, 2002; Smart et al., 2003; Moon et al., 2009; Ostroff et al., 2002; Bourne et al., 2007; Mitsuyama et al., 2008). Electrical stimulation of neurons activates many intracellular signaling cascades, such as ERK and mTOR, to control protein synthesis (Banko et al., 2006; Kelleher, III et al., 2004; Tsokas et al., 2007; English and Sweatt, 1997; Wu et al., 1999). Interestingly, various neuromodulators can influence synaptic plasticity by upregulating the translational machinery and hence modulate some types of memory formation. Thus, local protein synthesis is connected to synaptic plasticity as well as memory through a yet-to-be clarified mechanism involving many intracellular signaling molecules.

Initial reports on transcriptional regulation of LTP maintenance appeared in studies in which cell bodies were cut from dendrites, resulting in decremental LTP (Frey et al., 1989). Furthermore, inhibitors of transcription have also been shown to block L-LTP maintenance (Frey et al., 1996; Nguyen et al., 1994) supporting the notion that transcription is required for maintenance of LTP. Phosphorylation of the transcription factor CREB by upstream kinases such as PKA, PKC and MAPK is crucial for transcriptional regulation (Impey et al., 1998; Impey et al., 1996; Bito et al., 1996; Pokorska et al., 2003), and inhibiting CREB phosphorylation with PKA/MAPK blockers prevents L-LTP (Davis et al., 2000; Sweatt, 2004; Shaywitz and Greenberg, 1999; Johannessen and Moens, 2007). High, but not low, frequency stimulation phosphorylates CREB to bind with CREB binding proteins (CBP) of various immediate early genes to stimulate transcription (Lee and Masson, 1993; Tian et al., 1996). The possibility of cell-wide distribution of translation products challenges the idea of input specificity in synaptic plasticity. However, recent theories suggest that these gene products are available to be captured only by previously activated (“tagged ") synapses (Sossin et al., 1994; Frey and Morris, 1997). A model for different phases of LTP is shown in **Fig. 1.6**.

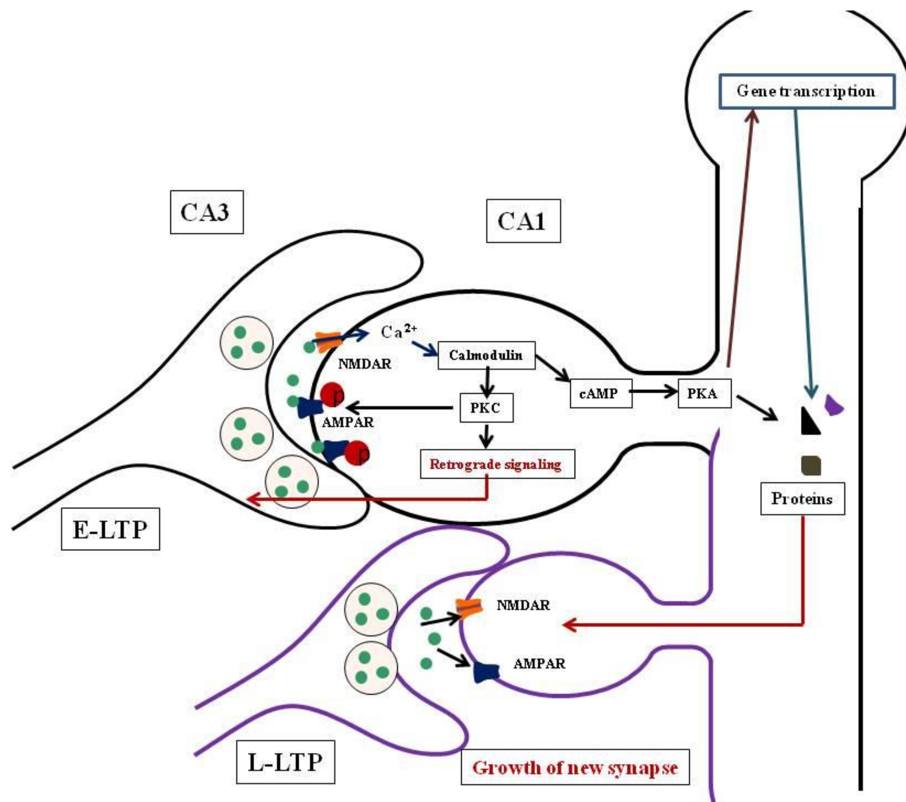


Fig. 1.6: Molecular mechanisms of phases of LTP (Adapted and modified from Kandel et al., 2001).

1.5 Signature of LTP/LTD in learning and memory

The link between synaptic plasticity and memory was formalized by Morris and colleagues as the synaptic plasticity and memory (SPM) hypothesis: "Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed" (Martin et al., 2000). Decades of research have established a possible connection between synaptic plasticity, such as LTP or LTD, and memory formation; however, a direct link has been difficult to prove. Although we have many interesting studies in vitro establishing the electrophysiological and molecular events responsible for activity-dependent changes in synaptic strength (Bliss and Collingridge, 1993; Engert and Bonhoeffer, 1999; Malenka and Nicoll, 1999; Lynch, 2004), the data demonstrating a role for synaptic plasticity during actual learning *in vivo* are sparse. However, several landmark experiments (described below) have provided evidence for changes in synaptic strength which coincide with learning and memory processes.

If LTP is a mechanism that supports the formation of a spatial cognitive map of the external world which can be later retrieved, then disruption of LTP should interfere with spatial memory formation. Experiments with two types of mutant mice provided more direct evidence for a possible role of LTP in spatial memory formation. In the first type of mutant mouse, the NR1 subunit of NMDA receptors was knocked out in the CA1 region of hippocampus, which resulted in disruption

of LTP and simultaneous impairment of spatial memory formation in the Morris water maze (MWM) test (Tsien et al., 1996). In a second mutant, expression of a persistently active form of Ca^{2+}/calmodulin-dependent protein kinase can be turned on and off at will. Activation of this transgene selectively impaired LTP in the frequency range of 1-10 Hz and caused instability of place fields. The mutant mice also performed poorly in spatial tasks. However, LTP was restored and the animal's capability to form spatial memory was re-established when the transgene was turned off (Mayford et al., 1996). These two sets of early genetic experiments on mutant mice established a foundation for LTP in the Schaffer collateral pathway as an important mechanism for spatial memory.

More recently, Whitlock et al. (2006) have shown that animals (rats) that have undergone inhibitory avoidance (IA) training (a test of memory trace formation) displayed an immediate NMDA receptor-dependent enhancement of phosphorylation at Ser 831 (but not Ser 845) of the GluR1 AMPAR subunits. IA training causes trafficking of GluR1/2 subunit of AMPA receptor in the hippocampus of trained animals compared to naive or control. Moreover, using multielectrode recordings, it has been shown that IA training induces fEPSP enhancements which occlude subsequent LTP induction *in vivo* in CA1. These data support the hypothesis that learning actually does induce LTP, a necessary corollary to the notion that LTP underlies learning.

Activity-dependent synaptic plasticity, such as LTP and LTD at glutamatergic synapses in hippocampal neurons, is considered a cellular mechanism for information encoding and consolidation of memory. LTP and LTD mechanisms are NMDA receptor subunit-specific, and pharmacological or genetic disruption of different subunits leads to changes in LTP or LTD. Blocking NMDA receptors pharmacologically prevents formation of the associative memories required for performing the MWM test (Morris et al., 1986). However the exact role of LTP and or LTD in MWM performance remained poorly understood and required additional experimental approaches that could selectively inhibit either LTP or LTD in freely moving rats. To this end, Ge et al. (2010) found that in freely moving rats, blocking LTP with the NR2A subunit-specific antagonist NVP-AAM077 leaves spatial memory intact, whereas preventing LTD with the NR2B-specific antagonist Ro25-6981 impaired performance. For further confirmation, they performed bilateral intrahippocampal injections of a membrane-permeable peptide, Tat-GluA23Y, which prevents LTD expression by inhibition of AMPA receptor endocytosis. Similar to the effects of Ro25-6981, injection of the Tat-GluA23Y peptide prevented spatial memory consolidation. Hence, this study supports the importance of LTD in CA1 in the consolidation of long-term spatial memories in the intact animal.

The hippocampus is involved in a variety of learning paradigms, including classical conditioning of eye blink responses (Berger et al., 1983; Sanchez-Andres and Alkon, 1991; McEchron et al., 2003; Munera et al., 2001). Bilateral

hippocampal lesions lead to impairment of the acquisition of trace eye blink conditioning, leaving delay conditioning unaltered (Thompson, 1988; Moyer, Jr. et al., 1990). Based on this, Gruart et al. (2006) set out to test the hypothesis that the strength of the hippocampal CA3–CA1 synapse can be modified by acquisition of associative learning. Using classical conditioning of eye blink responses, they have shown that the hippocampal CA3-CA1 synapse is involved in the acquisition, extinction, recall, and reconditioning of conditioned responses (CRs). CA3-CA1 synaptic strength can be enhanced or decreased in parallel with the acquisition or extinction of eye blink conditioning. They have also shown that LTP, evoked by HFS of the Schaffer collaterals, interferes with both the acquisition of CRs and the linear relationships between learning scores and fEPSP slopes. Saturating CA3-CA1 synapses with LTP-inducing stimulation prevented additional synaptic changes in plasticity (Barnes et al., 1994; Otnaess et al., 1999), leading to both anterograde and retrograde amnesia. Finally, Gruart et al. showed that an NMDA-receptor antagonist is not only able to prevent LTP induction *in vivo*, but also interferes with both the formation of eye blink CRs and functional changes in strength at the CA3–CA1 synapse. Thus, they concluded that functional transformations of CA1 pyramidal cells are necessary for the proper acquisition, extinction, recall and reconditioning of eyelid CRs.

While it was quite difficult to delineate a causal link between hippocampal LTP/LTD and memory formation, two groups came up with evidence by directly demonstrating the occurrence of LTP in association with behavioural training in

animals (McEchron et al., 2003; Rogan et al., 1997). Both of these groups directly measured LTP, either through *ex vivo* or *in vivo* recording techniques, respectively, in the amygdala in response to behavioural training (fear conditioning training). Both groups eventually arrived at the same conclusion: fear conditioning induces synaptic potentiation of CS inputs into the amygdala. Further research indicated that this type of LTP is similar to hippocampal synaptic plasticity. These papers were the first to demonstrate that LTP could be triggered by endogenously occurring, natural patterns of neuronal firing, initiated by environmental signals.

While it has been difficult to directly demonstrate LTP physiologically in association with spatial learning, biochemical markers for LTP induction such as ERK, CaMKII and PKA/PKC activation, and altered gene expression, have been demonstrated to occur with spatial learning. Such a broad spectrum of molecular changes occurring with both LTP *in vitro* and spatial learning *in vivo* strongly suggest a co-occurrence of LTP with hippocampus-dependent memory formation. In conclusion, we can say that although there is a need for extensive study to pinpoint the exact mechanism correlating LTP or LTD to memory, our current understanding from the previous studies strongly suggests a causal link between LTP or LTD and memory formation.

1.6 Epigenetics in synaptic plasticity and learning and memory

1.6.1 Defining epigenetic mechanisms

Waddington coined the term epigenetics to explain some concepts in developmental biology (Waddington, 1957). Somatic cells of multi-cellular organisms have identical genomes, but cells of different systems differ from each other in cellular structure and function, which is due to use of different genes: that is epigenetics. For example, a liver cell differs from a neuron in structure and function although they have the same DNA. To explain the central dogma of variation of functions in different cells having the same DNA, Waddington suggested that some mechanisms above the level of genes encoded by DNA exist that controlled DNA readout to produce different gene products for different cells even though all cells carry the same DNA. This is what we now refer to as epigenetics. During the cell fate determination of the cell cycle, these epigenetic marks are tagged on the DNA to serve as a cellular phenotype over the cell's lifespan. Wolffe later coined the term epigenetics to describe "heritable changes in gene expression that occur without a change in DNA sequence" (Wolffe and Matzke, 1999). However, evidence from recent studies on the nervous system (containing terminally-differentiated, non-dividing cells) is accumulating to prove that epigenetic mechanisms are not only responsible for phenotypic determination of cells, but also play a role in gene expression in response to memory-inducing events.

1.6.2 Epigenetic marks and tags: A brief molecular mechanism

Epigenetic mechanisms are the key regulators of transcription. A nucleosome, consisting of a long stretch of DNA along with histone proteins such as H2A-H2B dimers or H3-H4 tetramers, exists as a highly compressed structure in the nucleus (Quina et al., 2006) and connects to the next nucleosome through linker histone H1 to form what is known as chromatin (Happel and Doenecke, 2009). The chromatin can exist in an active or inactive state. In its inactive state, known as heterochromatin, characterized by a closed, highly compacted structure, it is restrictive to transcription, whereas active euchromatin, characterised by an open state, is amenable to transcription (Arney and Fisher, 2004). The switching between these two states and the beginning of transcription is mediated through changes in DNA or post-translational modification of histone proteins, collectively known as epigenetic modification **(Fig. 1.7).**

The changes in DNA occur through the enzyme DNA methyltransferase (DNMT) by the addition of a methyl group from S-adenosyl-methionine (SAM) onto 5'-cytosine positioned adjacent to guanine nucleobases (CpG) (Chiang et al., 1996; Turker, 1999; Bird, 2002; Price et al., 2010). There are different subgroups of DNMTs to carry out different functions. The *de novo* DNMTs (3a and 3b) create new methylation marks when the cell fate is determined, and the maintenance DNMTs (DNMT1) maintain previously marked methylation on DNA (Nakao, 2001) by propagating epigenetic marks in dividing cells. It was initially shown

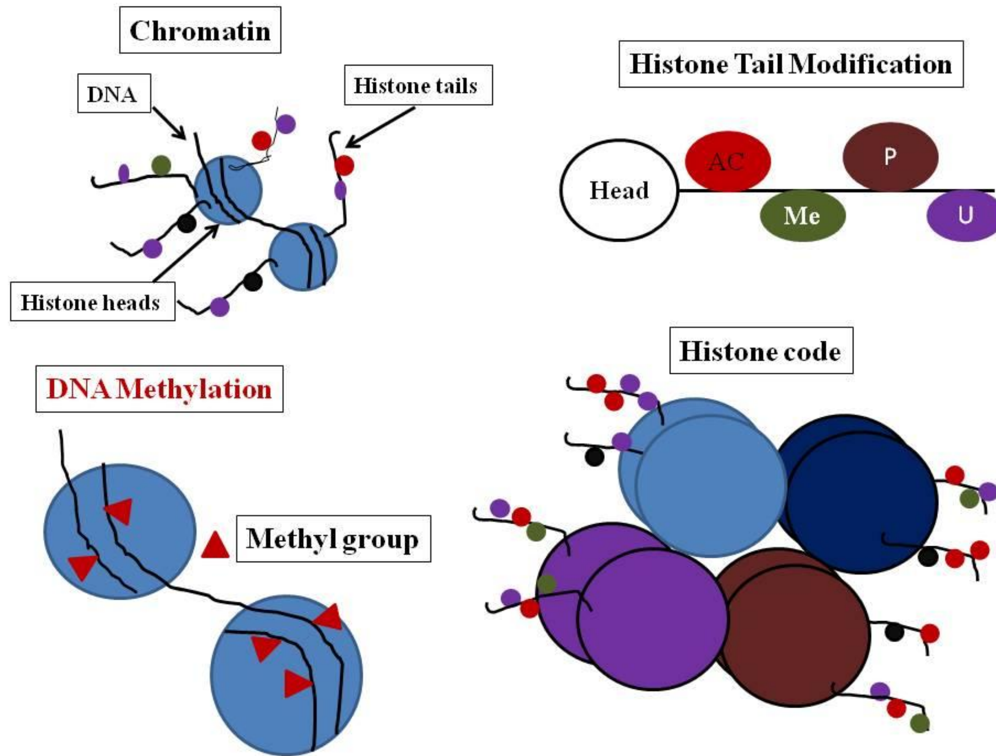


Fig. 1.7: Epigenetic marks and tags (adapted and modified from Jiang et al., 2008; Levenson et al., 2005)

that DNA methylation represses transcription by blocking transcription factors to bind to regulatory sites on DNA (Iguchi-Ariga and Schaffner, 1989) and by promoting closed chromatin structures through recruitment of transcriptional repressors (Karymov et al., 2001; Fuks et al., 2003; Drewell et al., 2002). Sometimes, extensive methylation can completely silence a gene. The methylation of cytosine residues of DNA recruits DNA binding proteins that have a methyl-DNA binding domain (MBD) and transcription regulatory domain (TRD). The TRD also recruits histone deacetylases (HDAC) to the site through adapter/scaffolding proteins. HDACs alter chromatin (DNA/protein complex) structure by removing an acetyl group from the core histone protein which leads to compacting of DNA and suppression of transcription. However, current studies suggest that methyl-CpG-binding protein 2 (MeCP2) can also activate transcription by interacting with CREB (Chahrour et al., 2008; Cohen et al., 2008). Recent studies also report a duality of function for *de novo* DNMTs (3a and 3b), whereby they are associated with heterochromatin and euchromatin (Chen et al., 2002; Kotini et al., 2011). Growing evidence indicates that DNA methylation is a dynamic and bidirectional mechanism in response to several experience-dependent events, such as neural activity, estrogen's effect on human cells, and exercise in muscle (Kangaspeska et al., 2008; Metivier et al., 2008; Guo et al., 2011a; Guo et al., 2011b; Barres et al., 2012).

A second major category of epigenetic markers is post-translational modification of histone proteins. Modification of histone proteins is a mechanism of epigenetic

tagging and can be mediated independent of DNA methylation. Histones are highly basic proteins with two distinctive portions. The interaction between histone proteins and the DNA molecule of chromatin is mediated through a long stretch of the N-terminal tail of histone proteins. Structural studies have found that the N-terminal tail of histone proteins protrudes from the chromatin core and is the site of post-translational modifications (PTMs) of histone proteins (Luger et al., 1997). The PTMs of histones are the critical regulators of DNA compaction and gene expression. The unmodified, positively charged histone proteins facilitate interaction with negatively charged DNA and inhibit gene expression by promoting the closed chromatin state (Muhlbacher et al., 2006). The N-terminal tail of histone proteins can undergo several covalent modifications, namely acetylation, phosphorylation, methylation, ubiquitination and sumoylation which alter the overall chromatin structure and binding properties of histone proteins (Strahl and Allis, 2000; Muhlbacher et al., 2006; Sanchez and Gutierrez, 2009). These combined PTMs of histone proteins serve as a "histone code" which directs gene expression by engaging transcriptional machinery (Strahl and Allis, 2000). Acetylation is the best and most widely studied PTM of histones and is characterized by the neutralization of the positive charges of amino groups of lysine residues by a group of enzymes known as histone acetyl transferases (HATs), which transfer an acetyl group from acetyl coenzyme A to the lysine residues of the histone tail (Tanner et al., 1999; Tanner et al., 2000b; Tanner et al., 2000a; Lau et al., 2000; Hebbes et al., 1988). The acetylation of histones is a reversible process, and the enzymes that mediate the reversal process are known

as histone deacetylases (HDACs). Histone acetylation is generally associated with the activation of transcription by recruiting transcription factors and RNA polymerase II and is considered a mark of the active state of chromatin or euchromatin (Mujtaba et al., 2007). CREB binding protein (CBP) is one of the best examples of HAT activity in regulation of transcription in learning and memory (Oliveira et al., 2007; Alarcon et al., 2004; Korzus et al., 2004; Martin and Sun, 2004; Vecsey et al., 2007).

