MOLECULAR BASIS FOR MEMORY STORAGE AND ADDICTION

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Under the guidance of

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CERTIFICATE

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DECLARATION

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Karan Bhatt

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List of Symbols and Abbreviations

CK	concentration of $CaMKII$ (M)
P	concentration of $PP1$ (M)
K_m	michaelis-menten constant (M)
T	total concentration of $CaMKII$
S	concentration of sGC (M)
N	concentration of NO (M)
K	rate constant for the particular reaction
I	concentration of inhibitor (M)
K_i	logarithmic concentration of inhibitor when $6C - sGC - NO$ formation is halved (M)
MO	concentration of morphine (M)
D	rate of dissociation (s^{-1})
R	rate of removal from the cell (s^{-1})

Dimensionless Numbers

C	turnover number

Subscript

ia	inactive
t	transient
a	active
I	inhibitor
MO	morphine
NO	nitric oxide
N	normalized
U	upper limit
L	lower limit

Abstract

Recent cellular and molecular studies of memory storage suggest that experience dependent modulation of synaptic strength and structure is a fundamental mechanism by which the diverse forms of memory are encoded and stored. For memory storage, some type of synaptic growth is thought to represent the stable cellular change that maintains the long-term process. In its most general form, the synaptic plasticity and memory hypothesis states that 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.

This thesis is mainly divided into two parts. In the first part, a mathematical model of the bistable switch is developed with the help of which a zone is defined where the Ca^{2+}/CaM-kinase II-protein phosphatase 1 ($CaMKII-PP1$) switch remains bistable. For each pair of $CaMKII$ and $PP1$, the critical stimulus concentration and the active $CaMKII$ concentration is calculated which leads to autophosphorylation of $CaMKII$. The change in the critical stimulus and active $CaMKII$ with respect to $CaMKII$ and $PP1$ is also plotted. The critical stimulus concentration increases in a linear manner with change in $CaMKII$ and $PP1$ for the upper limit while it changes randomly for the lower limits. For active $CaMKII$, the change is of sigmoidal nature in case of both upper and lower limits for $CaMKII$ and $PP1$. A novel correlation is developed for measuring the critical stimulus intensity required for $CaMKII$ to undergo direct autophosphorylation which leads to long term memory formation with a goodness of fit 99.74 %.

In the second part of the thesis, a mechanism is proposed for the inhibition of morphine on long-term potentiation of $GABA_A$ (γ-aminobutyric acid-A receptor) mediated synaptic transmission (LTP_{GABA}). Morphine binding on $\mu-opioid$ receptors on the presynaptic GABAergic cells results in inhibition of activation of soluble guanylate cyclase (sGC) by blocking the nitric oxide (NO) binding site. Retrogradely travelling NO is not able to bind sGC and activates it in the presence of morphine which results in the inhibition of activation of cyclic guanosine monophosphate ($cGMP$) and protein kinase G (PKG). As a result, LTP_{GABA} is not produced which increases the chances of addiction. A mathematical model is presented for morphine inhibition on LTP_{GABA} and its implication in addiction. A two step model of sGC activation is used, where morphine inhibits the NO during the first step and consequently blocks sGC activation. The dependence of morphine inhibition on various parameters such as morphine dissociation, morphine concentration, NO removal and rate of inhibition is also studied.

Keywords: memory storage, $CaMKII$, bistable switch, sGC, addiction.

CHAPTER 1

Introduction

1.1 Background

The capacity to form, retain, and use memories is a fundamental property of the brain essential for survival in all organisms. Humans have a rich array of memories associated with emotion, acquired skills and habits, facts about life and addictions. How do we form memories; how are they encoded and stored in the brain? To process and store a lifetime of memories, some form of plasticity in the brain is required. Following Hebb's dual-trace theory [1], it is now believed that memories are encoded as dynamic spatio-temporal patterns of synchronized cellular activity within widespread neural networks and that this dynamic activity progressively results in altered patterns of connectivity among the neurons. Within this framework, any memory representation would correspond with specific sets of patterns of activity in overlapping networks. The neural code embedded within these patterns of activity in large part defies our understanding. Nonetheless, it has long been recognized that this dynamic activity, transient in nature, cannot persist long enough to be the actual substrate of long-term memory. Thus, it has been postulated that there should exist a second state of memory encoded as changes at the cellular level to store these representations. A process of stabilization or consolidation would lead to what Hebb called a "structural trace," a memory trace that is maintained in some form of a dormant state but has the capacity to return to an "active state" to evoke recall whenever a subset of the original information, or related information, is available. Although it has been suggested that once a long-term memory had been established it is stable and remains immune to any form of disruption, it was not the case. A so-called established, or consolidated, memory when reactivated enters a dynamic but fragile state, requiring further stabilization via synaptic changes to

be available once again for recall, a process now known as reconsolidation. The main point is that long-term memories are not, as was originally thought, stable and essentially "hardwired", that the mechanisms of plasticity in neural circuits that encode and store long-term memories are dynamic and ongoing throughout the life of a memory. The function of this form of ongoing plasticity has not been clarified yet but may well serve to update or modify existing memories.

1.2 Literature Review

1.2.1 Brain Systems and Forms of Memory

Events in 1949 signaled a new era in research on brain substrates of learning and memory. Hebb's book, The Organization of Behavior [1] offered novel ideas that attempted to provide an understanding of how brain cells might cooperate to provide a basis for learning. He proposed that distributed assemblies of neurons in the brain activated by stimulation engage in reverberatory firing and provide a basis for recent memory. With repeated or sustained activation the cell assemblies stabilize and provide a basis for lasting memory. A key assumption required for Hebb's "dual-trace" hypothesis is that some change is required at junctions between neurons in order to provide the stabilization. The process he proposed to account for that induced stability is now well-known as the Hebb's hypothesis [1]; it states, "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in its 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". By coincidence, another important influence appeared in 1949. Carl Duncan published a seminal paper reporting that electroconvulsive shock stimulation applied to rats after they were trained induced retrograde amnesia [2]. The findings of this study and a great many subsequent studies using other kinds of treatments administered post-training provided strong evidence that initially fragile memory traces stabilize or consolidate over time, as suggested by Müller and Pilzecker [3]. Such findings also stimulated studies investigating the neurobiological conditions that modulate (enhance as well as impair) memory consolidation [4] as well as mechanisms essential for such consolidation.