Like acetylation, histone methylation is another epigenetic tag, catalyzed by histone methyl transferases (HMTs) which transfer up to three methyl groups from S-adenosine methionine to the lysine residue of the histone tail (Murray, 1964). However, in contrast to acetylation, a seemingly reversible process, methylation is a stable process and involved in long-term maintenance of genes (Cheung and Lau, 2005; Peters and Schubeler, 2005). Another important feature of histone methylation is its dual functional nature in transcription activation or repression depending upon methylation pattern. For example, H3-lys 4 methylation causes transcriptional activation, whereas H3-lys 9 methylation is associated with suppression of transcription (Binda et al., 2010).

Histone phosphorylation, specifically H3, has gained more attention since it is associated with condensation of chromosomes during mitosis (Bradbury et al., 1973; Gurley et al., 1974; Gurley et al., 1978). H3 phosphorylation was first reported in response to the activation of mitogenic signaling pathways

(Mahadevan et al., 1991). Phosphorylation of H3 on Ser 10 residue is mediated by ribosomal protein S6 kinase 2 (RSK2), which is downstream of several other kinases, including extracellular signal-regulated kinase (ERK), mitogen- and stress-activated protein kinase 1 (MSK1), and the aurora kinase family member increase in ploidy 1 (IPL1) (Sassone-Corsi et al., 1999; Thomson et al., 1999; Hsu et al., 2000). Recent studies also indicate aurora kinases in H3 Serine 28 phosphorylation (Goto et al., 2002). Histone phosphorylation is a reversible process in which phosphatases remove phosphate groups from histones (Ajiro et al., 1996; Mahadevan et al., 1991). Protein phosphatases 1 (PP1) and 2A (PP2A) have been indicated to regulate H3 phosphorylation (Hsu et al., 2000; Nowak et al., 2003). Taken together, H3 phosphorylation works in concert with other histone modifications to modulate essential cellular functions by regulating transcriptional machinery to bind with the chromatin molecule.

Histone protein 2A (H2A) is the first of its kind identified to be ubiquitylated in the cell (Goldknopf et al., 1975). Like other proteins, histone proteins such as H1, H2A, H2B and H3 are ubiquitylated through the addition of an ubiquitine on the amino terminal of the lysine residue (Goldknopf et al., 1975; West and Bonner, 1980; Chen et al., 1998a; Pham and Sauer, 2000). Ubiquitylated histones take part in transcription regulation (Ogawa et al., 2002; Gearhart et al., 2006) and many other cellular processes.

1.6.3 Epigenetics in the nervous system

Neurons are structurally and functionally different than other cells of the body because of the presence of a neuron-restrictive silencer element (NRSE) portion at their promoter region which can completely silence a gene in non-neuronal cells (Maue et al., 1990; Li et al., 1993; Mori et al., 1992). The repression of neuron-specific gene expression in non-neuronal cells is achieved through interplay of a transcription factor known as RE1-silencing transcription factor (REST), which presents ubiquitously in non-neuronal cells (Chong et al., 1995) and NRSE. Experimentally, deleting REST in non-neuronal tissue causes lethal expression of neuron-specific genes whereas ectopic expression of REST in the nervous system leads to silencing of neuron-specific genes and developmental disorders (Chong et al., 1995; Chen et al., 1998b; Paquette et al., 2000). Another important feature of neuronal phenotype determination is the requirement for two other identical expressed transcription co-repressors such as REST binding protein SIN3A and REST co-repressor (Co-REST) (Andres et al., 1999; Abrajano et al., 2009; Lakowski et al., 2006; Battaglioli et al., 2002; Huang et al., 1999; Naruse et al., 1999). Interestingly, chromatin modification through epigenetic mechanisms such as histone acetylation/deacetylation and DNA methylation is required for REST-dependent gene silencing in such a way that REST/SIN3A repressor complexes are associated with HDAC1 whereas REST/Co-REST complexes are associated with HDAC2 (Naruse et al., 1999; Huang et al., 1999; Grimes et al., 2000; Roopra et al., 2000; Qureshi et al., 2010). This complicated action of REST causes either

a decrease in histone acetylation or an increase in DNA methylation to epigenetically mark the neuronal gene.

1.6.4 Inhibitors of epigenetic modifications

With recent advancements in pharmacology, it is now possible to design and screen multiple small molecules which target specific kinases of epigenetic mechanisms such as DNA methylation and histone modification (acetylation, deacetylation, methylation, and phosphorylation). Currently available DNMT inhibitors (5-AZA and zebularine) are cytosine analogues with similar modes of action (Christman, 2002; Stresemann et al., 2006; Stresemann and Lyko, 2008). These compounds are rapidly incorporated into DNA during replication and interfere with covalent binding of DNMTs with DNA which leads to demethylation and gene reactivation (Christman, 2002; Liu et al., 2003; Momparler, 2005; Zhou et al., 2002; Cheng et al., 2004; Weisenberger et al., 2004). With FDA approval (Issa, 2005), ongoing clinical trials have shown promising results in treatment of diseases including myelodysplastic syndrome (MDS) and other leukemias (Gore et al., 2006; Tsujioka et al., 2013; Ghoshal and Bai, 2007) using these drugs.

A number of HAT family members, such as the p300/CBP family and PCAF family (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996) have been identified to regulate gene expression (Mizzen and Allis, 1998; Struhl, 1998) through acetylation of histones (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996) or other substrates (Imhof et al., 1997; Gu and

Roeder, 1997). Several cell permeable, small molecule modulators with minor homology in sequence and structures have been designed to specifically inhibit HATs (Vetting et al., 2005). Prior to the specific HAT inhibitors, several cell-impermeable non-specific HAT inhibitors, such as polyamine CoA conjugates (Cullis et al., 1982; Erwin et al., 1984) and natural plant derivatives (Balasubramanyam et al., 2003; Balasubramanyam et al., 2004a; Balasubramanyam et al., 2004b) were found to block HAT activities. Despite their application in past studies, many challenges still exist in terms of the potency, bio-availability and cell permeability of these drugs until recently. I used a more selective p300/CBP HAT inhibitor known as C646 to probe the role of histone acetylation. C646 is a reversible, cell-permeable p300/CBP HAT inhibitor (Ki = 400 nM), which competes with acetyl-CoA for the p300 Lys-CoA binding pocket (Bowers et al., 2010). The steady state level of histone acetylation depends on the balance between the activity of HATs that add acetyl groups and HDACs that remove acetyl groups from histone proteins. The HDACs are broadly divided into two classes of isoforms. The class I isoform includes HDACs 1, 2, 3, and 8, while class II are HDAC isoforms 4, 6, 9, 10 and 11. There are several commonly used HDAC inhibitors (HDI): trichostatin A (TSA) inhibits both class I and class II; sodium butyrate (NAB) and suberoylanilide hydroxamic acid (SAHA) are specific for class I. HDI blocks the reversible removal of acetyl groups from the lysine residue of the histone tail resulting in hyperacetylation of histones and altered gene expression (Thiagalingam et al., 2003; Dokmanovic et al., 2007; Martinez-Iglesias et al., 2008; Marks et al., 2000; Xu et al., 2007).

Phosphorylation at Ser 10, Ser 28, and at Ser 11 position of the histone (H3) is associated with condensation of chromosomes in mammalian cells (Gurley et al., 1978; Goto et al., 2002; Goto et al., 1999; Preuss et al., 2003). The Aurora kinase family of enzymes has been found to be involved in H3 phosphorylation at Ser 10 (de la Barre et al., 2000; De Souza et al., 2000; Giet and Glover, 2001). Phosphorylation at Ser 10 then recruits HATs and HMTs to activate transcription. I used the recently developed, specific Aurora kinase B inhibitor AZD1152 (Mori et al., 2011) to probe its role in histone phosphorylation.

1.6.5 Influence of upstream signaling in epigenetic regulation

The influence of upstream signaling on gene expression and cellular differentiation has been shown in both non-neuronal and neuronal tissue. Signaling through the MAPK pathway (Bading and Greenberg, 1991; English and Sweatt, 1996; Fiore et al., 1993) is one such influence. The MAPK pathway is critical for learning and memory (Atkins et al., 1998; Schafe et al., 2000; Sweatt, 2001). The ERK/MAPK pathway is the central integrating mechanism of many posttranslational modification of histones (Brami-Cherrier et al., 2009; Borrelli et al., 2008; Reul et al., 2009; Swank and Sweatt, 2001). One of the mechanisms of ERK is phosphorylation of the transcription factor CREB (Eckel-Mahan et al., 2008; Impey et al., 1998; Roberson et al., 1999) and subsequent recruitment of transcription co-activator CBP (Vecsey et al., 2007), which has intrinsic HAT activity. It has been shown that this ERK/MAPK pathway is also involved in histone (H3) acetylation and phosphorylation through mitogen- and stress-

activated protein kinase 1 (MSK1) (Levenson et al., 2004a; Chwang et al., 2006a; Chwang et al., 2007; Davie, 2003). More recently, the enhancing effect of HDAC inhibitors on object recognition memory was prevented by PKA inhibitors (Roozendaal et al., 2010) which again implies that specific signaling is required to alter chromatin structure and to enhance memory. Likewise, DNA methylation also appears to be dependent on the ERK/MAPK pathway, as evident by impaired DNA methylation with an intrahippocampal injection of a NMDA receptor antagonist (Lubin et al., 2008; Miller et al., 2008) and decreased DNMT3 expression with ERK/MAPK inhibition in amygdala (Monsey et al., 2011). Overall, these findings indicate a regulated mechanism in which upstream extracellular or environmental influences can be read out through a specific signaling cascade (ERK/MAPK) to induce transcription and epigenetic modification of chromatin for the purpose of memory formation.

1.6.6 Epigenetic code in synaptic plasticity and learning and memory

As previously discussed, the epigenetic code consists of two important modification of chromatin structure: DNA methylation and post translational modification (i.e., acetylation, deacetylation, phosphorylation, methylation) of histone proteins. I will discuss how these mechanisms play a role in synaptic plasticity and learning and memory. For the purpose of simplicity, my discussion will be limited to only those epigenetic mechanisms whose role in NE-mediated synaptic plasticity I probed. Griffith and Mahler (1969) first proposed the role of DNA modification in memory storage. The principle behind this postulation was

that DNA acts as an information storage unit upon continuous molecular turnover. Supporting this view, Crick (1984) postulated a mechanistic theory of preservation of information in DNA through a maintenance molecule (matching the function of DNMT1) against constant dissipation of acquired changes by molecular turnover. Holliday (1999) supported and extended this theory by suggesting that modification of the cytosine residues of DNA provides stability for long term memory storage. Later on, several studies showed an active DNA methylation in several brain regions (Feng et al., 2005; Feng and Fan, 2009; Feng et al., 2010; Hutnick et al., 2009; Veldic et al., 2005) in a time-dependent way. Recent studies also indicate a cortical layer specific distribution of DNMTs in the adult human brain (Veldic et al., 2005; Veldic et al., 2004). The presence of DNMTs in post-mitotic neurons raises the question of their role in the adult brain. To this end several neuroscientists have begun to address this question by investigating the role of DNMTs in learning and memory. Early studies found a change in DNA methylation of genes in the hippocampus upon learning (Miller and Sweatt, 2007; Lubin et al., 2008). Specifically, the upregulation of DNMT gene expression in the hippocampus has been found in the contextual fear conditioning test and inhibiting DNMT expression interfered with contextual fear memory formation (Lubin et al., 2008; Feng et al., 2010; Miller and Sweatt, 2007). Furthermore, a global inhibition of DNA methylation by DNMT inhibitors modifies methylation of specific memory-related genes including *reelin, bdnf* and *protein phosphatase 1* (*PP1*) and hence alters synaptic plasticity and learning and memory (Levenson et al., 2006a; Lubin et al., 2008; Miller and Sweatt, 2007).

Since LTP is thought to be a cellular signature of memory formation, it was expected that epigenetic modifications such as DNA methylation should alter, or be altered by, stimuli inducing plasticity. Indeed, the DNA methylation of memory enhancing gene *bdnf* has been found to be altered by synaptic depolarization (Chen et al., 2003; Martinowich et al., 2003). Since maintenance of remote memory requires separate structures such as the anterior cingulate cortex (Frankland et al., 2004), a recent study investigated the role of DNA methylation in the maintenance of remote memory (Miller et al., 2010). In this study, intracortical infusion of DNMT antagonists 29 days after training blocked memory retention. Observations from the above study indicated that altered DNA methylation of memory-inducing (*reelin* and *bdnf*) and memory-repressing (*PP1*) gene promoters in the CNS occurs in memory formation and retention. Furthermore, several novel studies also indicated DNMT3A and DNMT3B as demethylating enzymes (Kangaspeska et al., 2008; Metivier et al., 2008) and hence complicated our understanding of DNA methylation in learning and memory. In addition, other researchers reported the Gadd45 family as a key regulator of DNA demethylation in the CNS (Barreto et al., 2007; Ma et al., 2009a).

In biological systems, it is difficult to establish the incidence of one event independent of others. Likewise, it has been shown that DNA methylation and histone modification work in parallel to regulate transcription in the formation and storage of memory in the rat hippocampus (Barrett and Wood, 2008; Graff and

Mansuy, 2008; Lubin and Sweatt, 2007; Wood et al., 2006b). The cross talk between DNA methylation and histone modification has been nicely demonstrated in a recent study in which hypermethylation of the Zif268 gene promoter is correlated with an increase in H3-methylation upon contextual fear conditioning (Gupta et al., 2010a). Taken together, the balance between methylation and demethylation of DNA and the coordinated action of DNA methylation and histone modifications may engage several transcription molecules not understood properly, to form and maintain memory.

Recently, several studies have indicated a role for post translational modification (PTM) of histone proteins in synaptic plasticity and learning and memory (Levenson and Sweatt, 2005; Reul and Chandramohan, 2007; Levenson et al., 2004a; Barrett and Wood, 2008; Graff and Mansuy, 2008; Roth and Sweatt, 2009). Prior to mammalian studies, several groups used *Aplysia* and crab models to elucidate the role of histone acetylation in memory formation. The *Aplysia* model has been used to demonstrate a role of 5-HT in memory formation by facilitating synaptic responses (Kandel, 2001). It was later shown that 5-HT also induces acetylation of H3 and H4 proteins at the C/EBP promoter region (Guan et al., 2002). Inhibition of HDACs by TSA causes long-term facilitation (LTF) with just 1 pulse of 5-HT, which proves that 5-HT induces LTF by regulating histone acetylation or deacetylation activity. In another study using the crab model, Federman et al. (2009) demonstrated that strong training in the context-signal memory paradigm enhances LTM formation by inducing H3 acetylation.

Interestingly, inhibition of HDACs by TSA also causes formation of LTM of a weak training protocol. Taken together, these studies suggest a role for histone acetylation and deacetylation in memory formation in invertebrates.

Using other experimental paradigms, studies have focused on histone acetylation and deacetylation in LTP modulation in the mammalian hippocampus to study memory formation. Investigating the role of HDAC inhibition on LTP modulation, Levenson et. al. in 2004 found that induction and maintenance of L-LTP by HDAC inhibition is transcription dependent. In another study, Vecsey et. al (2007) found that pairing a sub-threshold stimulus with HDAC inhibitor induces a PKA/CREB transcription-dependent L-LTP in hippocampal CA1 region. The CREB-binding protein (CBP) has intrinsic HAT activity and it has been found that CBP +/- mice are L-LTP deficient with normal E-LTP (Alarcon et al., 2004). Interestingly, HDAC inhibition was able to restore L-LTP, which indicates that the reduced L-LTP in those mice is due to a deficiency of HAT activity. In addition, it is also reported that application of TSA enhanced forskolin-induced LTP in amygdalar slices. Since LTP is thought to be a cellular mechanism of memory formation, these studies show that acetylation and deacetylation of histones play a major role in hippocampal and amygdalar synaptic plasticity, as well as memory formation.

Behaviourally, the contextual fear conditioning test in rodents has served as a model to study formation of LTM in mammals. It has been found that contextual

fear conditioning in rodents is associated with transient increase of H3 acetylation while H4 acetylation remains unchanged, and the LTM formation in this contextual test is NMDA and ERK2 dependent (Levenson et al., 2004). In addition, injection of HDAC inhibitor 1 hour before contextual fear conditioning caused increased freezing behavior when assessed 24 hrs after the test, suggesting long-term fear-enhanced memory formation.

CBP, with its intrinsic HAT activity, recruits many other transcriptional co-activators to induce gene transcription. Heterozygous mutation of CBP causes cognitive disorders including Rubinstein-Taybi syndrome, characterized by severe mental retardation (Petrij et al., 1995). Considerable advances in genetic engineering allow us to alter specific genes of interest. Using this, Korzus et al. (2004) have generated transgenic mice carrying a dominant-negative CBP transgene which specifically blocks HAT activity with an inducible tet system. These mice were deficient in declarative and spatial memory formation, while their contextual fear memory formation was intact. The behavioral phenotype was reversible upon turning off the transgene. Similarly, Alarcon et al. (2004) used CBP $^{+/-}$ heterozygous mice to assess the role of CBP HAT activity in memory formation. They have found that CBP $^{+/-}$ heterozygous mice froze less than control animals in the contextual fear conditioning test, but showed no difference in latency and path length in MWM spatial memory test. Administration of the HDAC inhibitor restored the deficit in LTM formation in both transgenic and mutant mice. Beside CBP, two other transcriptional co-activators, p300 and

p300/CBP associated factor (PCAF), also have acetyltransferase activity and play roles in LTM formation (Maurice et al., 2008; Olivera et al., 2007). These studies underpin the importance of CBP and other transcription co-activators with HAT activity in gene transcription in memory formation.

Many other studies also examine the role of histone acetylation in memory formation. Training for eye-blink conditioning and object recognition memory induces H3 acetylation, and inhibition of HDAC causes enhanced memory formation with this training (Fontan-Lozano et al., 2008). Several studies have found an increase in histone acetylation in the BDNF promoter region in the hippocampus and the prefrontal cortex upon a fear conditioning test (Lubin et al., 2008; Bredy et al., 2007). More recently, it was shown that a weak training stimulus which is unable to form LTM, when paired with HDAC inhibitor, induces LTM formation (Stefanko et al., 2009). This is in line with the observation of Vecsey et al. (2007) who reported that a single train of high frequency stimuli which normally generates E-LTP, can induce transcription-dependent L-LTP when paired with HDAC inhibitors. Protein phosphatase 1 (PP1) acts as a memory suppressor gene and inhibition of PP1 has been shown to induce acetylation of H2B, H3 and H4 to promote LTM formation in the MWM task and object recognition test (Koshibu et al., 2009). Thus, this study underlines a mechanistic way in which histone acetylation by PP1 could support LTM formation. Considering the cross talk between DNA methylation and histone acetylation, it has been found that inhibition of DNMTs blocks training induced

H3 acetylation which could be rescued with HDAC inhibition (Miller et al., 2008), which points to a complex interaction between these two mechanisms in memory formation. These studies also suggest that HDAC might act as a negative constraint on memory formation (Abel et al., 1998). Indeed, accumulating evidence support this by showing that overexpression of the HDAC2 gene impaired, but deficiency of HDAC2 enhanced LTP as well as memory formation (Guan et al., 2009).

Histone phosphorylation is another PTM which provides a unique epigenetic mark to regulate chromatin dynamics (Graff and Mansuy, 2008). In this regard, the mitogen-and stress-activated protein kinase 1 (MSK1) plays a major role in bringing on the function of histone phosphorylation. Consistent with this, germline knockout of MSK1 impairs long-term spatial and contextual fear memory formation, leaving cued fear memory intact (Chwang et al., 2007). In contrast to the previous findings, HDAC inhibitors failed to rescue the memory deficit in MSK1 knockout mice, suggesting a critical interrelation between histone acetylation and phosphorylation through a common upstream regulator of both. In addition to MSK, another kinase complex known as the IκB kinase (IKK) complex also regulates histone phosphorylation in the hippocampus (Lubin and Sweatt, 2007). Taken together, these studies indicate a critical role of histone kinases in memory formation.

1.6.7 Epigenetics and neurodegenerative diseases

There is considerable evidence to indicate the role of epigenetic mechanisms in human cognition and behaviour. Rubinstein-Taybi syndrome (RTS) is an inherited autosomal-dominant disorder resulting from the mutation of transcription co-activators CBP HAT (Petrij et al., 1995; Blough et al., 2000). Several studies on animals indicate that CBP deficiency is a crucial factor in deficiency of memory formation in those animals and hence imply a molecular basis for RTS in humans (Korzus et al., 2004; Alarcon et al., 2004; Oike et al., 1999; Bourtchouladze et al., 2003). Rett syndrome (RT) is another inherited X-linked disease due to mutation of MECP2 (Ellaway and Christodoulou, 2001; Sirianni et al., 1998; Amir et al., 1999; Chen et al., 2001). Overexpression of MECP2 in animals causes an enhancement of hippocampal LTP as well as LTM formation (Collins et al., 2004). Fragile X syndrome is the most common form of inherited mental retardation, due to an abnormal sequence in the FMR1 and FMR2 genes (Turner et al., 1996; Ashley et al., 1993). The abnormal expansion of the CGG or CCG trinucleotide in Fragile X syndrome results in increased DNA methylation and histone acetylation that eventually leads to transcriptional silencing of FMR genes (Gecz et al., 1996; Gu et al., 1996). Alzheimer's disease (AD) is the most common form of dementia due to an increase in accumulation of soluble β-amyloid peptide in the brain (Kuo et al., 1996). Some of the pathology of AD is due to dysregulation of histone acetylation (Sastre et al., 2001; Kimberly et al., 2001; Cao and Sudhof, 2001; von Rotz et al., 2004). Finally, schizophrenia is a serious cognitive disorder, leaving individuals almost incapable of

maintaining social life and performing daily cognitive activity. Extensive research has indicated that deficiency of memory promoting genes such as *reelin*, is the causative factor in schizophrenia (Costa et al., 2002). The promoter region of *reelin* contains several sites of DNA methylation, and inhibitors of HDACs and DNMTs increase *reelin* expression, supporting the notion that different epigenetic mechanisms regulate *reelin* expression (Chen et al., 2002). These findings indicate that alterations in epigenetic marks of the genome can cause severe abnormality in gene expression, which eventually leads to cognitive impairment.

1.7 Noradrenergic neuromodulation in synaptic plasticity and learning and memory

Communication between neurons is achieved by synaptic transmission in which neuroactive chemicals, upon binding to their receptors, generate a brief and temporally-restricted post-synaptic potential. Neuromodulators, in contrast, are a class of neuroactive agents that act upon either pre-or post-synaptic sites without generating synaptic potentials. These neuromodulators act through second messenger systems, instead of binding to a ligand-gated ion channel as in case of neurotransmitters, to bring widespread and long-lasting cellular effects (Hasselmo, 1995). Recently, it has been reported that neuromodulators too can generate synaptic potentials like neurotransmitters, which opens up a new avenue for further research on neurophysiology. Irrespective of their mode of action, these modulators are important candidates for normal and pathological brain function.

1.7.1 Noradrenergic system in the mammalian brain

The central noradrenergic neurons originate primarily from the locus coeruleus (LC) and lateral tegmental regions (Cooper et al., 2003), and project diffusely to the cerebral cortex, midbrain, thalamus, cerebellum, amygdala, hippocampus (Moore and Bloom, 1979; Morrison and Foote, 1986; Barone et al., 1981) and many subcortical structures. This wide noradrenergic projection supports the idea of its role in information procession mediated through activity of different brain regions (Berridge and Waterhouse, 2003).

1.7.2 Noradrenaline biosynthesis

Noradrenaline, also called norepinephrine (NE), is a catecholamine which has multiple roles in the body as a hormone or neurotransmitter in the central nervous system (Vogt, 1954). NE is released from the sympathetic nervous system as a neurotransmitter and increases heart rate and force of contraction. The hormonal effects of NE consist of the fight or flight response upon its release from the adrenal medulla.

NE is synthesized from dopamine through the enzymatic action of dopamine β-hydroxylase in the chromaffin cells of the adrenal medulla. The rate-limiting step in the biosynthesis of NE is tyrosine hydroxylase, which is again regulated by PKC, PKA and CAMKII. This allows a short term alteration of NE synthesis. The stepwise biosynthesis of NE is shown in the next figure **(Fig. 1.8).**

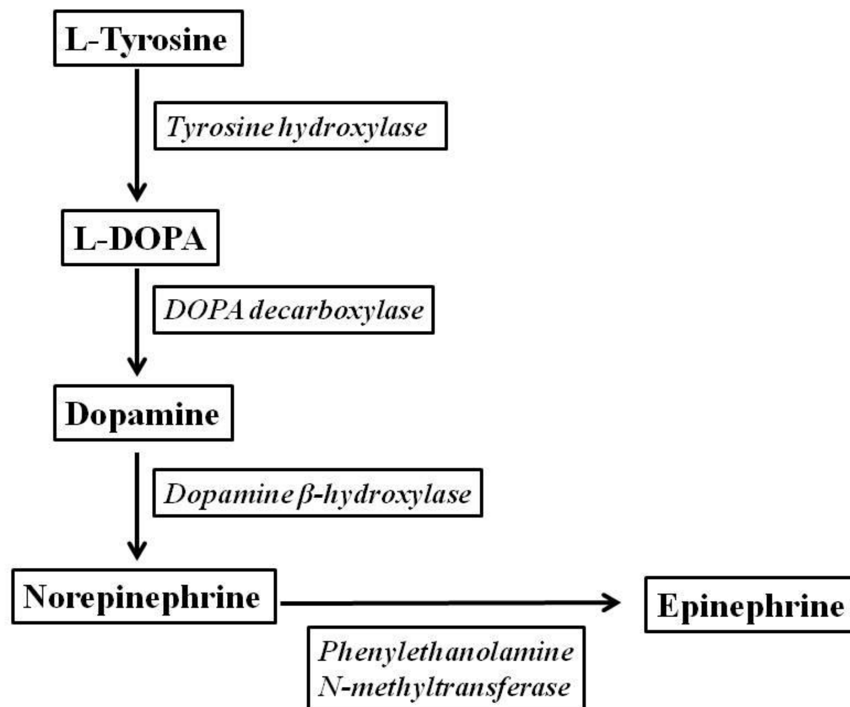


Fig. 1.8: Biosynthesis of norepinephrine

NE is rapidly degraded once it is released presynaptically by calcium-dependent exocytosis. The release of NE is regulated by presynaptic autoreceptors which can monitor the concentration of NE at the synaptic cleft (Langer, 1976; Dixon et al., 1979).

1.7.3 Noradrenergic receptor subtypes

NE receptors are a large family of G-protein coupled receptors which initiate diverse physiological actions depending upon the type of subunit attached to it **(Fig. 1.9).** Based on the diverse actions of NE, the receptors are classified as α1-, α2- and β-adrenergic receptors.

α1-adrenergic receptors are diffusely distributed in the CNS neuron (Domyancic and Morilak, 1997), glia (Lerea and McCarthy, 1989), interneurons and subclassified again into α_{1A}, α_{1B}, and α_{1D} subtypes based upon their affinity to various pharmacological agents and their physiological actions upon activation (Harrison et al., 1991; Mizobe, 1997). Interestingly, these receptors are present in pyramidal and granular cells of various hippocampal regions (Jones et al., 1985; Day et al., 1997; Pieribone et al., 1994; Nicholas et al., 1993). These receptors are present in the human brain at CA3 and the dentate gyrus region (Zilles et al., 1991). Generally, α1-adrenergic receptors are coupled to the $G_{q/11}$ protein, which, upon activation, cleaves the membrane protein phospholipase C into the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG). These second messengers then mediate diverse physiological actions by elevating the

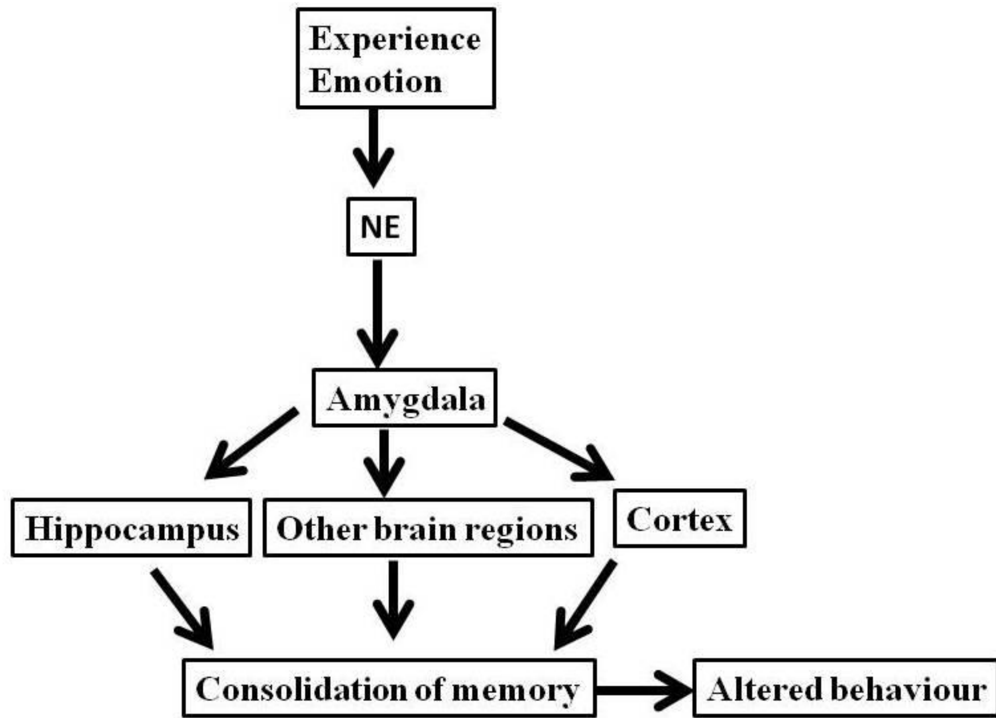


Fig. 1.9: Noradrenergic regulation of memory (adapted and modified from McGaugh, 2000)

intracellular calcium concentration from the cellular calcium store (Cotecchia et al., 1990; Sirvio and MacDonald, 1999). The α2 receptors can be further divided into α_{2A}, α_{2B}, and α_{2C} subtypes depending upon sensitivity to pharmacological activation and tissue-specific distribution (Harrison et al., 1991). In hippocampus, these receptors are mainly located at presynaptic sites to regulate release of NE (Dismukes et al., 1977), though their presence in the dendritic spines of hippocampal neurons and glial cells have also been observed (Milner et al., 1998). These receptors are coupled to the inhibitory G-protein G_i and thus reduce the activity of adenylyl cyclase, which leads to a decrease in intracellular cAMP concentration (Dismukes and Mulder, 1976).

β-adrenergic receptors are coupled to the G_s stimulatory protein to induce intracellular signaling, and are broadly classified into β1-, β2-, and β3-subtypes depending upon reactivity with specific agonists. For example, β1 and β2 ARs are more reactive to the agonist isoproterenol than the endogenous agonists NA or adrenaline, while β3 responds more potently to NA. These receptors are present in various regions of the CNS, including the cortex, hippocampus, amygdala, thalamus, and cerebellum (Nicholas et al., 1993; Wanaka et al., 1989). The human brain has a subregional specificity of β-AR expression (Reznikoff et al., 1986). For example, pyramidal cells and dentate granular cells contain mainly β1- and β2-ARs, whereas glial cells in the CA1 region contain only β2-ARs. Additionally, interneurons do not have β-ARs. These receptors have a common second messenger, cAMP, which is enhanced upon activation of β-ARs and causes

physiological actions (Raymond, 1995; Morris and Malbon, 1999). These different adrenergic receptors have physiological functions in different hippocampal regions, which is discussed next.

1.7.4 Functional aspects of adrenergic receptors in hippocampal neurons

1.7.4.1 α-adrenergic receptors and hippocampal neuronal excitability

The α1-adrenergic receptors mediate cell-specific functions in the hippocampus. Activation of these receptors generally decreases the excitability (Harley, 1991; Pang and Rose, 1987; Mynlieff and Dunwiddie, 1988) of principal neurons in the hippocampal regions of dentate gyrus, CA3 and CA1 as evidenced by lowered population spike amplitude. The possible mechanism for this phenomenon is activation of inhibitory interneurons in the hippocampus which in turn hyperpolarize the principal neurons. When released from the LC, noradrenaline causes a biphasic response in pyramidal neurons: an initial suppression, which is thought to be due to α1-AR activation, followed by activation of pyramidal neurons, which is β-AR-mediated (Curet and de, 1988). In line with this, this biphasic action of NA has also been observed in *in vitro* studies. The specific action of ARs is concentration-dependent; high concentrations of NA activate α1, and lower concentrations activate the β-AR (Rutecki, 1995; Mueller et al., 1981; Mueller et al., 1982). The α1-ARs have a minor role in synaptic plasticity in the dentate gyrus as they do not cause changes in field potential recordings (Chaulk and Harley, 1998). In the CA3 region, it causes presynaptic inhibition and thus affects LTP induction at mossy fibre synapses as LTP is presynaptic in this region

(Zalutsky and Nicoll, 1990). Activation of α1-ARs, however, has a different action in the CA1 region of hippocampus. When paired with weak electrical stimuli, α1-AR agonists facilitate LTP induction as well as maintenance (Izumi and Zorumski, 1999; Pussinen and Sirvio, 1998). Together with β-ARs, α1-ARs confer the immunity to depotentiation of LTP when induced by low-frequency stimulation (Katsuki et al., 1997). These receptors are also involved in LTD in the CA1 region when a high concentration of NA or α1-agonists is applied (Scheiderer et al., 2004). Many interneurons also express α1-ARs, and activation of these receptors by NA or α1-agonists causes depolarization of these cells, which eventually influence the firing properties of pyramidal neurons in the CA1 region (Bergles et al., 1996). The α2-ARs of CA3 and CA1 regions suppress the firing properties of pyramidal neurons; studies suggest that this post-synaptic effect of α2-ARs is extrasynaptic (Curet and de, 1988). In the hippocampus, α2-ARs mediating synaptic plasticity are mainly presynaptic, as these receptors act as auto receptors at the presynaptic terminal to regulate NA release.

1.7.4.2 β-adrenergic receptors and hippocampal neuronal excitability

Stimulation of β-adrenergic receptors modulates many cellular functions in synaptic plasticity, and is one of the primary mechanisms through which the brain noradrenergic system consolidates new information. The rise of the intracellular calcium concentration through calcium channels is critical for cell signaling pathways including PKC and CAMKII (Malenka and Bear, 2004). β-adrenergic receptors can enhance calcium influx through NMDA receptors during excitatory

synaptic transmission (Raman et al., 1996; Vanhoose and Winder, 2003). β-adrenergic receptors also modulate the properties of VDCCs and hence cellular calcium dynamics (Fisher and Johnston, 1990; Hoogland and Saggau, 2004). Therefore, β-adrenergic receptors are an important component in the regulation of calcium dynamics in the dendrites of hippocampal neurons. Besides its role in calcium dynamics, many researchers also indicate a role of β-adrenergic receptors in the phosphorylation of the GluR1 subunit of AMPA receptors (Vanhoose and Winder, 2003; Vanhoose et al., 2006), which has a major role in LTP and its β-adrenergic modulation (Shi et al., 2001).

Stimulation of β-ARs generally increases the excitability of hippocampal principle neurons. This increased excitability can be seen as an enhancement of fEPSP population spikes, as in the case of application of NE in the dentate gyrus region (Stanton and Sarvey, 1987), and also in the areas of CA3 and CA1 (Dunwiddie et al., 1992). The effect of β-AR stimulation is pathway-specific in the hippocampal region, suggesting a distinct role in information processing in various hippocampal subregions (Dahl and Sarvey, 1989).