Besides contributing the dual-trace hypothesis and the "Hebb synapse", Hebb made an additional crucially important contribution to research on memory: He suggested that his graduate student, Brenda Milner, conducted neuropsychological testing of patients of the neurosurgeon W.B. Scoville who were treated with bilateral medial temporal lobe surgery. Her studies [5] revealed that the lesions resulted in blocked or significantly attenuated ability to form new explicit (declarative) memories but they left the patient's primary (recent) memory and memory proper (remote) intact. Milner's findings pro-

vided a new target for investigations of brain regions and memory: the medial temporal lobe — especially the hippocampus. More than two decades later, Mishkin published the first evidence of similar symptoms in monkeys with medial temporal lobe lesions [6]. With renewed insight, research on medial temporal lobe involvement in memory focused on the effects of brain lesions on the learning of explicit and declarative information. Experiments using rats found that hippocampal lesions impaired their ability to learn to swim to a specific location in a pool of water [7] and to remember the location of recently rewarded and non-rewarded alleys of a maze [8]. Such studies led to a growing acceptance of the idea that the hippocampus is involved in the learning of some kind of information, especially those concerning contextual cues and the relationships among cues [9].

1.2.2 Cells and Systems

As was fully recognized by Hebb, a major problem in the neurobiology of memory is discovering how the activation of neurons in the brain leads to the formation of knowledge and actions. How, that is, do cells collude with brain systems to produce memories that enable changes in behaviour? He proposed that experience-induced changes in neuronal firing could provide a starting point for an explanation. Part of the answer was given by Bliss and Lomo [10] who showed that brief activation of hippocampal cells induced a change in the connectivity of existing synaptic connections with other cells — a finding now well-known as long-term potentiation (LTP). Various forms of LTP and the reverse effect, long-term depression (LTD) have been the subjects of extensive investigations for several decades. The quest of such research is to find synaptic mechanisms mediating the creation of Hebb synapses that may provide cellular bases for memory. Progress in understanding molecular genetics has led to the development of new methods for investigating cellular processes mediating such neuroplasticity. However, creating memory involves more than changing synaptic connections. Sets of neurons must become interconnected with other sets of neurons to create organized systems that serve to represent memory. Although memory is no doubt based on experience-induced neuronal changes, the consequences of the changes must also depend on the functions of the brain system of which they are a part. Collusion of cells and systems is required.

Within a system, the firing of some cells is no doubt involved in inducing synaptic changes enabling memory. The activity of other cells that project to other brain regions can act to modify the functioning in those distal regions. For example, the firing of cells in the basolateral complex of the amygdala (BLA) of a cat is increased greatly by a single foot shock and the increased firing lasts at least 2 hours [11]. Such increased firing may serve to modulate memory processing in efferent brain regions including the entorhinal

cortex and hippocampus [12, 13]. In support of this view, electrophysiological studies have reported that noradrenergic stimulation of the BLA enhances the induction of LTP in the hippocampus and that disruption of the BLA with lesions or a β-adrenoceptor antagonist blocks the induction of such LTP [14, 15]. Other recent findings indicate that noradrenergic stimulation of the BLA that enhances memory consolidation also increases dorsal hippocampal levels of activity-regulated cytoskeletal Arc protein [16], an immediate-early gene implicated in hippocampal synaptic plasticity and memory consolidation processes [17]. Additionally, inactivation of the BLA with infusions of lidocaine impairs memory consolidation and decreases Arc protein levels in the dorsal hippocampus [16]. Clearly, the BLA is a major player in the collusion of cells and brain systems involved in memory consolidation.

1.2.3 Long-lasting Forms of Synaptic Modulation

Long-Term Potentiation (LTP)

Tim Bliss and Terje Lomo [10, 18] first reported the phenomenon of LTP, an increase in synaptic efficacy following synaptic activity, 40 years ago. Since then, LTP has generated enormous interest as a potential mechanism of memory, primarily because it exhibits numerous properties expected of a synaptic associative memory mechanism, such as rapid induction, synapse specificity, associative interactions, persistence, and dependence on correlated synaptic activity.

Many features of LTP as a phenomenon make it a compelling candidate for the synaptic processes underlying neural information storage. First, LTP is induced rapidly. Soon after its induction, LTP appears within minutes. Hanse and Gustafsson [19] suggested that it develops incrementally, reaching asymptotic levels by approximately 5 to 20 s, depending upon the synapse studied. LTP is not always rapidly expressed and can show incremental growth over a period of 10 to 20 min. The precise reasons why such incremental LTP is observed in some cases and not others is unknown. Our experience is that the methodology used to induce LTP can determine initial LTP magnitude. For example, rapid LTP induction is seen with direct stimulation of afferents in both commissural and perforant path inputs to the CA3 region. However, a slowly developing, incremental LTP often is observed when LTP is induced in an associative manner by pairing weak commissural or perforant path trains with a strong tetanus to a convergent CA3 afferent system [20]. Thus, while LTP develops relatively rapidly, it can take some time to develop fully.

Another feature is that LTP is associative. If high frequency stimulation of one set of afferents induces LTP, individual active synapses can also be recruited to express LTP — provided that the synapse is coactive. Associativity can be derived from the requirements for activation of the N-Methyl-D-aspartic acid (NMDA) receptor. The property

of associativity can be derived directly from and is essentially identical to the property of cooperativity [21], indicating that LTP has a threshold and a threshold number of afferents must be active to induce LTP. Input specificity is a crucial property of virtually all forms of LTP and refers to the fact that LTP is synapse-specific and restricted only to synapses of activated afferents.