Electrical stimulation of the medial and lateral perforant pathway requires β-AR activation to generate LTP in the dentate gyrus region (Munro et al., 2001). However, the population spike is unaffected while β-AR antagonists inhibit HFS induced EPSPs in that region, suggesting a distinct mechanism of action for modulating plasticity in the dentate gyrus region. LTP in the CA3 region is β-AR

dependent as blockade of these receptors prevents L-LTP expression and maintenance by multiple trains of HFS (Huang and Kandel, 1996). Correspondingly, β-AR stimulation causes a frequency-dependent increase in the magnitude, duration and probability of LTP induction (Hopkins and Johnston, 1988; Hopkins and Johnston, 1984). Conversely, in area CA1, the induction of LTP by HFS does not require β-AR activation (Dunwiddie et al., 1982; Murchison et al., 2004; Swanson-Park et al., 1999; Sarvey et al., 1989). In addition, β-AR agonists do not alter baseline synaptic activity persistently (Thomas et al., 1996). In the CA1 area, β-AR stimulation modulates the effects of LFS on synaptic strength. Pairing LFS with β-AR activation overcomes the inhibitory effects of protein phosphatases to induce LTP (Thomas et al., 1996; Winder et al., 1999). The CA1 pyramidal cells fire bursts of action potentials known as "complex spikes", which could also enhance LTP during β-AR stimulation in a PKA-dependent manner (Hoffman and Johnston, 1999). Furthermore, β-AR-mediated LTP enhancement is observed during theta-burst stimulation, an *in vivo* firing pattern in the pyramidal cells of rodents during spatial navigation (Otto et al., 1991; Swanson-Park et al., 1999). Norepinephrine can alter synaptic responses by influencing the state of the synapse in a process known as metaplasticity (Abraham, 1999). In this process, β-ARs inhibit the metaplastic mechanisms, and permit subsequent LTP induction at previously activated synapses (Moody et al., 1999). Taken together, these studies suggest that β-ARs recruit various distinct mechanisms such as channel modification, neuronal

excitation and metaplastic processes in hippocampal subregions to induce and express LTP.

Consistent with its role in LTM formation, β-AR antagonists impair the formation of both spatial and contextual fear memory (Sara, 2009; Ji et al., 2003; Cahill et al., 2000). Conversely, β-AR activation facilitates the hippocampal long-term potentiation necessary for memory formation (Kemp and Manahan-Vaughan, 2008). It has been implicated that β-ARs play a crucial role in memory retrieval (Cahill et al., 1994; Barros et al., 2001; Murchison et al., 2004). A recent study found that restoring NE levels in genetically mutant mice lacking NE improved memory retrieval through a β-AR mechanism (Murchison et al., 2011). Direct injection of NE into the hippocampus preferentially facilitates LTM consolidation (Izquierdo et al., 1998). Additionally, β-ARs also have a role in extinction, a mechanism of formation of new associations to an altered stimulus pattern (Ouyang and Thomas, 2005). Thus, β-ARs engage various physiological mechanisms to encode and store new information.

1.8 Objectives of the current study

Neuromodulatory influences are diffuse in the mammalian brain and strongly influence the cellular and molecular mechanisms of brain functions. Many behavioural or cognitive outcomes of the nervous system are thought to be due to the influences of various neuromodulators on the dynamics of ion channels, the biophysical properties of neurons as a unit, or alteration of function of the neural network as a whole. Learning and memory is a higher cognitive function of the brain which is impaired in many conditions. Norepinephrine, a neuromodulator, is involved in the consolidation and retrieval of memory in humans and other animals. In many neurodegenerative disorders, the level of neuromodulators like NE is altered and causes memory-related cognitive impairment. In addition, emerging studies suggest a role for epigenetic mechanisms in cognitive disorders related to memory impairment. Thus, the present study would be able to narrow down a unique noradrenergic signaling cascade and epigenetic regulation of genes which could be recruited upon a novel experience and facilitate memory encoding. The current study will facilitate the understanding of the different cellular and genetic mechanisms responsible for NE-related cognitive disorders associated with learning and memory deficits, and could have important therapeutic implications.

To this end, my first objective was to characterize the role of NE in long-term synaptic potentiation in area CA1 of mice hippocampal slices. I looked for requirements of macromolecular synthesis, such as translation and transcription,

in NE-induced synaptic plasticity. The second objective was to determine the engagement of epigenetic mechanisms, with the primary focus on histone acetylation in NE-induced LTP. Specifically, I attempted to address the following questions for my thesis:

1. Does NE facilitate the induction and maintenance of LTP in the CA1 area?

2. Which receptor subtype(s) are involved in NE-LTP?

3. Is there any specific intracellular signaling molecule(s) that is recruited upon NE-LTP?

4. Does activation of beta-adrenergic receptors by NE engage translation and transcription mechanisms to stabilize LTP?

5. Are epigenetic mechanisms involved in NE-LTP? If so, then what role do they play?

Chapter 2: Materials and methods

2.1 Animals

Male C57BL/6 mice (7-12 weeks) were used for all experiments described in this thesis for their robustness in hippocampal synaptic plasticity and other behavioral learning and memory tests (Schimanski et al., 2002). Animals were housed in the University of Alberta's animal facility center under the guidelines of the Canadian Council on Animal Care (CCAC). Animals were kept on a 12-hr light/dark cycle, with all experiments conducted during the light portion of the cycle. Animals received no environmental enrichment in cages.

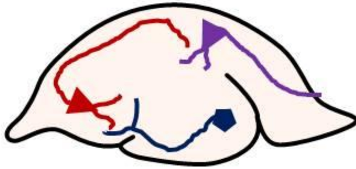
2.2 Hippocampal slice preparation and two pathway extracellular field potential recording

Following cervical dislocation and decapitation, the intact brain was removed quickly and placed in a beaker of ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1.0 $NaH2PO_4$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 glucose, aerated with 95% O_2 and 5% CO_2. After an initial few minutes of cooling, the brain was hemisected into two lobes containing two hippocampi and one hippocampus was removed from its surrounding tissue and placed on a manual tissue chopper (Stoelting, Wood Dale, IL, USA). Transverse hippocampal slices (400 µM) were collected and transferred to an interface recording chamber (for details of method, please refer to Nguyen and Kandel, 1997) and maintained at 30°C with the help of a heating coil within the recording chamber. The slices were under continuous perfusion (1-2 mL/min) of

ACSF. Electrophysiological recording of extracellular field potentials (fEPSPs) from slices began after an initial 90 min recovery period. A glass microelectrode (pulled by a borosilicate electrode puller, resistances of 2–3 MΩ) filled with aCSF was positioned in the stratum radiatum of area CA1 and fEPSPs were recorded. The pyramidal cell layer of CA1 acts as a visual guide for electrode replacement during experimental set up **(Fig. 2.1).** The hippocampal Schaffer collateral commissural fibers were stimulated at two separate sets of inputs (S1 & S2) converging onto the same postsynaptic population of neurons using two bipolar nickel-chromium electrodes (diameter 130 µm; AM Systems, Carlsborg, WA, USA). fEPSPs of 40% of maximal amplitude (Gelinas and Nguyen, 2007; Woo and Nguyen, 2003) were evoked by adjusting the stimulus intensity (0.08 ms pulse duration) and constituted our baseline responses. Subsequent fEPSPs were obtained at the rate of once per minute at this test stimulation intensity, with S2 stimulation following S1 stimulation by 200 ms. To confirm independence of pathways, interpathway paired-pulse facilitation elicited by successive stimulation through the two electrodes (S1 & S2) at 50, 100, 150 and 200 ms intervals was used and the absence of paired-pulse facilitation was used as the criterion to determine stimulation of independent pathways.

After establishing a 20 min baseline recording, NE-LTP was induced on S1 alone through application of one train of high-frequency stimulation (HFS; 100 Hz, 1 s duration at test strength) following a 10 min application of NE (10 µM). fEPSPs were measured as percentage of baseline, and these measurements were used as

C57BL/6 Mouse



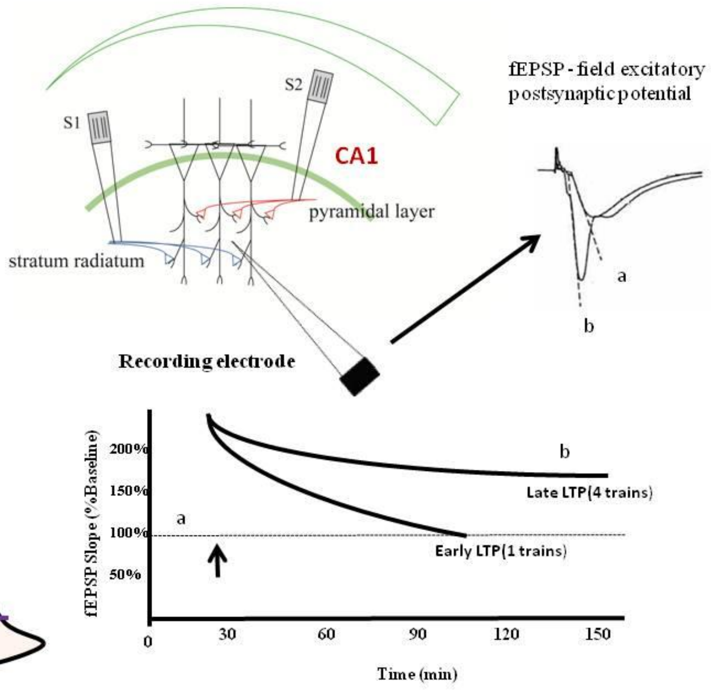


Fig. 2.1: Field EPSP and LTP recording model

an indicator of LTP in response to 1 x 100 Hz stimulation as this procedure induces early LTP (E-LTP) in mouse hippocampal slices (Duffy et al., 2001). NE was applied for an additional 5 mins following HFS.

All fEPSPs were measured by an amplifier and low pass filtered at 2 kHz. Responses were then digitized at a rate of 20 kHz by a Digidata 1200 system and recordings were analyzed offline with pClamp 10 software (Axon Instrument Inc., Union City, CA, USA).

2.3 Tissue collection and quantitative Western blot

Hippocampal slices were prepared and placed on the recording chamber as previously described. Following drug application or electrical stimulation, slices were harvested from the recording chamber. Area CA1 of the slices was dissected by a razor blade under an upright dissecting microscope **(Fig. 2.2).** CA1 subregions were then flash-frozen in liquid nitrogen and stored at -80°C until assayed as described in Gelinas and Nguyen, 2007.

2.4 Data analysis

Axon Clampex (10.2, Molecular Devices) was used for fEPSP analysis. The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu, 1995). fEPSP slopes were averaged from 20 min of stable baseline recording to obtain a baseline mean value for each experiment. All subsequent slopes were expressed as percentages of these baseline slopes. To compare LTP levels between two groups, I used data points at 120 or 180 min after LTP

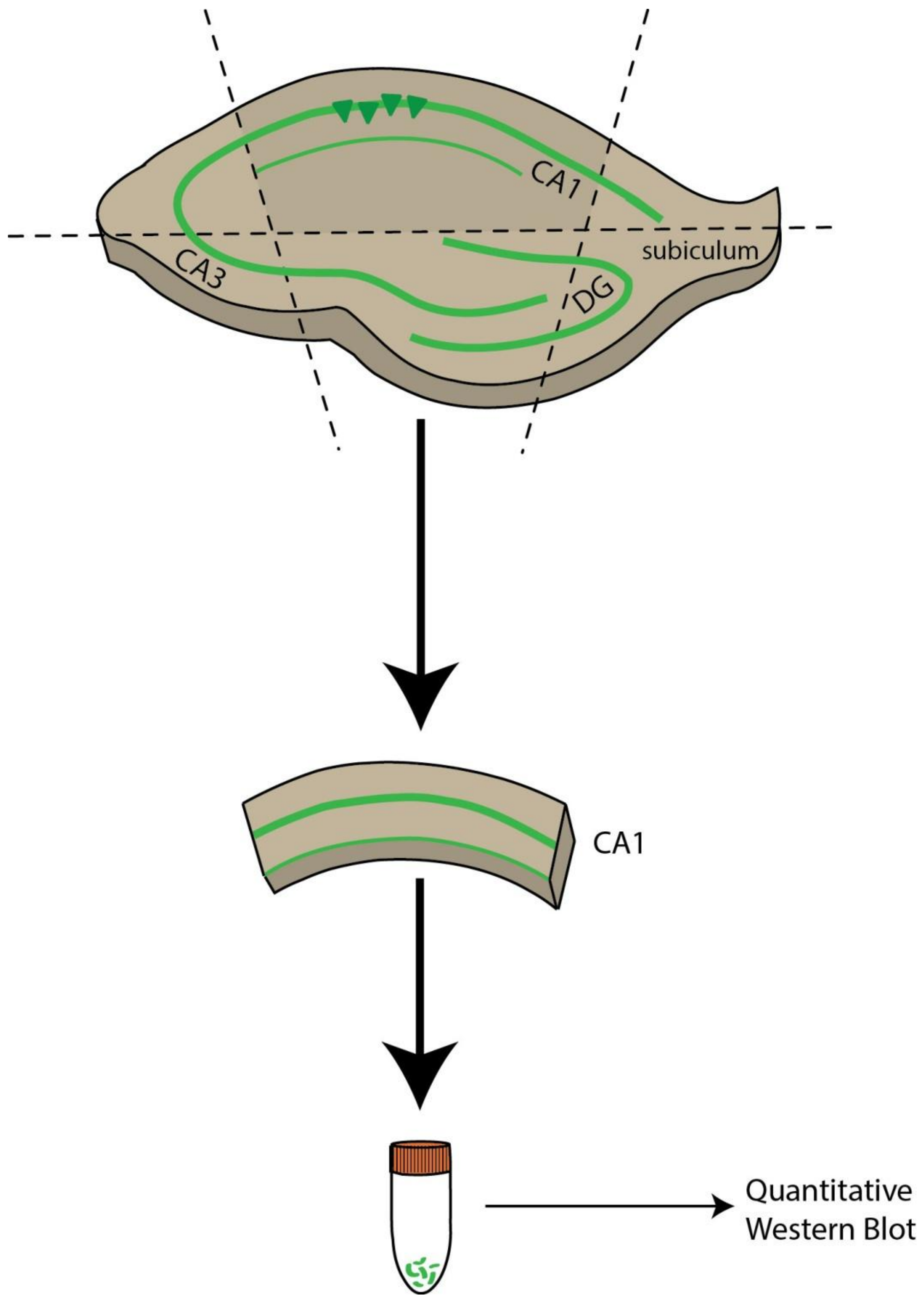


Fig. 2.2: Tissue collection and quantitative Western Blot

induction. Student's *t* test was used for statistical comparison of mean fEPSP slopes between two groups, with a Welch correction if standard deviations were significantly different between groups. $p < 0.05$ was set as criteria of significance in all experiments. One-way ANOVAs (Graphpad Instat Software, San Diego, CA, USA) were conducted to determine significant difference between more than two groups. Subsequent Tukey-Kramer post-hoc tests were performed to determine which groups were significantly different from others. Data are reported as means ± SE, with n = number of slices.

2.5 Drugs and protocol

The drugs and their concentrations used in my experiments are shown in **table 1**. Drugs were dissolved in appropriate solutions according to MSDS and literature. Stock solutions of drugs were stored at -20°C. Aliquots of stock solution were thawed, mixed properly, and diluted in ACSF to the final working concentration immediately before experimentation. Fresh stock solutions of NE were made daily to avoid oxidization by oxidizing agents. NE was applied for a total duration of 15 min, starting 10 min before 100Hz stimulation protocol. Drugs such as antagonists and inhibitors were applied 20 min before NE application and were present during NE application and 10 min after NE application. Due to light sensitivity of the drugs, experiments were done under dimmed light conditions.

Name	known for	abbreviation	company	stock concentration	working concentration
L-(-)-norepinephrine bitartrate salt monohydrate	adrenergic receptor agonist	NE	Sigma	1mM in aCSF	10 µM
ICI 118,551 hydrochloride	β2 antagonist	ICI	Sigma	1mM in aCSF	1 µM
Betaxolol hydrochloride	β1 antagonist	Betax	Sigma	1mM in aCSF	1 µM
Prazosine hydrochloride	α1 antagonist	Prazo	Sigma	150 µM in dH_2O	10 µM
Yohimbine hydrochloride	α2 antagonist	Yohim	Sigma	1mM	3 µM
DL-2-amino-5-phosphonopentanoic acid	NMDA receptor antagonist	APV	Sigma	50 mM in aCSF	50 µM
PKI 14-22 Amide	Cell permeable PKA antagonist	PKI	Calbiochem	1 mM in distilled water	20 µM
Brefeldin A	Epac	BFA	Sigma	50 mM in	50 µM

	inhibitor			DMSO	
ESI09	Epac inhibitor	ESI	Biolog Life Sciences	50 mM in DMSO	10 µM
Anisomycin	protein synthesis inhibitor	Aniso	Sigma	50 mM in DMSO	25 µM
Cycloheximide	protein synthesis inhibitor	CHX	Sigma	25 mM in DMSO	80 µM
Actinomycin D	transcription blocker	Act-D	Sigma	25 mM in DMSO	25 µM
5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside	transcription blocker	DRB	Sigma	50 mM in DMSO	50 µM
5-Aza-2′ deoxycytidine	DNA methylation blocker	Aza	Sigma	100 mM in DMSO	30 µM
Zebularine	DNA methylation blocker	Zeb	Sigma	65 mM in DMSO	25 µM

C646	p300/CBP (HAT) inhibitor	C646	Sigma	10 mM in DMSO	5 µM
AZD1152-HQPA	Aurora kinase B inhibitor	AZD1152	Sigma	5 mM in DMSO	1 µM
PD 98,059	MAPK inhibitor	PD98	Sigma	10 mM in DMSO	50 µM
Trichostatin A	HDAC inhibitor	TSA	Sigma	16.5 mM in DMSO	1.65 µM
Rapamycin	mTOR inhibitor	Rap	Sigma	1 mM in DMSO	1 µM

Table 1: Drugs and concentrations used

Chapter 3: Results

3.1 Norepinephrine facilitates the induction of LTP through mechanisms requiring both β-adrenergic and NMDA receptors

NE has been shown to induce LTP while paired with specific stimulation protocols (Katsuki et al., 1997). Application of HFS alone generates LTP in the CA1 region which returns to pre-stimulation levels within 120 min (Fig. 3.1A: fEPSP slopes were 108 ± 2%); all following statistics are also taken at 120 min post-HFS. To examine the effect of NE (10 μM) on LTP generation, I paired HFS with NE application, which induced LTP that persists for several hours (>3 hr, Fig. 3.1B: fEPSP slopes were 150 ± 11%). To determine if NMDA receptors are required for NE-mediated LTP (NE-LTP), I applied the general NMDA receptor antagonist APV (50 μM) overlapping with NE + HFS. Application of APV inhibited the induction and expression of NE-LTP (Fig. 3.1C: fEPSP slopes were 104 ± 5%). An ANOVA comparing fEPSPs of the three groups (HFS alone, NE + HFS and NE + APV + HFS) revealed a significant difference between groups ($F(2,16) = 6.48$; $p < 0.01$) (fig. 3.1D). Subsequently, a Tukey-Kramer *post hoc* test revealed that persistent LTP was only observed when HFS was paired with NE application, and this LTP was blocked by APV ($p < 0.05$; Fig. 3.1D).