Another feature of LTP is that it is remarkably persistent. LTP in the hippocampal formation can persist from hours to weeks or months, depending upon the stimulation parameters. In intact animals, LTP is decremental and usually decays within 1 to 2 weeks [22]. While this is certainly too brief a period for the storage of long-term memory, several points should be made with respect to LTP longevity. First, LTP in the hippocampus need not be permanent. Current findings support the view that, as is suspected in humans, the hippocampus has a time-delimited role in memory; persistent long-term memory is gradually consolidated in neocortical areas [23]. In this view, memories formed by the hippocampus are transferred to and consolidated in the neocortex, possibly during slow wave or sleep states [24, 25]. This usually occurs within 2 weeks in rats, as indicated by both lesion and imaging data [26]. Thus, if the hippocampus indeed serves as a temporary repository of information, LTP may not last long simply because it may not need to.

Long-Term Depression (LTD)

LTP is particularly noteworthy in that its induction follows the rule of pre- and postsynaptic associativity as formalized by Donald Hebb [1]. However, a mechanism serving to increase synaptic strength cannot operate alone; otherwise the strength of synapses could only increase, eventually reaching a point of saturation. Other mechanisms that permit either the reversal or the inverse of LTP are likely to be necessary. Such a phenomenon is observed at the same synapses that display LTP and is termed LTD. LTD was noted in early studies, although its possible role in information storage was only suggested by Barrionuevo et al. [27] in the early 1980s. As it became apparent that any device that serves as a temporary repository for information must have some way to decrease synaptic strength, LTD became a focus of many studies in the 1990s [28].

In contrast to LTP, distinct forms of LTD were noted early on in these studies, as evidenced by the distinct mechanisms of their induction. Homosynaptic LTD is used to describe LTD that follows synaptic activity and typically is induced by repetitive low frequency (0.5 to 5 Hz) stimulation. In most synapses, homosynaptic LTD is, like LTP, input-specific, dependent upon NMDA receptor activation [29], associative [30, 31] and also requires calcium, although the levels of calcium influx necessary for LTD induction appear to be lower than those for LTP. This may reflect the modulation of phosphatases associated with LTD induction by calcium, which require much smaller changes in calcium concentration. LTD also is observed when either synaptic activity or LTP occurs

at neighboring synapses. This form of LTD is referred to as heterosynaptic in that it is observed at synapses that are not potentiated. Heterosynaptic LTD is usually most evident in the perforant path projections to the dentate, where induction of LTP in one set of afferents can induce heterosynaptic LTD of responses evoked by a separate inactive set of afferents, and vice versa. Here, LTD induction appears sensitive to both NMDA receptors and the voltage-dependent calcium channels (VDCCs) [32, 33], suggesting that low levels of calcium necessary for LTD may be provided by VDCC activated in response to NMDA receptors [34].

The diversity of types or forms of LTD induction mechanisms may reflect distinct roles for these forms of plasticity in hippocampal function and memory. As graceful degradation rather than catastrophic interference appears to be characteristic of many neural systems [35], this serves as another indicator that synaptic potentiation within the hippocampus is tightly regulated and likely utilizes an activity-dependent mechanism that serves to weaken synaptic strength. Thus LTD may play a role in reversing LTP (also referred to as depotentiation).

1.2.4 Ca^{2+} and its Transducer

After the activation of neurotransmitter receptors, several downstream signals are triggered. Probably the most prominent signal for synaptic plasticity is calcium which has the ability to interact with the actin cytoskeletons of dendrites and through this interaction regulate structural synaptic plasticity [36]. However, after synaptic activation, the influx of calcium ions (Ca^{2+}) into cells through ligand- and voltage-gated calcium channels or from internal reservoirs results in a complex set of transitory and oscillatory signals. This complex signal requires a molecular device to transform it into a more stable message. Such a device should be capable of activating the intracellular cascades involved in the stabilization of synaptic plasticity. The $CaMKII$ is a ubiquitous and broad specificity Ser/Thr protein kinase highly enriched in the central nervous system. This enzyme is highly concentrated in the post-synaptic density and is considered an important Ca^{2+} detector in the postsynaptic region [37]. The unique regulatory properties of $CaMKII$ make it an ideal interpreter of the diversity of Ca^{2+} signals. Evidence has shown that $CaMKII$ can interpret messages coded in the amplitude and duration of individual Ca^{2+} spikes and translate them into distinct amounts of long-lasting Ca^{2+}-independent activity [38].

In a nonactivated state, $CaMKII$ is auto-inhibited, but when it interacts with Ca^{2+}/CaM complexes, the blockade is released. After activation, $CaMKII$ phosphorylates other proteins but also displays an important autophosphorylation activity. When $CaMKII$ is autophosphorylated, the dissociation rate with CaM decreases; the enzyme is able to remain active even after CaM has dissociated from it. Thus, autophosphorylation gen-

erates a constitutive active form of $CaMKII$ able to translate a transient Ca^{2+} signal into a persistent and independent one [39]. The ability of $CaMKII$ to maintain phosphorylation activity for a prolonged period through autophosphorylation [40] represents an important way to sustain signalling and may have great relevance for the consolidation of long-term synaptic plasticity. The active form of $CaMKII$ is found in the postsynaptic density [41] where it interacts with different molecules important for the structures and functions of the postsynapses [42]. After $CaMKII$ is activated in the postsynaptic density, it interacts with NMDA glutamate receptors [43]. This interaction is very important because it increases $CaMKII$ autophosphorylation and its ability to become hyperphosphorylated [44]. Hyperphosphorylation can also increase the period of activation by saturating local phosphatase molecules, preventing dephosphorylation [45].