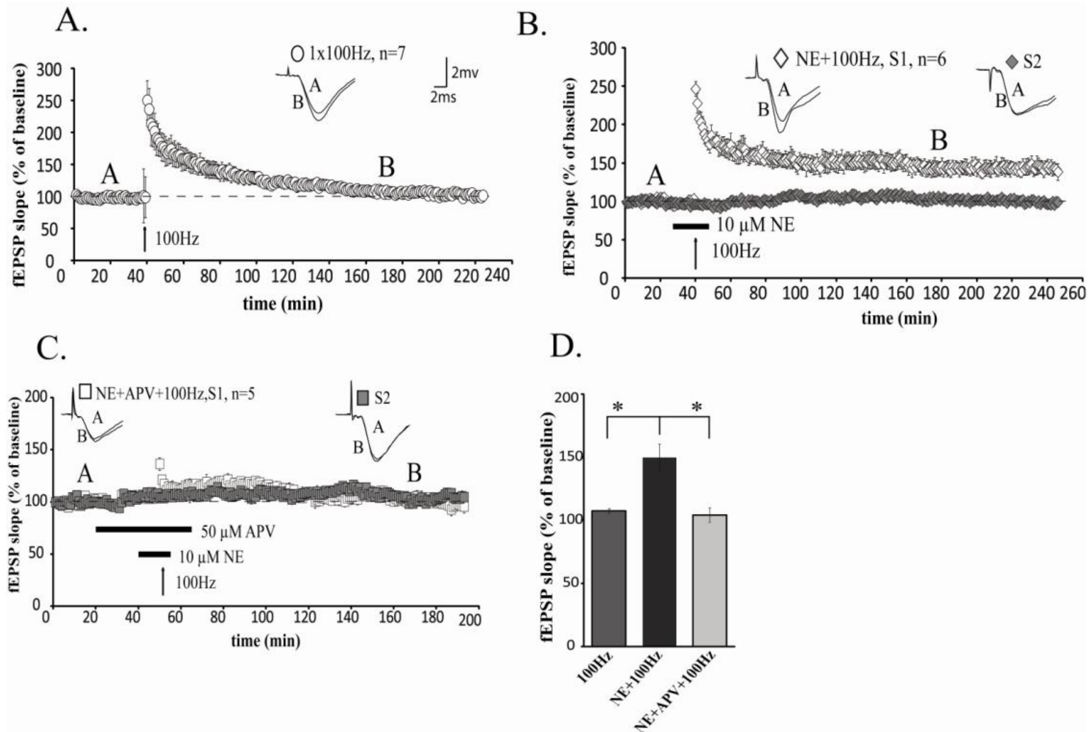


Figure 3.1: **Norepinephrine-induced LTP is maintained for several hours and mediated through NMDA receptors.**

A: 100 Hz stimulation alone (open circles) induces transient (< 2 hr) LTP.
B: Pairing 1 x 100 Hz stimulation with NE application induces L-LTP (S1, open diamonds) which lasts for several hours (>3) after stimulation.
C: Application of the NMDA receptor antagonist APV inhibits LTP generated by pairing 100 Hz stimulation with NE application (S1, open squares).
D: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation at S1. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after stimulation at S1. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in D represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.2 Adrenergic receptor subtypes in NE-LTP

To determine which adrenergic receptors (ARs) are required for NE-induced LTP, I used different NE receptor subtype specific antagonists, overlapping with NE + HFS. NE, when paired with HFS, induced long-lasting LTP (Fig. 3.2A: mean fEPSPs were 154.6 ± 10.8% of baseline; all following statistics given for 120 min post-HFS). Application of the β1-specific antagonist betaxolol (Betax: 1 µM, Fig 3.2B: mean fEPSPs were 101.7 ± 8% of baseline recording) and the β2-specific antagonist ICI 118,551 (ICI: 1 µM, Fig. 3.2C: mean fEPSPs were 106.3 ± 8% of baseline recording) blocked the expression of NE-LTP. In contrast, inhibition of α1-ARs with prazosin (Praz: 10 µM, Fig. 3.2C: mean fEPSPs were 143.7 ± 10% of baseline recording) or α2-ARs with yohimbine (Yohim: 3 µM, Fig. 3.2D: mean fEPSPs were 137 ± 5% of baseline recording), failed to inhibit expression of LTP. An ANOVA comparing fEPSPs of different antagonist-treated groups revealed a significant difference between groups ($F(4,29) = 7.8$; $p < 0.001$) (Fig. 3.2E). Subsequently, a Tukey-Kramer *post hoc* test revealed that persistent LTP was prevented significantly only by overlapping co-application of betaxolol and ICI ($p < 0.01$) but not by prazosin and yohimbine ($p > 0.05$). Thus, NE induces LTP by engaging β- but not α-ARs in mouse hippocampal slices.

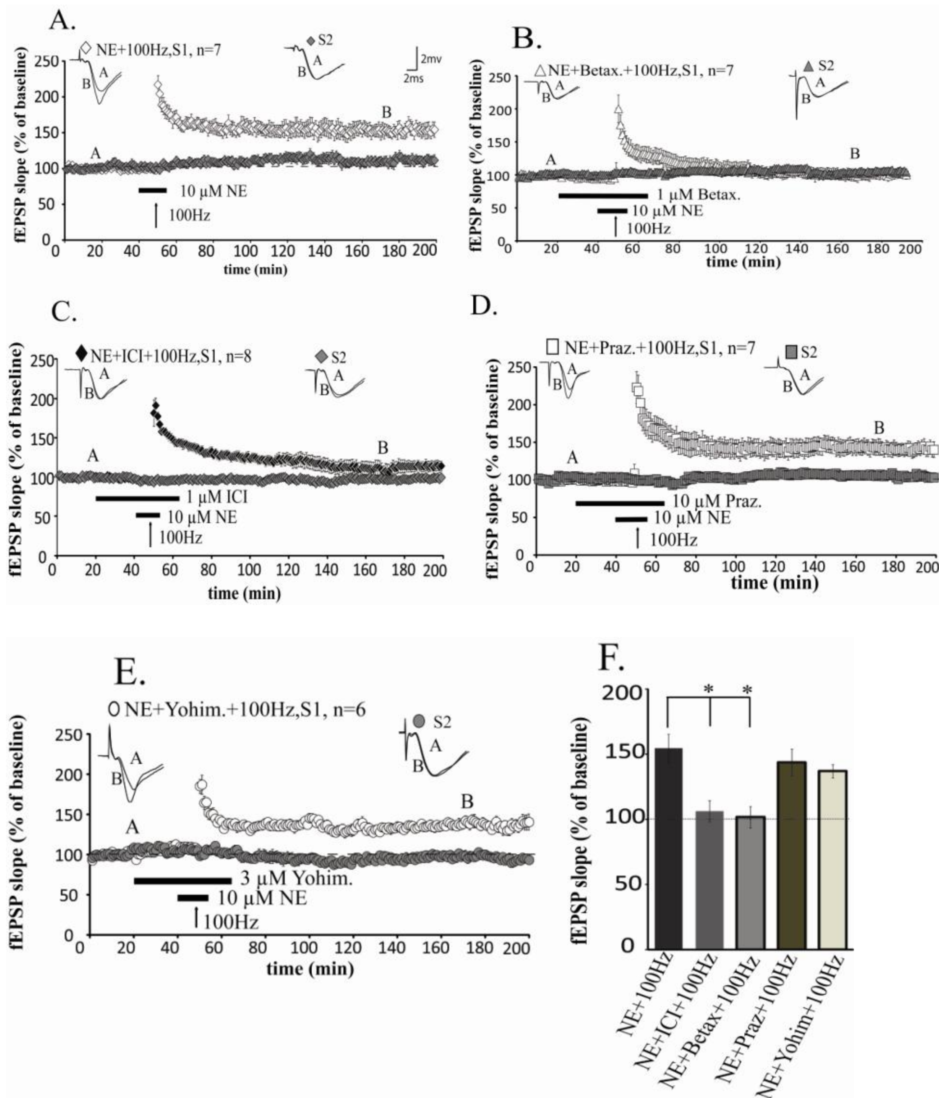


Figure 3.2: LTP elicited by NE application during 100 Hz stimulation requires β-adrenergic receptors but not α.

A: Application of 1 x 100 Hz stimulation paired with NE (open diamonds) elicits long-lasting LTP. β1 adrenergic receptor antagonist betaxolol (B, open triangles) and β2 adrenergic receptor antagonist ICI 118551 (C, filled diamonds) inhibit maintenance of LTP generated by 1 x 100 Hz stimulation with NE application. α1 receptor antagonist prazosin (D, open squares) and α2 receptor antagonist yohimbine (E, open circles) do not inhibit LTP induced by NE + 1 x 100 Hz paired protocol.
F: Summary histogram of fEPSP slopes obtained 120 minutes after 1 x 100 Hz stimulation at S1. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 1 x 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 1 x 100 Hz stimulation. Results in F represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.3 Intracellular signaling pathway for NE- LTP

Previous studies have implicated a role of cAMP dependent protein kinase A (PKA) in β-AR mediated LTP (Thomas et al., 1996) and long-term memory formation (Nayak et al., 1998). To determine if PKA is required for NE induced L-LTP, I co-applied a cell permeable cAMP-dependent protein kinase A (PKA) inhibitor, PKI 14-22 Amide (PKI, 1 μM), with NE + HFS. PKA activation was not necessary for NE-induced synaptic enhancement, as treatment with PKI did not alter the maintenance of NE-LTP (Fig. 3.3B: mean fEPSPs were 148 ± 11% of baseline; all following statistics reported for 120 min post-HFS) compared to PKI free control (Fig. 3.3A: mean fEPSPs were 146 ± 7% of baseline). An alternative route through which cAMP couples to downstream effectors through Epac has been implicated in β-AR-induced LTP (Gelinas et al., 2008) and long-term memory (Ma et al., 2009b). The bath application of the Epac signaling inhibitor brefeldin-A (BFA: 50 μM), returned NE-LTP to baseline levels (Fig. 3.3C: mean fEPSPs were 98.4 ± 4% of baseline recording). I confirmed these results using a membrane permeant, highly specific inhibitor of Epac, ESI-09 (Almahariq et al., 2013) (Fig. 3.3D: mean fEPSPs were reduced to 102 ± 7% of baseline). As a preliminary step toward characterizing the downstream signaling of the cAMP-Epac pathway, I examined the role of MAPK by using PD 98059, a selective inhibitor of MAPK. Pairing HFS with overlapping PD 98059 application inhibited NE-LTP (Fig. 3.3E: mean fEPSPs were reduced to 101.28 ± 5.12% of baseline). An ANOVA comparing fEPSPs of the Epac inhibitor-treated slices with inhibitor-free controls revealed a significant difference between groups

($F(4,31) = 7.84$; $p < 0.01$) (Fig. 3.3F). Subsequent Tukey-Kramer *post hoc* tests revealed that maintenance of NE-LTP was prevented by application of BFA, ESI-09 and PD 98059 ($p < 0.05$) but not by PKI ($p > 0.05$). Thus, these data indicate that NE-mediated synaptic potentiation is dependent upon the Epac intracellular signaling pathway.

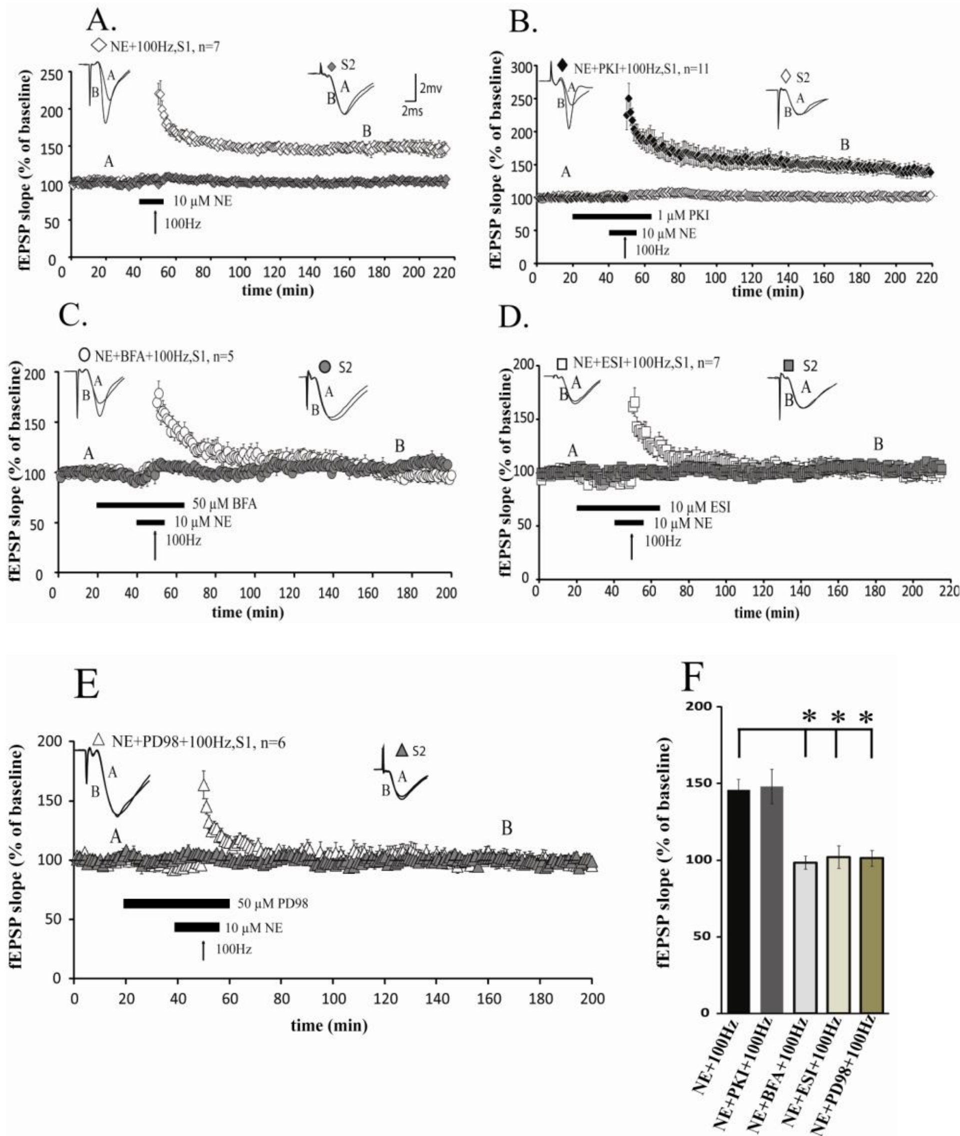


Figure 3.3: L-LTP induced by NE paired with 1 x 100 Hz stimulation requires Epac enzyme activity but not PKA.

A: L- LTP induced by NE paired with 100 Hz stimulation (open diamonds). B: Application of PKI did not cause NE-induced L-LTP to decay (filled diamonds). C: brefeldin A (BFA) inhibits persistence of NE-generated L-LTP maintenance (open circles). D: ESI-09 (ESI) prevented the expression of NE-induced L-LTP (open squares). E: PD98 blocked NE-LTP (open triangles). F: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of PKI, BFA, ESI and RAP on 100 Hz stimulation at S1 paired with NE application. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2)

that did not receive 100 Hz stimulation. Results in E represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.4 mTOR-dependent protein synthesis is upregulated by NE

The mechanisms underlying the persistence of memory and LTP share a requirement for translation regulation (Stanton and Sarvey, 1984; Frey et al., 1988; Nguyen and Kandel, 1996). Furthermore, upregulation of protein synthesis following neuromodulatory receptor activation has been demonstrated (Navakkode et al., 2007; Raymond et al., 2000; Huber et al., 2000). Mammalian target of rapamycin (mTOR) activation is increased by β-AR stimulation during translation-dependent LTP (Gelinas et al., 2007). I sought to determine if NE similarly upregulates mTOR to bolster LTP. Application of the mTOR inhibitor rapamycin (RAP, 1 μM) decreased the maintenance of NE-dependent LTP (Fig. 3.4B: mean fEPSPs were 114 ± 6% of baseline; all the following statistics are reported for 120 min post-HFS). NE-LTP also decayed (Fig. 3.4D: mean fEPSPs were 120 ± 6% of baseline) compared to drug-free control (Fig. 3.4A: mean fEPSPs were 155 ± 10% of baseline) when the translation repressor anisomycin (Aniso: 25 μM) was bath applied with NE + 100 Hz stimulation. The validity of various translation inhibitors has recently been called into question due to off-target effects (Routtenberg and Rekart, 2005; Alberini, 2008). To address this issue, I conducted a second series of experiments with cycloheximide (CHX: 80 μM), another protein synthesis inhibitor which arrests translation through alternative mechanisms. Consistent with anisomycin, CHX treatment resulted in decaying LTP (Fig. 3.4C: mean fEPSPs were 100 ± 3% of baseline). An ANOVA comparing fEPSPs of treatments revealed a significant difference between groups ($F(3,23) = 10.43$; $p < 0.001$) (Fig. 3.4E). Tukey-Kramer *post hoc* test revealed that

inhibiting translation with either anisomycin or CHX reduced the duration of NE-LTP ($p < 0.05$). No significant differences were found between anisomycin and CHX treated groups ($p > 0.05$).

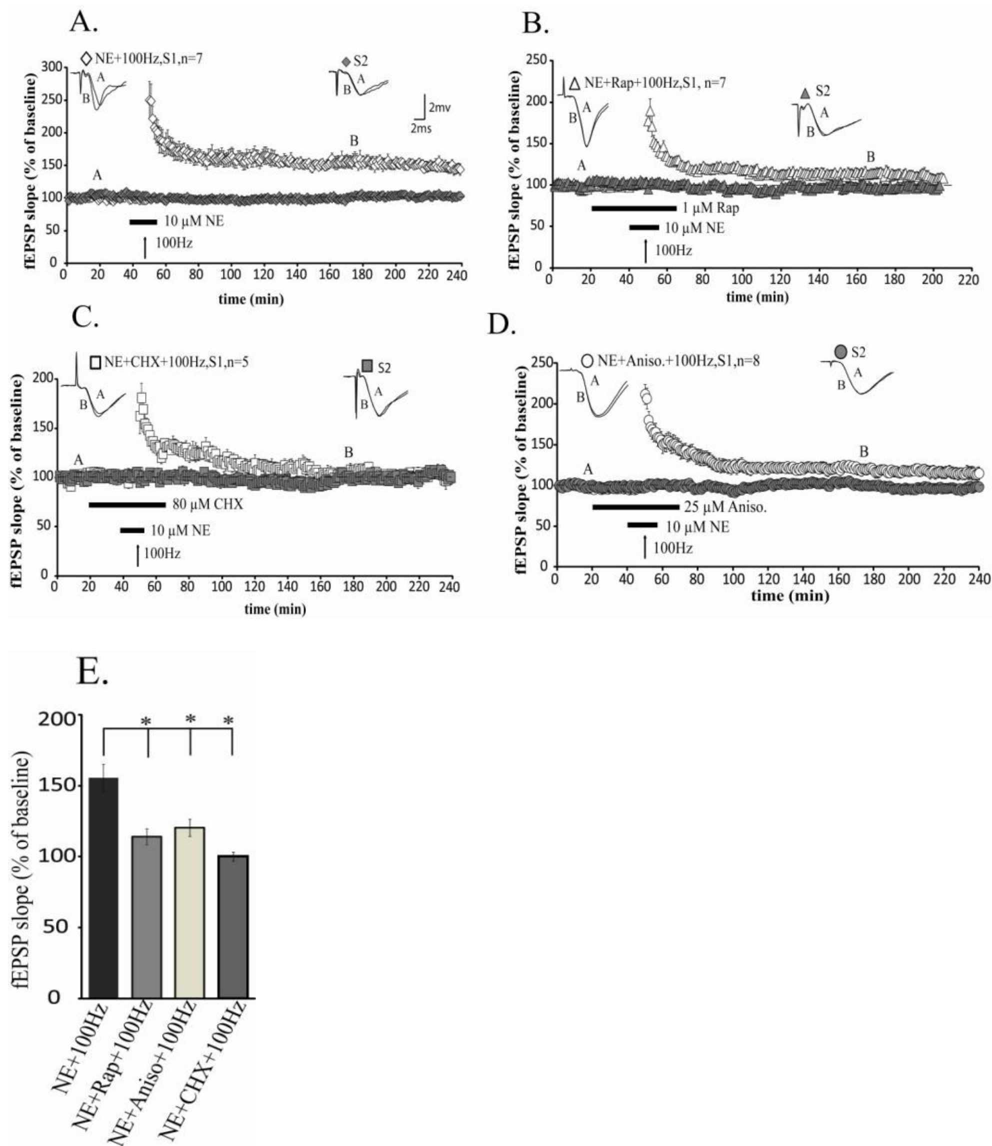


Figure 3.4: Protein synthesis through mTOR is required for NE-induced L-LTP.

A: L-LTP elicited by pairing 1x100 Hz stimulation with NE (open diamonds). B: rapamycin (RAP) caused decaying LTP when applied with NE + 1 x 100 Hz stimulation (open triangles). C: CHX (open squares) and Aniso (D, open circles)) inhibited the maintenance of L-LTP generated by NE paired with 1 x 100 Hz stimulation. E: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of RAP, Aniso and CHX on 100 Hz stimulation paired with NE application at S1. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in E represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.5 Nuclear signaling:

Transcription and NE-LTP

Neuronal stimulation associated with enduring forms of memory and synaptic plasticity results in modified gene expression (Sossin, 1996; Abraham et al., 1993; Nguyen et al., 1994; Frey et al., 1996; Sossin, 1996). To determine if NE stimulation recruits transcriptional components of L-LTP, I applied two different inhibitors of transcription: actinomycin D (Act-D: 25 μM) and 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB: 50 μM). Bath application of Act-D (Fig. 3.5B: mean fEPSPs were 106 ± 7% of baseline; all statistics given for 180 min post-HFS) or DRB (Fig. 3.5C: means fEPSPs were 118 ± 6% of baseline) repressed NE-LTP relative to inhibitor-free controls (Fig. 3.5A: means fEPSPs were 189 ± 16% of baseline). An ANOVA comparing fEPSPs of Act-D & DRB treated slices revealed a significant difference between groups ($F(2,14) = 20$; $p < 0.001$) (Fig. 3.5D). Tukey-Kramer *post hoc* tests revealed that both Act-D and DRB prevented NE-LTP indicative of a transcription-dependent component ($p < 0.05$). No significant difference was observed between Act-D and DRB treated groups ($p > 0.05$).

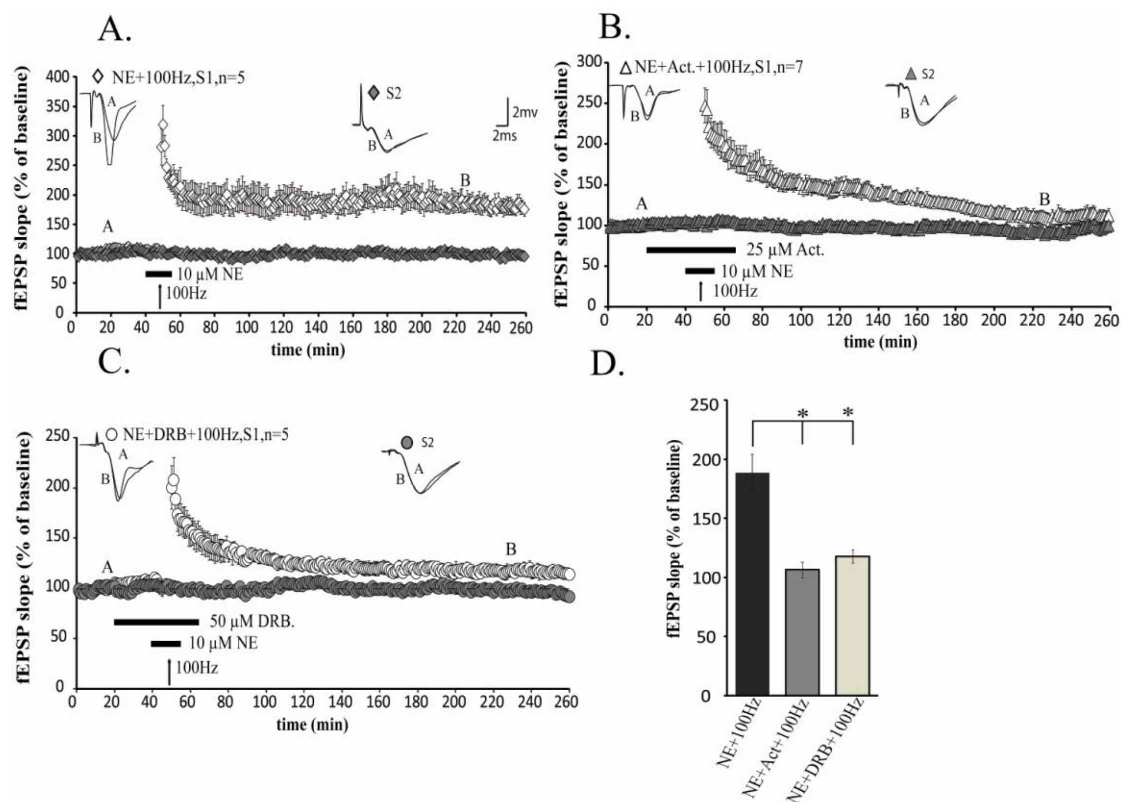


Figure 3.5: L-LTP induced by NE paired with 1 x 100 Hz stimulation is transcription dependent.

A: Pairing 1 x 100 Hz electrical stimulation with NE application elicited L-LTP lasting several (>3) hours (open diamonds). Application of Act-D (B, open triangles) and DRB (C, open circles) prevented the L-LTP maintenance initiated by NE paired with 1 x 100 Hz electrical stimulation. D: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of Act-D and DRB on 100 Hz stimulation paired with NE application at S1. All sample traces were taken 10 min after commencement of baseline recording and 180 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in D represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.6 Epigenetic modification in NE- LTP

Accumulating evidence suggests that epigenetic mechanisms are engaged during synaptic plasticity and learning and memory (Levenson et al., 2004b; Chwang et al., 2006b; Levenson et al., 2006b; Gupta et al., 2010b; Kramer et al., 2011; Monsey et al., 2011; Biergans et al., 2012). Recent evidence in non-neuronal tissue has indicated a putative interaction between noradrenergic receptor stimulation and epigenetic regulation (Ha et al., 2010; Haworth et al., 2012; Li et al., 2012; Chang et al., 2013). To probe a role for regulation of the epigenome in NE-mediated synaptic plasticity, I investigated several prominent epigenetic modifications including DNA methylation, histone acetylation and histone phosphorylation in response to NE + HFS.

DNA methylation in NE-LTP

To determine whether DNA methylation was recruited in NE-mediated synaptic plasticity, I used two specific inhibitors of DNA (cytosine-5) methyltransferases (DNMT): 5-Aza-2′ deoxycytidine (AZA: 30μM) and zebularine (ZEB: 25μM). Treatment of slices with AZA resulted in decremental LTP when co-applied with NE + HFS (Fig. 3.6B: mean fEPSPs were 104 ± 5% of baseline; all statistics given for 120 min post-HFS) compared to AZA-free control (Fig. 3.6A: mean fEPSPs were 141 ± 3% of baseline). Slices exposed to ZEB similarly blocked LTP expression (Fig. 3.6C: mean fEPSPs were 113 ± 6% of baseline). An ANOVA comparing fEPSPs of AZA and ZEB treated slices revealed a significant difference between groups ($F(2,19) = 17$; $p < 0.001$) (Fig. 3.6D). *Post hoc* tests

revealed that both AZA and ZEB significantly reduced the enhancement of L-LTP by NE ($p < 0.05$). AZA and ZEB treated groups did not significantly differ ($p > 0.05$).

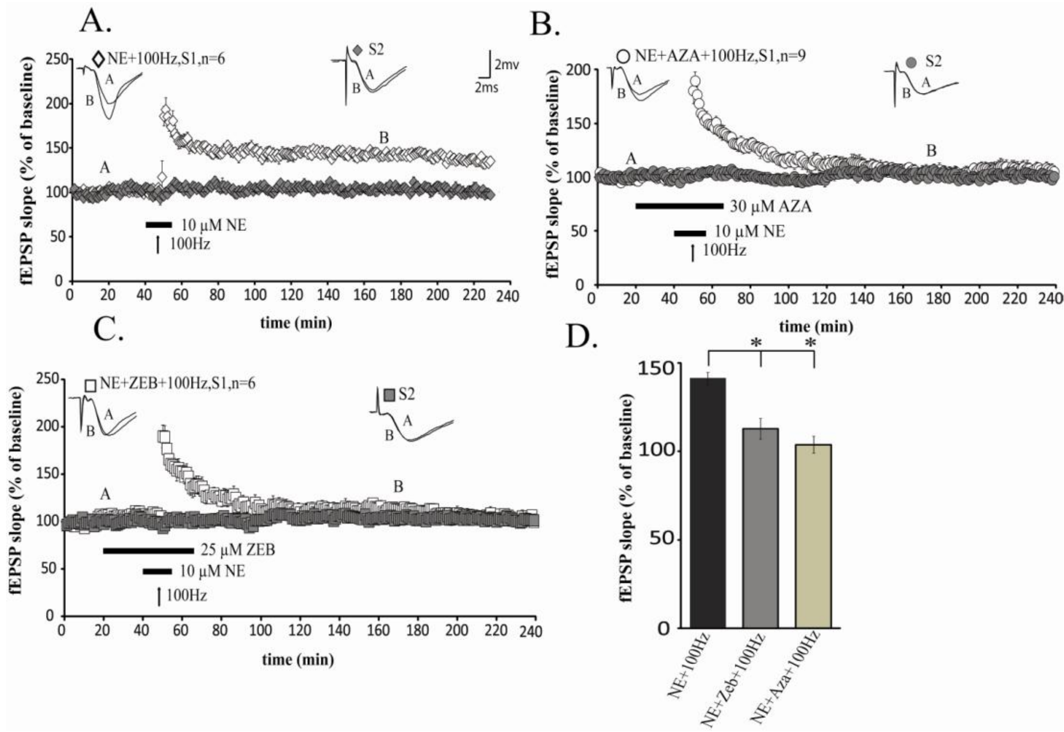
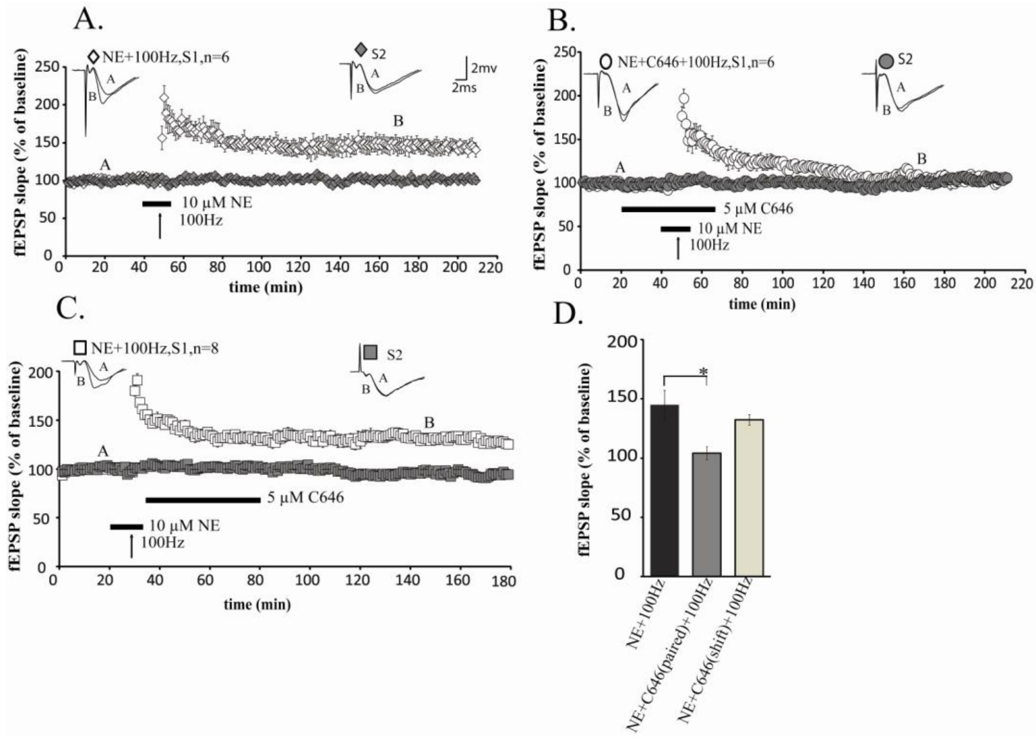


Figure 3.6: L-LTP induced by NE pairing with 1 x 100 Hz stimulation requires DNA methylation.

A: L-LTP elicited by pairing 1 x 100 Hz stimulation with NE (open diamonds). DNA methylation inhibitors AZA (B, open circles)) and ZEB (C, open squares) blocked NE-induced potentiation. D: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of AZA and ZEB on 100 Hz stimulation paired with NE application at S1. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in D represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.7 Acetylation of histones is required for NE-LTP

Recent data suggest intrinsic histone acetyl transferase (HAT) activity of CBP and its homolog (p300) boosts synaptic plasticity and long-term memory formation (Wood et al., 2005; Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2006a; Oliveira et al., 2007; Oliveira et al., 2011). In the present study, I investigated whether acetylation of histone protein is required for maintenance of LTP induced by NE, using a potent and specific CBP/p300 inhibitor, C646 (Bowers et al., 2010). Pairing C646 (5 μM) application with NE + HFS reduced the magnitude of NE-LTP (Fig. 3.7B: mean fEPSPs were 104 ± 5% of baseline; all statistics given for 120 min post-HFS) compared to control slices (Fig. 3.7A: mean fEPSPs were 145 ± 12% of baseline). Shifting C646 application 5 min after NE+HFS had no effect on LTP induced by NE (Fig. 3.7C: mean fEPSPs were 132 ± 4% of baseline). My western blot results complement my electrophysiological data by showing that acetylation of histone (H3) at Lys-14 is enhanced upon NE+HFS compared to control (Fig. 3.7E). An ANOVA was conducted to compare fEPSPs of slices treated with C646, either paired with or shifted relative to NE application. A significant effect of treatment was observed ($F(2,19) = 8$; $p < 0.01$) (Fig. 3.7D). *Post hoc* analysis revealed only a significant decrease ($p < 0.05$) in NE-LTP when C646 application was paired with NE but not when shifted to after NE application ($p < 0.05$). My result indicates that a transient activation of CBP/300 HAT enzymatic activity is sufficient for the induction of L-LTP by NE + HFS.



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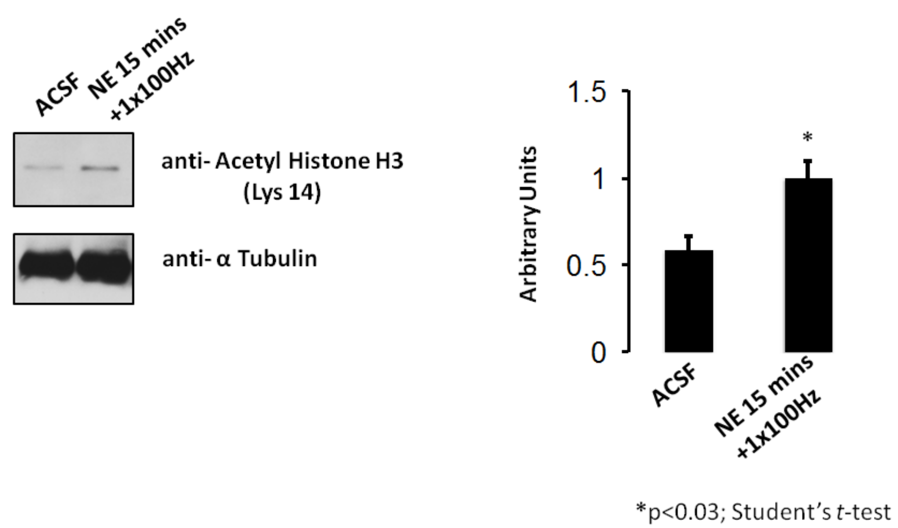

*p<0.03; Student's *t*-test

Figure 3.7: Histone acetylation and its time restricted role in NE-LTP.