These functional properties of $CaMKII$ are dependent on it working as a bistable memory switch, in which $CaMKII$ activity changes between a transitory to a stable state depending on the interaction between $CaMKII$ and the NMDA receptor [37]. These bistable nature of $CaMKII$ is beacuse of its property to autophosphorylate [46]. This autophosphorylation results in Ca^{2+}/CaM-independent kinase activity [47]. Autophosphorylated $CaMKII$ phosphorylates LTP related targets even after $[Ca^{2+}]$ returns to its resting level [48]. Dephosphorylation of $CaMKII$ is carried out by $PP1$ which decreases the amount of active $CaMKII$. The mechanism by which the $CaMKII - PP1$ system acts as a bistable switch is as follows: when $CaMKII$ is in inactive form, there is no autophosphorylation and $PP1$ rapidly dephosphorylates any phosphorylated $CaMKII$. The 'off state' is thus stable because the rate of phosphorylation is low compared to the rate of dephosphorylation. This situation is reversed in the 'on state'. In this case, the kinase reaction is faster and the high concentration of phosphorylated subunits saturates the phosphatase, which results in low rate of dephosphorylation. The 'on state' is thus stable because the rate of phosphorylation is high compared to the rate of dephosphorylation.

In 1985 Lisman [49] developed a model which showed that a bistable switch consisting of kinase and phosphatase takes part in memory storage which was later confirmed to be $CaMKII$ and $PP1$ [50, 51]. The complex regulatory properties of $CaMKII$ were also studied with the help of models [52, 53] while Zhabotinsky [54] developed a model based on autophosphorylation of $CaMKII$ and its dephosphorylation by $PP1$. He defined bistability of the switch with respect to varying levels of Ca^{2+} inside the PSD. Miller et al. [55] studied the stable nature of the switch by using a stochastic model. With the help of Monte-Carlo simulations, they showed that switch stability depends on the rate of kinase and phosphatase reactions. Pi and Lisman [56] proposed that a minimal tristable system consisting of $CaMKII$ and protein phosphatase 2A ($PP2A$) bistable switches is required to build a model of synapse. Pepke et al. [57] developed a model based on the interactions of Ca^{2+}, CaM and $CaMKII$ and showed the de-

pendence of activation on the frequency of Ca^{2+} transients. These data indicate that $CaMKII - PP1$ is an important molecular switch whose activity can be related to persistent forms of synaptic plasticity and may play a prominent role in long-term memory formation. Therefore understanding the bistable nature of the switch depending on the relative concentrations of $CaMKII$ and $PP1$ may well serve to update or modify existing theories of memory formation.

1.2.5 Synaptic Plasticity and Addiction

It is not surprising that the evidence accumulated over the last decade demonstrates that drugs of abuse can co-opt synaptic plasticity mechanisms in brain circuits involved in reinforcement and reward processing. Indeed, an influential hypothesis is that addiction represents a pathological, yet powerful, form of learning and memory [58–63]. Although the brain circuitry underlying addiction is complex, it is unequivocal that the mesolimbic dopamine system, consisting of the ventral tegmental area (VTA) and nucleus accumbens (NAc), as well as associated limbic structures, are critical substrates for the neural adaptations that underlie addiction. It is also clear that the interactions between addictive drugs and synaptic plasticity in different brain regions will contribute to specific aspects of addiction, such as craving, withdrawal and relapse.

Addiction is not triggered instantaneously upon exposure to drugs of abuse. It involves multiple, complex neural adaptations that develop with different time courses ranging from hours to days to months. Work to date suggests an essential role for synaptic plasticity in the VTA in the early behavioural responses following initial drug exposures, as well as in triggering long-term adaptations in regions innervated by dopamine (DA) neurons of the VTA [60]. By contrast, downstream synaptic changes in the NAc and other brain regions, are likely to represent the formation of powerful and persistent links between the reinforcing aspects of the drug experience and the multiple cues (both internal and external) associated with that experience [58–63]. Of course, the brain adaptations that underlie addiction are complex and involve drug-induced changes in essentially every parameter that has been studied including gene transcription, membrane excitability and neuronal morphology. Moreover, because of advances in our understanding, and the societal importance, of the neurobiology of addiction, this topic has been the subject of numerous reviews in both the basic science and clinical literatures.

Opioids such as morphine are hypothesized to induce addiction by taking part in the synaptic plasticity of the reward learning process at the mesolimbic dopamine system [58, 60–64]. Several studies have shown that there is an increase in the synaptic transmission of excitatory inputs on DA neurons following drug exposure [65–71]. In addition, the drugs are also found to depress inhibitory synaptic plasticity on the DA

neurons in the VTA [72–74]. Thus, these drugs promote excitatory transmission or block inhibitory transmission or perform both the actions simultaneously and therefore influence long-term storage of reward-related memories in the VTA that may lead to addiction [63, 75]. These changes in the synaptic plasticity are seen due to changes in the LTP and LTD of both the excitatory as well as inhibitory neurons. By releasing GABA from the presynaptic neurons onto $GABA_A$ receptors present at the postsynaptic DA neurons, inhibitory interneurons oppose postsynaptic excitation and limit the spread of neural activity by generating LTP_{GABA} [76, 77]. This long-lasting potentiation of GABAergic synapses onto DA neurons in the VTA (LTP_{GABA}) is blocked by in vivo administration of morphine [74, 78] which seems to be the major cause for addiction.