A: NE-induced L-LTP maintenance (open diamonds). B: Histone acetyl transferase (HAT) inhibitor C646 prevents L-LTP when co-applied with NE and 1 x 100 Hz stimulation (open circles). C: Shifting C646 application did not block L-LTP induced by NE paired with 1 x 100 Hz stimulation (open suqares). D: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of C646 during and after NE+100 Hz stimulation at S1. E: NE paired with 100 Hz increases H3 acetylation at lys-14 of polypeptide chain. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in D represent means ± SEM, $p < 0.05$. Calibration: 2 mV, 2 ms.

3.8 Inhibiting HDAC does not cause any further enhancement of NE-LTP

Previous research has shown that inhibition of HDACs by TSA causes a stabilization of E-LTP of hippocampal slices induced by HFS which is dependent on transcription. In fact, consolidation of contextual fear memory is also increased by enhancing acetylation of histones while TSA is injected intrahippocampally (Vecsey et al., 2007). Since I have shown that NE-LTP also recruits transcription mechanisms, I wondered if another signaling cascade could be recruited upon adrenergic receptor activation by NE. I used occlusion of NE-LTP by TSA.

The stabilization of NE-LTP (Fig. 3.8A: mean fEPSPs were 130.5 ± 3.6% of baseline; all statistics given for 120 min post-HFS) was not altered compared to either TSA alone (Fig. 3.8B: mean fEPSPs were 132.1 ± 4.1% of baseline) or when TSA was paired with NE application (Fig. 3.8C: mean fEPSPs were 135.1 ± 6.7% of baseline). A one-way ANOVA revealed no significant difference between groups (Fig. 3.8D: $F(2,16) = 0.26$; $p > 0.05$). This occlusion experiment further strengthens the notion that NE-LTP recruits the same signaling pathway as histone acetylation, since the HDAC inhibitor TSA did not enhance NE-LTP any further.

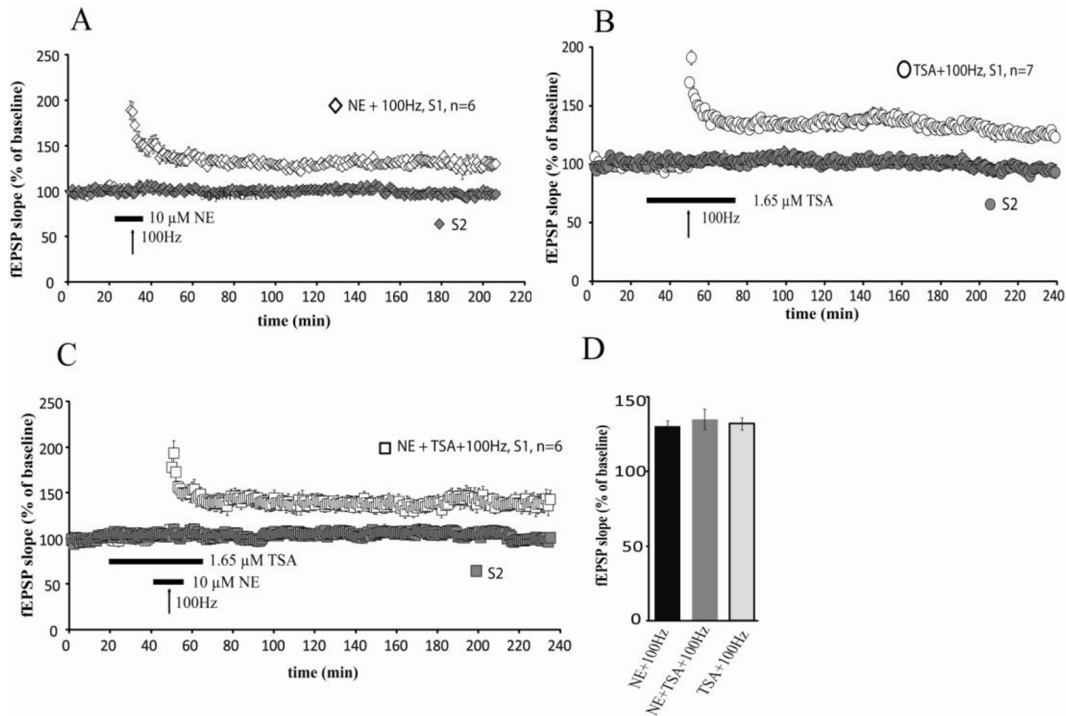

Figure 3.8: Occlusion of NE-LTP with HDAC inhibitor TSA.

The maintenance of NE-LTP (A, open diamonds) was not different from either TSA alone (B, open circles) or TSA paired with NE + 100 Hz (C, open squares). D: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effects of NE alone, TSA alone, and TSA + NE at S1. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in D represent means ± SEM, $p > 0.05$. Calibration: 2 mV, 2 ms.

3.9 Norepinephrine triggers histone phosphorylation through Aurora kinase

To further probe what epigenetic mechanisms are recruited by NE, I assayed histone phosphorylation through a previously identified histone phosphorylation substrate, Aurora kinase. Importantly, Aurora kinase drives transcription of genes associated with synaptic modifications subserving memory genesis through phosphorylation of histones (Wei et al., 1999; Carmena and Earnshaw, 2003). The recent introduction of a novel, highly specific Aurora kinase B inhibitor AZD1152 (AZD) (Mori et al., 2011) allowed me to more directly determine the importance of Aurora kinase B relative to other isoforms of Aurora kinase (which include the Aurora A and C isoforms). Application of AZD (1 μM) overlapping with NE + HFS prevented the maintenance of NE-LTP (Fig. 3.9B: mean fEPSPs were 121.5±5% of baseline; all statistics given for 120 min post-HFS) compared to AZD-free controls (Fig. 3.9A: mean fEPSPs were 155.5 ± 8% of baseline). Student's *T*-test revealed a significant difference between groups (Fig. 3.9C: $p < 0.01$), suggesting that histone phosphorylation by Aurora kinase B is required for NE-induced synaptic plasticity.

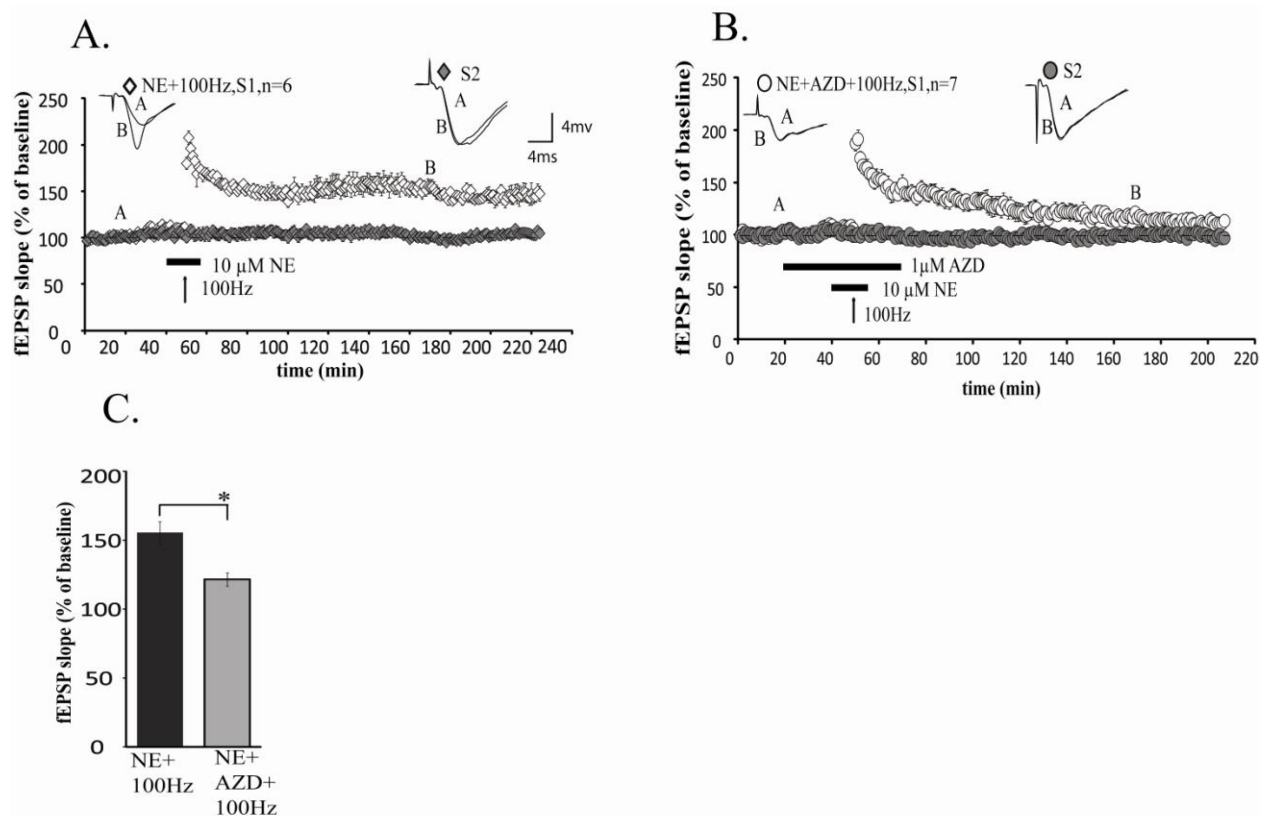

Figure 3.9: Histone phosphorylation is required for NE-induced L-LTP.

A: Pairing 1 x 100 Hz stimulation with NE application causes LTP to maintain for hours (open diamonds). B: Inhibition of histone phosphorylation by AZD1152 (AZD) prevents the maintenance of LTP initiated by NE paired with 1 x 100 Hz stimulation (open circles). C: Summary histogram of fEPSP slopes obtained 120 min after 1 x 100 Hz stimulation comparing effect of AZD on 100 Hz stimulation paired with NE application at S1. All sample traces were taken 10 min after commencement of baseline recording and 120 min after 100 Hz stimulation. The addition of drugs did not alter basal synaptic transmission in a second independent pathway (S2) that did not receive 100 Hz stimulation. Results in C represent means ± SEM, $p < 0.05$. Calibration: 4 mV, 4 ms.

Chapter 4: Discussion

4.1 Noradrenergic modulation of cognitive function

The LC noradrenergic system projects extensively to many other cortical and subcortical structures of the brain. Importantly, it is a part of the brainstem reticular activating system, which controls the sleep-wake cycle and vigilance state of individuals (Roussel et al., 1967; Aston-Jones and Bloom, 1981; Aston-Jones et al., 1991). In fact, inhibition of adrenergic receptors causes a deficit in attention and arousal responses (Stone and Quartermain, 1999). The extensive innervation of the forebrain by the LC noradrenergic system has been implicated in many cognitive functions including attention, sensory information processing, anxiety responses, reorganization of neuronal networks, memory formation and memory retrieval (Berridge and Waterhouse, 2003; Bouret and Sara, 2005). Interestingly, an early theory by Amaral et al. (1977) described the noradrenergic system as the "cognitive arm" of the central nervous system. Korsakoff's syndrome, characterized by memory impairment, was the first pathophysiological study to link LC-noradrenergic function with cognitive ability (Mair and McEntee, 1983). Careful observation of Korsakoff's patients found a significant decrease in 3-methoxy-4-hydroxyphenylglycol (MHPG), the major metabolite of noradrenaline. The model of Korsakoff's syndrome has given rise to the hypothesis of noradrenergic function in mnemonic processing. Subsequently, several studies indicated a central noradrenergic role in memory processing (Brown and Silva, 2004; McGaugh, 2002; Harley, 2004).

4.1.1 Noradrenergic modulation of declarative memory: A role in consolidation and retrieval

Kety (1972) initially proposed a role for noradrenaline in transmission of "novel or significant stimuli" for learning. Later, pharmacological studies indicated that the noradrenergic system in the brain influences the consolidation of memory events (for review, see McGaugh and Roozendaal, 2009). Interestingly, consolidation of memory has been found to be β-AR dependent. For example, intracerebal injection of a β-AR antagonist 2 hr after learning impaired memory in rats when tested 48 hr later. However, there was no effect on memory when the rats were injected immediately after the learning session. This proves that a β-AR-dependent critical time period is required for consolidation of memories (Sara et al., 1999; Tronel et al., 2004). Additionally, the LC-noradrenergic neurons in the rat brain have been shown to fire during slow wave sleep after a period of learning (Eschenko and Sara, 2008). This study strengthens the notion of noradrenergic influence in memory consolidation.

Noradrenaline is also involved in the retrieval of contextual and spatial memories. Increasing noradrenaline release either by pharmacological or electrical activation of LC enhances retrieval of memory in rats (Sara and Devauges, 1989; Sara and Devauges, 1988). This enhancement of memory retrieval is β-AR dependent (Devauges and Sara, 1991). In line with the evidence that the noradrenergic system plays a crucial role in memory retrieval, Murchison et al. (2004), by using the $dbh^{-/-}$ mouse model, found that β-AR signaling is necessary for retrieval of

contextual information. In brief, mice lacking genes for dopamine β-hydroxylase, an enzyme of the noradrenaline biosynthesis pathway, showed a deficit in retention of contextual memory 48 hours after a training session. Interestingly, these mice were able to learn the task perfectly. The deficit in memory retention was restored by injection of a noradrenaline precursor between the training and test sessions. Additionally, injection of propranolol, a β-AR antagonist, before the training or test session impaired the retention of memory after 24 hr of training but not after 1 hr or 1 week. This further proves the selective role of the noradrenergic system in retrieval of recent but not remote memories.

4.1.2 Noradrenergic influence in emotional memory

Adrenergic and noradrenergic hormones have been implicated in the storage of emotionally charged events in animals as well as humans (Gold et al., 1977; Chamberlain et al., 2006). Initial studies found that blocking biosynthesis of noradrenaline in mice results in impairment of retention of aversive memories (Fernandez-Tome et al., 1979; Rainbow et al., 1976; Randt et al., 1971). Meanwhile, Gallagher et al. (1977) reported the same results with an intra-amygdaloid injection of propranolol. Additionally, they also observed that the impairment of memory was reversed upon noradrenaline administration. This suggests that β-ARs are involved in emotional memory retention, which was later supported by Ellis et al. (1983) and Liang et al. (1986).

Subsequently, literature provides evidence of an interaction between the LC-amygdaloid system as well as modulation of emotional memory (Cahill and McGaugh, 1996). Studying healthy human volunteers, Cahill et al. (1994) investigated the effects of propranolol on formation of memories related to either an emotionally charged event or a neutral event. They reported that propranolol selectively impaired the emotional memory but not the neutral one. This further confirms the associativity of β-AR activation with emotional memory formation. Inspired by studies of noradrenergic influence on emotional memory, several groups have investigated and reported that propranolol applied at encoding or retrieval impaired recall for emotional events in humans (For reviews see van Stegeren, 2008; Chamberlain et al., 2006).

4.2 Novel signaling in noradrenaline-induced long term potentiation

Considering the importance of the noradrenergic system in learning and memory across species, I sought to characterize the signaling mechanisms that are involved in NE-induced synaptic plasticity in the mouse. My results indicate a novel signaling mechanism in which NE, upon binding to β-ARs, engages the Epac signaling pathway to recruit translation, transcription and epigenetic mechanisms for endurance of NE-induced synaptic plasticity **(Fig.4.1)**.

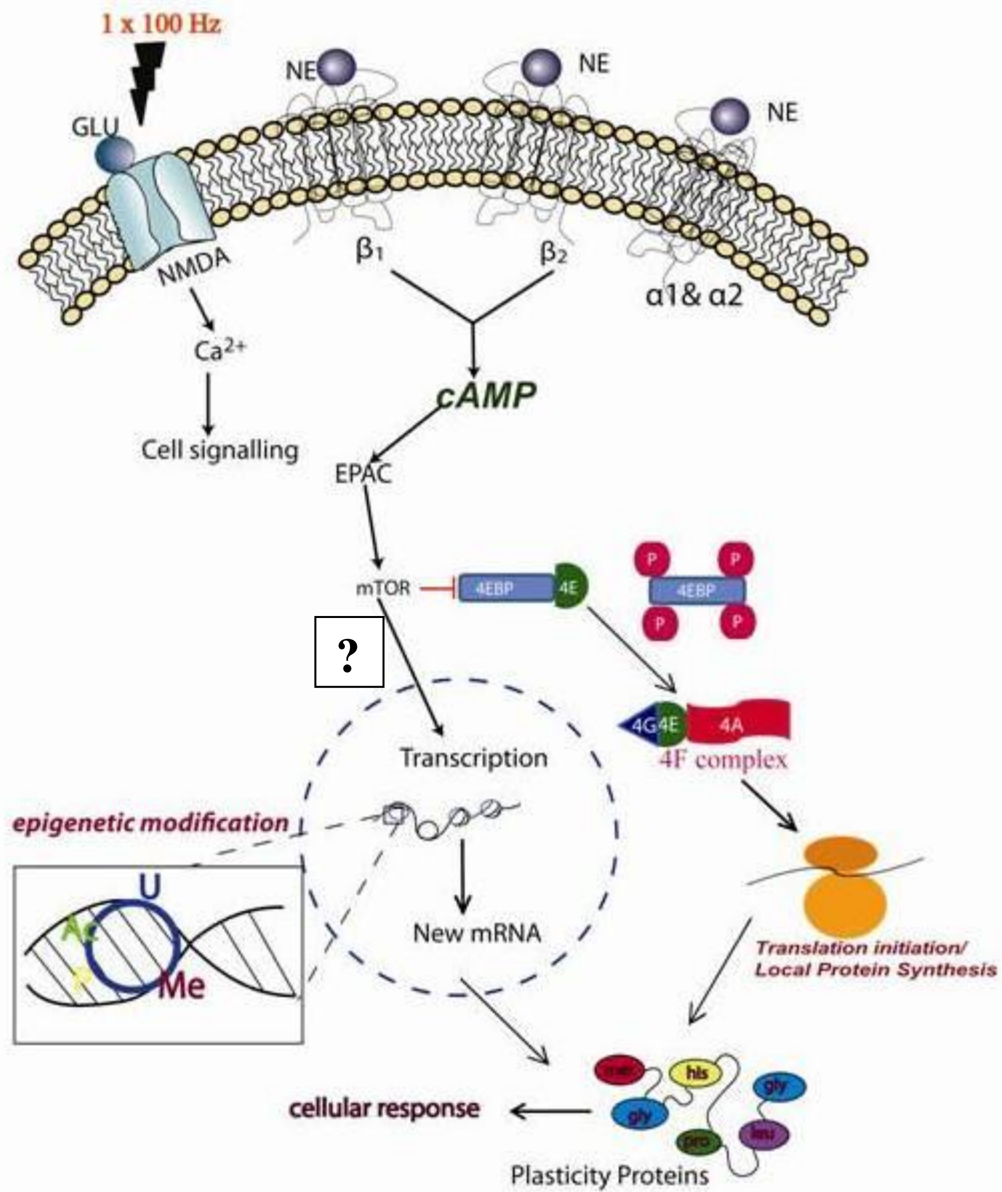

Fig. 4.1: Schematic representation of signaling mechanisms in NE-LTP

4.2.1 Adrenergic receptor specificity and the role of NMDARs in NE-induced plasticity

Accumulating evidence indicate that adrenergic receptors play a crucial role in transmission of cell surface signals to the intracellular molecules to induce synaptic plasticity (Izumi et al., 1992; Puumala et al., 1998; Pussinen and Sirvio, 1998; Izumi and Zorumski, 1999; Ferry et al., 1999; Doze et al., 2011; Gazarini et al., 2013). The

enhancement of NE-induced synaptic plasticity and consolidation or retention of emotionally charged events has mostly been attributed to the activation of β-ARs (Cahill et al., 1994; Gelinas et al., 2005; Sara et al., 1999; Przybyslawski et al., 1999; McGaugh and Roozendaal, 2002). However, several studies also showed interesting results on the selectivity of adrenergic receptors for NE-induced plasticity and learning and memory. For example, Puumala et al. (1998) reported that stimulation of α1-ARs facilitates formation of new memories in rats. This is in line with studies by Sternberg et al. (1985) and Sternberg et al. (1986); both studies have shown a receptor specificity (α1-ARs) in NE-induced memory enhancements. More recently, Doze et al. (2011) observed that long term α1-AR activation causes enhancement of synaptic plasticity and improvement in spatial cognitive tasks. These interesting discrepencies encouraged me to find the adrenergic receptor specificity in NE-induced synaptic plasticity using my stimulus protocol.