Administration of in vivo morphine completely blocked the ability of the inhibitory synapses to undergo LTP_{GABA} within 2 hours and 24 hours prior to brain slice preparation, but not after 5 days [74, 78]. A single administration of morphine, therefore, potentiates excitatory synaptic transmission [66] while, at the same time, it prevents a complementary increase in inhibitory transmission that normally could have counterbalanced the increased excitation. Thus, blockade of LTP_{GABA} by morphine induces long lasting excitability of DA neurons which contributes to the reinforcing effects of opioids and development of addiction. LTP_{GABA} is heterosynaptic; it is initiated by glutamate release onto the N-Methyl-D-aspartate (NMDA) receptors on the postsynaptic DA neuron. NMDA receptor induces increased uptake of Ca^{2+} which activates NO synthase leading to the production of intracellular NO, which then travels retrogradely to presynaptic GABAergic neurons and activates sGC. Further downstream processing leads to increased levels of cyclic Guanosine Monophosphate ($cGMP$) and Protein Kinase G (PKG), responsible for promoting long-lasting potentiation of GABA release at these synapses [74]. Morphine-induced blockade of LTP_{GABA} specifically affects the $sGC - cGMP - PKG$ pathway, presumably at the level of sGC [74]. Interestingly, activation of sGC with a sGC activator in slices from morphine-treated rats is also able to induce LTP_{GABA}, providing indirect evidence for the presence of adequate levels of sGC in morphine-treated slices to produce enough $cGMP$ and thus mimic LTP_{GABA} [78]. Whether morphine directly or indirectly interacts with sGC to disrupt LTP_{GABA} is still not known and requires further investigation.

1.3 Objectives

The objectives of the current study are as follows:

- To study the bistable nature of $CaMKII-PP1$ molecular switch depending on different parameters, to define a zone of bistability depending on the concentrations of $CaMKII$ and $PP1$ respectively and to calculate the critical stimulus concentra-

tion for the switch to initiate autophosphorylation and lead to long term memory formation.

- To understand the mechanism of morphine addiction by proposing a novel mechanism of LTP_{GABA} inhibition by morphine via $sGC - cGMP - PKG$ pathway and to understand the dependence of various parameters on the proposed mechanism of inhibition.

1.4 Outline of Thesis

The main work of this thesis is the development of models based on the above mentioned objectives. This thesis is divided into four chapters. The main outline of all the chapters is given below:

Chapter 1: This chapter consists of general background of long term memory formation processes, overview of $CaMKII - PP1$ bistable switch in general & the mechanism of addiction based on synaptic plasticity and literature review.

Chapter 2: A model for $CaMKII - PP1$ bistable switch is developed in this chapter. Parameters like $CaMKII$ concentration, $PP1$ concentration, kinase activity and phosphatase activity are taken into consideration. The range of $CaMKII$ and $PP1$ concentrations are calculated wherein the switch remains bistable. The critical stimulus concentration is also calculated and a correlation is developed for calculating it based on the concentration of $CaMKII$ and $PP1$ respectively.

Chapter 3: In this chapter, a novel mechanism of morphine inhibition on the $sGC - cGMP - PKG$ pathway is proposed which leads to the development of addiction. The dependence of inhibition on various parameters such as morphine concentration, rate of inhibition, rate of morphine removal and rate of NO removal from the cell is also studied.

Chapter 4: Summary of the whole thesis is in this chapter. The basic conclusions are drawn. The direction of future work has been presented.

CHAPTER 2

Effect of $CaMKII$ and $PP1$ in Long Term Memory Formation

2.1 Introduction

According to the author's best knowledge, none of the investigators have studied the bistable nature of the switch depending on the relative concentrations of $CaMKII$ and $PP1$. Here, we develop a simple model of the $CaMKII-PP1$ switch and study it using finite differential methods. In this study, a zone of bistability is defined where the $CaMKII-PP1$ molecular switch maintains bistability. Whenever the concentration of $CaMKII$ and $PP1$ falls between the upper and lower limit of the zone then the switch shows bistability which has implications in memory formation. If the concentration of either $CaMKII$ or $PP1$ falls outside this zone then the switch does not remain bistable and neither does it activate further downstream pathways for memory storage. The dependence of $CaMKII$ and $PP1$ on the amount of criitical stimulus concentration and the active $CaMKII$ concentration required for autophosphorylation of $CaMKII$ is also measured. This dependence is defined for both the upper and lower limit of the zone. Based on the above results, a mathematical correlation is developed for calculating the critical stimulus concentration required for autophosphorylation of $CaMKII$ depending on respective $CaMKII$ and $PP1$ concentrations.

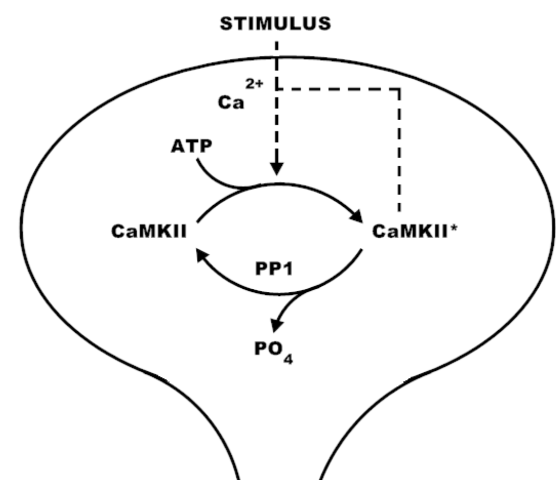


Figure 2.1: The model of $CaMKII - PP1$ bistable switch present in the dendritic spine. The switch is made up of two proteins $CaMKII$ and $PP1$. $CaMKII$ is present in two forms: active ($CaMKII^*$) and inactive ($CaMKII$) and the transition between the two states is based on phosphorylation.

2.2 Methods

2.2.1 The model

In this model we have considered $CaMKII$ consisting of two subunits: one subunit ($CaMKII_\alpha$) gets phosphorylated by the incoming Ca^{2+} while the other subunit ($CaMKII_\beta$) gets activated by autocatalytic phophorylation. $CaMKII_\beta$ later undergoes autophosphorylation and remains active for a long period of time. As the stimulus is received, there is an intake of intracellular Ca^{2+} in the dendritic spine; this increase in $[Ca^{2+}]$ leads to phosphorylation of $CaMKII_\alpha$ resulting in its activation. Simultaneously, $PP1$ also starts dephosphorylating the activated $CaMKII_\alpha$ and deactivates it. After a critical concentration of $CaMKII_\alpha$ is activated, $CaMKII_\beta$ gets autophosphorylated. As the concentration of active $CaMKII$ increases, phosphatase becomes saturated and the $CaMKII$ remains active for a long period of time. The reactions shown below constitute a bistable switch, one in which the $CaMKII$ is phosphorylated and other in which it is unphosphorylated.