I observed that NE-LTP is long-lasting (>3 hr minimum). The NE-LTP was significantly reduced in the presence of betaxolol (β1 antagonist) and ICI (β2 antagonist) but was unaltered in the presence of prazosin (α1 antagonist) and yohimbine (α2 antagonist). This showed that NE-LTP is dependent on β- (both β1 & β2) but not α-ARs. These discrepancies with previously published data on rats could be due to species variance, the stimulation protocol used to induce LTP, or the recording period after LTP induction. For example, I used a cut-off time of 2 or 3 hr as an indicator of LTP maintenance and HFS (1 x 100 Hz) to induce LTP, which is in contrast with a total 60 min recording period and θ-burst stimulation used by previous studies as a method of LTP induction. Together, my results suggest a unique signaling pathway of synaptic plasticity through β-ARs.

β-ARs in the CA1 region of hippocampus are located in such a strategic position on the cell membrane surface that upon activation, they can serve as a triggering event for multimodal cellular effects including activation of other cell surface molecules, such as PSD-95 and NMDA receptors (Hu et al., 2000). It has been shown that LTP induction requires activation of NMDA receptors (Collingridge et al., 1983; Morris et al., 1990). Additionally, the strength of excitatory synapses can be potentiated upon a specific pattern of stimulation through the coupling of cell-surface receptors such as β-ARs to NMDARs (Moody et al., 2011). In my study, the enhancement of synaptic strength by NE application seems to be an NMDA receptor-dependent event, as application of APV (NMDAR antagonist) blocked the induction of LTP, demonstrating its requirement in LTP. One of the

mechanisms of β-AR mediated synaptic plasticity is the phosphorylation and incorporation of AMPA receptors from extra-synaptic to synaptic sites (Tenorio et al., 2010), mediated through a transient period of NMDA receptor activation, known as metaplasticity (Abraham, 2008; Abraham and Bear, 1996). My results suggest that further investigation of the interaction between β-ARs and NMDARs should yield a mechanistic explanation of NE-induced synaptic plasticity as well as learning and memory.

4.2.2 Epac-dependent protein synthesis

β-ARs are the transmembrane receptors, coupled to G_s-proteins, which upon activation, stimulate adenylyl cyclase to increase levels of the intracellular second messenger cAMP (Maguire et al., 1977; Minocherhomjee and Roufogalis, 1982). The role of cAMP in synaptic plasticity, learning and memory was reported in early studies by many groups (Libet et al., 1975; Brunelli et al., 1976). Using advanced genetic engineering and pharmacological techniques, it has been established that cAMP is an important molecule in the formation of memories (Randt et al., 1982; Bernabeu et al., 1997; Byers et al., 1981).

Considerable evidence has indicated that PKA, the best characterized downstream target of β-AR stimulation or cAMP signaling, is a major signaling molecule for synaptic plasticity and memory formation (Abel et al., 1997; Duffy et al., 2001; Woo et al., 2003; Abel and Nguyen, 2008). However, not much is known about Epac, the second target of cAMP signaling, in synaptic plasticity and memory

formation. Ouyang et al. (2008) found that beside PKA, Epac was recruited for retrieval of memories. Studies have also indicated an important role for Epac in bidirectional synaptic plasticity as well as learning and memory (Gelinas et al., 2008; Ma et al., 2009; Ostroveanu et al., 2010). I am the first to show that an NE-mediated increase synaptic response is PKA-independent but Epac-dependent, as blocking Epac activity pharmacologically, either with BFA or the more specific Epac inhibitor (ESI), disrupted NE-LTP. Hence, β-ARs stabilize NE-LTP through the Epac signaling pathway.

The long-term enhancement of synaptic strength as well as the formation of memory requires synthesis of new proteins (Costa-Mattioli et al., 2009; Klann et al., 2004; Richter and Sonenberg, 2005). Additionally, activation of β-ARs can induce translation dependent long-lasting enhancement of synaptic strength through the mTOR signaling pathways (Gelinas et al., 2007; Gelinas and Nguyen, 2005; Tang et al., 2002). Here I found that inhibition of mTOR signaling (by rapamycin) and of translation (by anisomycin or CHX) results in blocking of NE-LTP. My results also reveal that NE activates ERK to stabilize LTP, as inhibition of ERK (by PD 98,059) prevents NE-LTP. ERK is an important intracellular molecule which has been implicated in translational control of synaptic plasticity and memory (Sweatt et al., 2004; Thomas and Huganir, 2004; Banko et al., 2004). Further studies will be necessary to find a causal link between ERK-mTOR signaling and translation in synaptic plasticity. My results indicate a novel β-AR mediated signaling which activates Epac to recruit ERK- and mTOR-dependent translation for the persistence of NE-LTP. The involvement of Epac in NE-LTP

has physiological and clinical relevance. Many neurodegenerative diseases are characterized by altered levels of Epac expression (McPhee et al., 2005; Maillet et al., 2003). My results establish a foundation where Epac may be a crucial molecule for long-lasting enhancement of synaptic plasticity.

4.2.3 Transcription and epigenetic mechanisms in NE-induced synaptic plasticity

Hippocampal synapses are capable of maintaining long-lasting potentiation due to the presence of polyribosomes and protein synthetic machineries in the dendritic shafts as well as spines (Steward, 1997; Steward and Schuman, 2001; Eberwine et al., 2001; Huang and Kandel, 2005). However, gene expression at the transcriptional level has also been implicated in long term synaptic plasticity and memory formation (Sossin, 1996; Nguyen et al., 1994). My data suggest that a somatic or transcriptional component is included in NE-LTP as inhibition of transcription (by Act-D or DRB) decreased the magnitude and duration of NE-LTP. My results extend previous work by showing that different signaling (i.e., Epac) may recruit transcription in addition to local translation to form a stable memory trace. To this end, my results shed light on communication between the synapse and nucleus upon cell membrane receptor (i.e., β-AR) activation as such that the two separate pathways (a proximal, synapse-based translation and a distal, soma-based transcription) can operate simultaneously to generate plasticity proteins or mRNAs for the endurance of synaptic strength. Further research will be necessary to define which specific genes are transcribed in NE-LTP.

The requirement of somatic transcription suggests that NE may interact with epigenetic mechanisms to boost LTP. The epigenetic mechanisms and NE-mediated transcription share the signaling pathway upon cell membrane receptor activation. For example, CBP has intrinsic HAT activity and inhibition of HDAC increases the expression of CRE reporter genes by cAMP (McManus and Hendzel, 2001; Fass et al., 2003). Thus, epigenetic modification (i.e., histone acetylation) could significantly alter NE-cAMP mediated transcription in synaptic plasticity. NE-induced epigenetic modification is reported in non-neuronal tissues (Ho et al., 2007; Price et al., 2009). However, few studies characterized the influence of neuromodulators on epigenetic mechanisms in hippocampal synaptic plasticity (Crosio et al., 2003). With recent advancements in the understanding of epigenetic mechanisms in synaptic plasticity and learning and memory (for review, see Roth et al., 2010), I explored this new avenue of NE-mediated epigenetic mechanisms in hippocampal synaptic plasticity. Many extracellular signaling paths integrate at the nuclear level by phosphorylating CREB and hence play an essential role in hippocampal plasticity and the memorial process in a broad spectrum of species (Frank and Greenberg, 1994; Yin et al., 1994; Lonze and Ginty, 2002). Phosphorylated CREB then recruits CBP/P300 (with HAT activity) for transcription of genes. In contrast to histone acetylation, DNA methylation works in a different way to alter transcription of genes. Generally, methylation of genes by DNMTs represses transcription and increases synaptic plasticity, learning and memory. On the other hand, acetylation of genes by CBP-HATs enhances transcription by removing transcriptional repressors and thus

enhances synaptic plasticity and memory (Nelson and Monteggia, 2011). These observations suggest that separate sets of genes are responsive to DNA methylation and acetylation.

My results reveal a unique mechanism by which NE can alter nucleosome structure by methylation of DNA and initiation of post-translational modifications (i.e., acetylation and phosphorylation) of core histone proteins. I found significantly reduced NE-LTP in the presence of inhibitors of DNA methylation (AZA and ZEB). This led me to hypothesize that NE activates DNMTs to methylate memory suppressor genes to transcriptionally silence them. The presence of AZA or ZAB blocks this effect of NE and transcriptionally activates the genes. Since I predicted that these genes are memory suppressor genes, transcription of these genes decreases synaptic strength and memory. Further studies will be needed to characterize the genes and to determine the expression pattern of DNMTs in response to NE+HFS. These data also suggest that neuromodulators such as NE can alter synaptic strength through two temporally congruous but mechanistically different mechanisms: one by upregulation of local protein synthesis at the synaptic site, and the other by modulation of mRNA through epigenetic mechanisms such as DNA methylation, histone acetylation and phosphorylation. My results have shown that blockade of HDAC activity by TSA did not enhance NE-LTP. This further strengthens the notion that NE engages histone acetylation for endurance of NE-LTP. I also found that shifting CBP/P300 HAT inhibitor (C646) application to shortly after NE+HFS did not block NE-

LTP. This indicates that modification of the histone protein is temporally restricted, which is in line with evidence that transient histone acetylation is recruited upon consolidation of fear memories (Federman et al., 2009). Here, I have provided an initial characterization of a novel β-AR mediated epigenetic response which bolsters LTP in hippocampal slices. My results identified DNA methylation, histone acetylation and histone phosphorylation as epigenetic regulators triggered when NE is paired with HFS. Though I used HFS to induce LTP, my results may provide a cellular mechanism in memory consolidation during different brain activities. Memory enhancer or repressor genes (i.e., *bdnf* and *pp1,* respectively) are modified by epigenetic mechanisms in memory formation (Bredy et al., 2007; Martinowitch et al., 2003). Thus, it is of great interest to discover which genes are transcribed through epigenetic mechanisms in NE-LTP. A model is shown to describe the suggested mode of epigenetic modification in NE-LTP **(fig. 4.2)**.

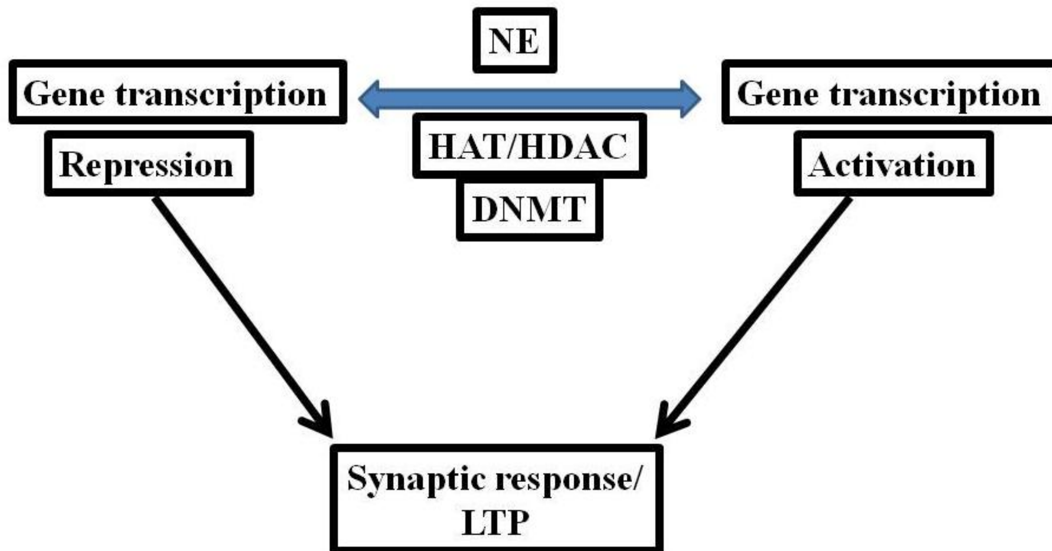

Fig. 4.2: Suggested model of epigenetic modification in NE-LTP

4.2.4 Therapeutic implication of noradrenaline signaling mechanisms

Synaptic plasticity is impaired in many neurodegenerative diseases which lead to cognitive dysfunction. Cell membrane receptors can initiate epigenetic modifications through intracellular signaling (i.e., ERK) to potentiate synaptic responses as well as to maintain proper cognitive abilities (Day and Sweatt, 2011). NE, a vital neurotransmitter, influences brain functions including attention, network reorganization, learning and memory. At the cellular level, NE acts on β-ARs to modulate synaptic plasticity as well as cognitive functions. My research aimed to determine how β-ARs engage translation and epigenetic mechanisms to modulate synaptic plasticity. To this end, my results will shed light on a new avenue of neuroscience by exploring the role of NE in epigenetic regulation of cognitive abilities. Altered noradrenergic neurotransmission has been implicated in many cognitive disorders as well as neurodegenerative diseases such as Alzheimer's disease, PTSD, ADHD, and depression (Berridge and Waterhouse, 2003). For example, NE concentration in the CSF is significantly higher than normal in PTSD patients (Geracioti, Jr. et al., 2001), and the β-AR blocker propranolol is used to reduce the probability of developing PTSD (Henry et al., 2007). Furthermore, the age-related dysfunction of the central noradrenergic function is implicated in memory loss (Leslie et al., 1985). On a different note, NE also acts as a neuroprotective agent by inducing neurogenesis (Jhaveri et al., 2010) and by modulating inflammatory gene expression in the brain (Feinstein et al., 2002).

A growing number of studies has revealed that epigenetic mechanisms are disrupted in memory-related cognitive impairments (Kosik et al., 2012; Day and Sweatt, 2012; Zovkic and Sweatt, 2013). Recent advancements in epigenetic research have made tremendous progress to the level that HDAC inhibitors are being used in clinical trials. The current century is the era, full of hopes and promises in both technologies and medical research. Developments in neuroscience have grown far beyond our modest expectations of the past. Modern psychopharmacology encompasses the knowledge of neuroscience, pharmacology, psychology and eventually, culminating into rational drug design. Besides other mainstream drugs, now we have access to drugs which are meant for enhancing cognitive function.

Overall, with previous data linking β-AR stimulation to translational upregulation, my results expand the knowledge of the noradrenergic signaling cascade and also identify key epigenetic modulators, such as CBP, that could be therapeutic targets in the treatment of neurological disorders related to memory dysfunctions.

4.3 Future directions

My results have opened up exploration of the role of epigenetic mechanisms (specifically, acetylation of histones by CBP) in NE-LTP. As histone acetylation and CREB-CBP activation are downstream to the ERK signaling pathway, which is also activated upon β-AR activation, CBP might then be an important target molecule in NE-LTP. The coupling of NE-LTP to epigenetic regulation of gene expression can be understood properly using both pharmacological and genetic approaches.

Currently, I am collaborating with Dr. Nahum Sonenberg from McGill University to characterize the acetylation pattern of H3-Lys-14 in response to different pharmacological treatments targeting signaling molecules in NE-LTP. For example, I have demonstrated that NE, preferentially through β-ARs, engages CBP histone acetylation and DNA methylation to stabilize LTP. Thus, treatment with propranolol or C646 should reduce the proportion of acetylated histones (H3). Considering the complicated nature of interactions between epigenetic modifications, a pharmacological intervention would not be enough to rule out a specific epigenetic modification such as histone acetylation in NE-LTP. Thus, a genetic approach where CBP-HAT activity is selectively disrupted should be used to determine if CBP-HAT is a critical component of NE-LTP.

To this end, CBPδ1 transgenic mice would be helpful to start NE-LTP experiments. These mice express a transgene for CBP lacking the HAT domain

and do not possess any developmental disorder (Wood et al., 2005). These features make it a unique model to test NE-LTP. My preliminary electrophysiology data in wild type mice indicate that CBP/P300-HAT activity is required for NE-LTP, which is again confirmed by my Western blot result that NE+HFS significantly enhances the proportion of acetylated histone compared to control. Thus, the study of NE-LTP in CBPδ1 mice would shed light on the requirement of CBP-HAT activity. The relative contribution of P300-HAT activity should also be considered if we find no significant difference in NE-LTP. For example, the fEPSPs might be the same in wild type and CBPδ1 mice after 2 hr of NE+HFS, which would indicate the masking effect of CBP-HAT by other HATs in NE-LTP. P300-HAT is another homologue of CBP-HAT, and P300δ1 or P300 conditional knockout mice (Olivera et al., 2007) have similar phenotypic characteristics to CBPδ1. Thus, we also need to compare NE-LTP in those mice. These experiments will identify specific signaling molecules as epigenetic regulators of NE-LTP.

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