The mode of memory storage by this switch is as follows: initially, when $CaMKII$ is inactive, stimulus will lead to increase in intracellular Ca^{2+} concentration which will result in $CaMKII_\alpha$ phosphorylation. Above a critical concentration, $CaMKII_\beta$ will start autophosphorylating itself and will remain active even in the absence of Ca^{2+} and the presence of phosphatase. This $CaMKII$ will remain active for a long period and further activate other downstream processes which will lead to memory formation (fig. 2.1).

Table 2.1: Parameters used in the model:

Parameter	Symbol	Value	Unit	Reference
Total concentration of $CaMKII$	T	0.1-30	μM	[79]
Concentration of CaMKII$_\alpha$	CK_α	0.25	μM	This paper
Turnover number of $CaMKII$	C_1	30	-	[49]
Turnover number of $PP1$	C_2	3	-	[49]
Turnover number of CaMKII$_\alpha$	C_3	30	μM	This paper
Michaelis-menten constant of $CaMKII$	K_{m1}	100	μM	This paper
Michaelis-menten constant of $PP1$	K_{m2}	0.4	μM	This paper
Michaelis-menten constant of CaMKII$_\alpha$	K_{m3}	100	μM	This paper

2.2.2 Mathematical formulation

The kinase activity is turned on by phosphorylation, which is produced either by Ca^{2+} stimulation or autocatalytic phosphorylation. The distinction between inter- and intramolecular autocatalytic reactions is not considered here to simplify the problem. $PP1$ deactivates activated $CaMKII$ by dephosphorylation. Assuming that the enzymes follow Michaelis–Menten kinetics, the rate equation is expressed a

$$\frac{dCK_a}{dt} = \frac{CK_{ia} \times CK_a \times C_1}{K_{m1} + CK_{ia}} - \frac{P \times CK_a \times C_2}{K_{\mathrm{m2}} + CK_a} + \frac{CK_\alpha \times CK_{ia} \times C_3}{K_{\mathrm{m3}} + CK_{ia}} \tag{2.1}$$

Here CK_{ia} and CK_a are the inactive and active forms of $CaMKII$; P is the concentration of $PP1$ for which bistability is observed; C_1, C_2 and C_3 are the turnover numbers for $CaMKII$, $PP1$ and $CaMKII_\alpha$ respecively; and K_{m1}, K_{m2} and K_{m3} are the michaelis-menten constants for $CaMKII$, $PP1$ and $CaMKII_\alpha$ respectively.The total concentration of $CaMKII$ is conserved inside the cell. It is equal to the sum of active and inactive $CaMKII$ in the cell and is given by T as shown below:

$$T = CK_{ia} + CK_a \tag{2.2}$$

A simple model of $CaMKII - PP1$ bistable switch is used to study the kinetics of long term memory formation. It is assumed that the relative concentrations of $CaMKII$ and $PP1$, where the bistable nature of the switch is observed, are responsible for long term memory formation.The activation of CaM by Ca^{2+} and the Ca^{2+} dependence of $PP1$ are neglected. The numerical simulations are carried out using fourth order runge kutta method. The parameters used are given in table 2.1.

2.3 Results and Discussions

A model based on biochemical reactions of $CaMKII$ phosphorylation, autophosphorylation and dephosphorylation is constructed. For concentration of inactive $CaMKII$

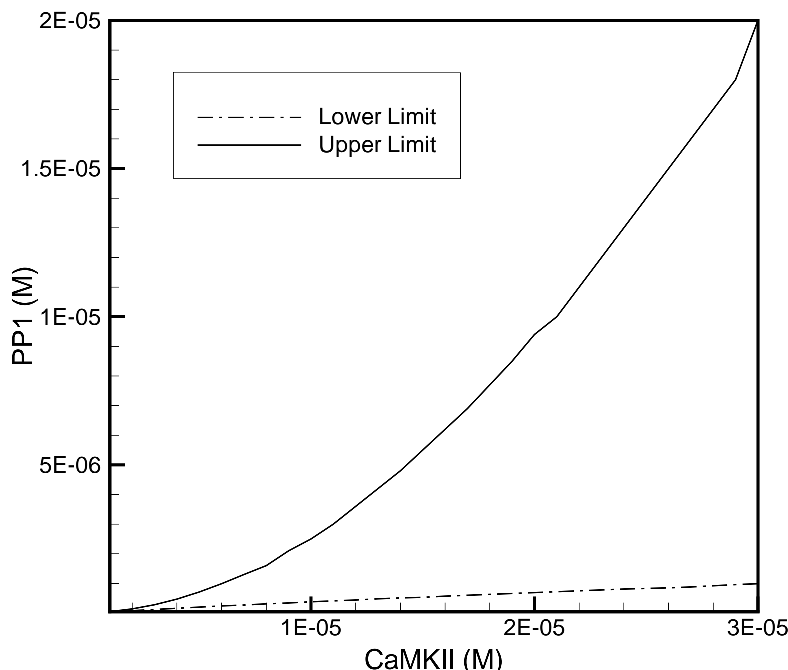


Figure 2.2: An area of $CaMKII$ and $PP1$ is shown under which the switch shows bistability. The $CaMKII$ concentration is in the range of 0.1 - 30 μM[79]. The upper limit is shown by solid line while the lower limit is shown by dashed line. As $CaMKII$ concentration increases, the range between upper and lower limit of $PP1$ increases for maintaining bistability. Parameters (in μM): C_1= 30, C_2= 3, C_3= 30; K_{m1}= 100, K_{m2}= 0.4, K_{m3}= 100; K_2= 0.25.

between 0.1-1μM, ten equally spaced concentrations were considered to find out the upper and lower limit of bistability. The same was done for inactive $CaMKII$ concentration between 1-30μM where thirty different values with equal intervals were used. For each set of inactive $CaMKII$ and $PP1$ concentration within the bistability zone, the critical stimulus concentration was found out which lead to autophosphorylation.

2.3.1 Zone of bistability

For the range of $CaMKII$ between 0.1 - 30 μM [79], a zone of bistability for the $CaMKII-PP1$ switch is identified as shown in fig. 2.2. It is observed that for each individual concentration of $CaMKII$, there is a range of $PP1$, bound by an upper limit and a lower limit, for which the switch shows bistability. Whenever the concentration of $PP1$ falls outside this range then the switch loses its bistable nature and eventually does not lead to memory formation. The range between upper and lower limit of $PP1$ expands with increase in the concentration of $CaMKII$ which implies that at higher concentration of

$CaMKII$ there is a high chance for the switch to maintain its bistability. These results are in a good agreement with the results observed in the hippocampal neurons [80].

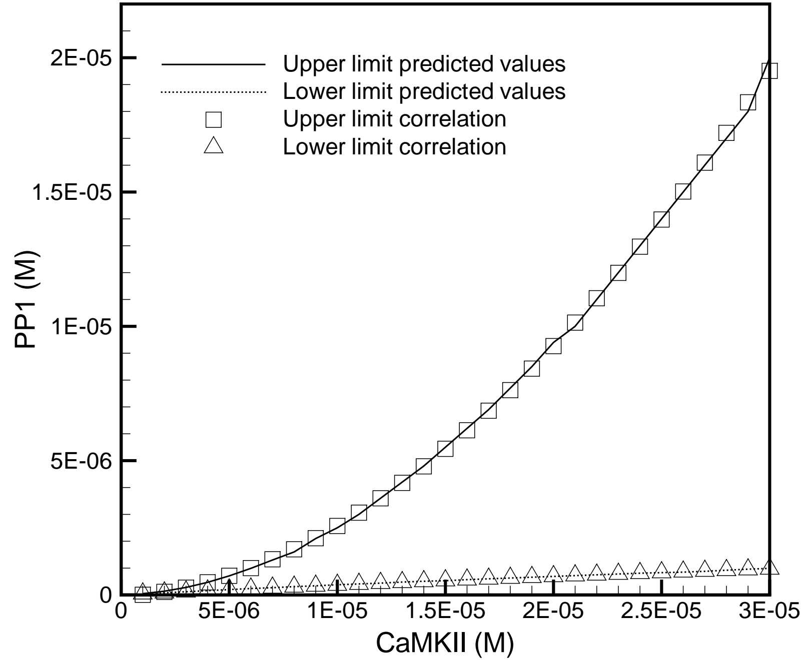


Figure 2.3: The goodness of fit observed for the upper and lower limits of $PP1$ with respect to $CaMKII$. The range of $CaMKII$ concentration used for the correlation is between 0.1-30 μM. The goodness of fit is 0.9995 and 0.9994 for the upper and lower limits respectively.

From the previous result, it can be observed that the change in the upper limit of $PP1$ concentration with respect to $CaMKII$ is sinusoidal in nature, while the same for the lower limit is cubic. Thus, the concentration of the upper and lower limit of $PP1$ can be estimated for all the individual concentrations of $CaMKII$ between which the switch remains bistable. So, a correlation is developed for both the upper and lower limits of $PP1$ with respect to $CaMKII$. The correlation for the upper limit of $PP1$ is given as per the following equation:

$$P_U = A + B \times CaMKII + C \times (CaMKII)^2 + D \times (CaMKII)^3 \tag{2.3}$$

where $CaMKII$ is its respective concentration at individual points and $A = -5.91 \times 10^{-8} M$, $B = 4.177 \times 10^{-2}$, $C = 2.296 \times 10^4 M^{-1}$and $D = -8.699 \times 10^7 M^{-2}$.

The correlation for the lower limit is as follows:

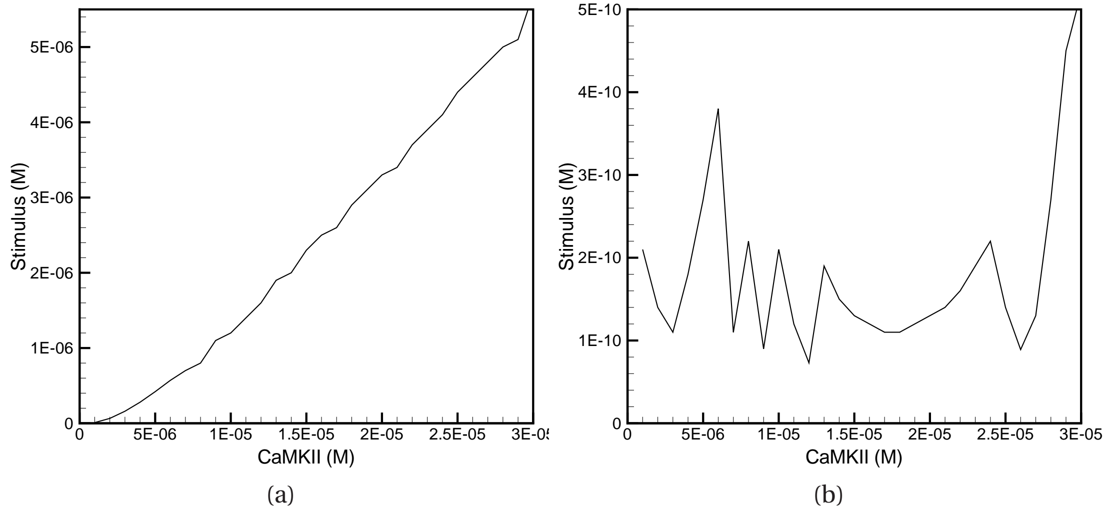


Figure 2.4: (a) For the upper limit of $PP1$ concentration, the dependence of $CaMKII$ on critical amount of stimulus required for $CaMKII$ to undergo autophosphorylation. The curve is monotonic in nature and the range of stimulus is in$\mu Moles$. (b) While for lower limit the curve is random in nature and the range of stimulus is even less than $nMoles$. The $CaMKII$ used is in the range of 0.1-30 μM.

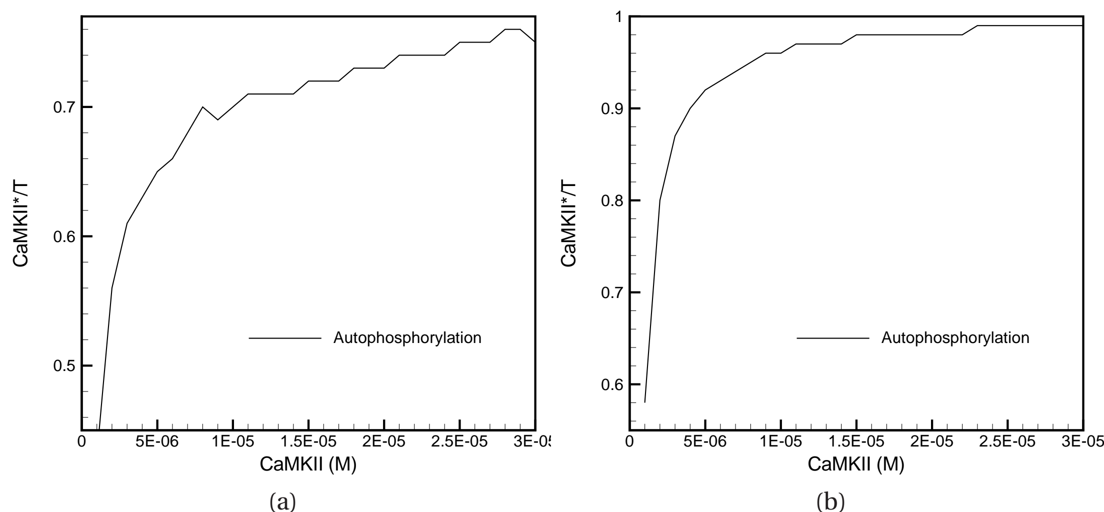
$$P_L = \sin[A + B \times CaMKII + C \times (CaMKII)^2] \quad (2.4)$$

where $CaMKII$ represents the concentration and $A = 1.164 \times 10^{-8} M$, $B = 3.808 \times 10^{-2}$and $C = -2.016 \times 10^2 M^{-1}$.

The goodness of fit for the upper and lower limits of $PP1$ is 99.95 % and 99.94 % as shown in fig. 2.3. Thus, the concentration of upper and lower limit of $PP1$ for $CaMKII$ can be calculated with the help of the above equations where switch bistability is maintained.

2.3.2 $CaMKII$ dependence on autophosphorylation

For each individual concentration of $CaMKII$, a region defined by an upper and a lower concentration of $PP1$ is identified to maintain bistability of the switch. It is also observed that for each concentration of $PP1$, lying inside the bistability zone, a critical amount of stimulus is required to induce autohosphorylation of $CaMKII$. The curves of $CaMKII$ vs stimulus, for which autophosphorylation is induced, are drawn for both the upper and lower limit of $PP1$ as shown in fig. 2.4a and 2.4b. It can be noticed that the critical concentration of stimulus required for inducing autophosphorylation, when considering upper limit of bistability zone, increases monotonically with the increase in the concentration of $CaMKII$ (see fig. 2.4a). However, this variation is random



(a) (b)

Figure 2.5: (a) Dependence of $CaMKII$ on the amount of active $CaMKII$ required for autophosphorylation on the upper limit of $PP1$ concentration. The amount of active $CaMKII$ required stabilizes approximately at 0.75, i.e. maximum of 75% active $CaMKII$ at any given time. (b) A similar curve for the lower limit as well and the amount of active $CaMKII$ required stabilizes approximately at 0.99 which means that almost all of the $CaMKII$ is in the active form. The $CaMKII$ used is in the range of 0.1-30μM.

when the required critical concentration of stimulus for inducing autophosphorylation is plotted against the concentration of $CaMKII$ as shown in fig. 2.4b. This signifies that at higher velocities of $PP1$, the stimulus required for autophosphorylation can be calculated; while at lower velocities, it becomes difficult.

For the upper and lower limits of $PP1$, fig. 2.5a and 2.5b show the dependence of the concentration of $CaMKII$ on the critical concentration of active $CaMKII$ required for autophosphorylation of $CaMKII$. In both the cases, initially there is an exponential increase in the amount of active $CaMKII$; after a certain limit, the concentration of active $CaMKII$ reaches a steady state. It is observed that for the lower limit, the total amount of $CaMKII$ is never present in its active form which shows that $PP1$ is simultaneously converting the active $CaMKII$ into its inactive form. However, on the other hand, almost all of the inactive $CaMKII$ gets converted into active $CaMKII$ for higher values of inactive concentration of $CaMKII$. Also, the part of inactive $CaMKII$ which gets converted into active $CaMKII$ at the time of autophosphorylation is always more for lower limit of $PP1$ as compared to the respective case with upper limit of $PP1$ for a given concentration of inactive $CaMKII$. It is because of two reasons: (1) with increase in $PP1$ velocity the stable point leading to autophosphorylation decreases and (2) with decrease in $PP1$ concentration, more $CaMKII$ becomes activated [49].

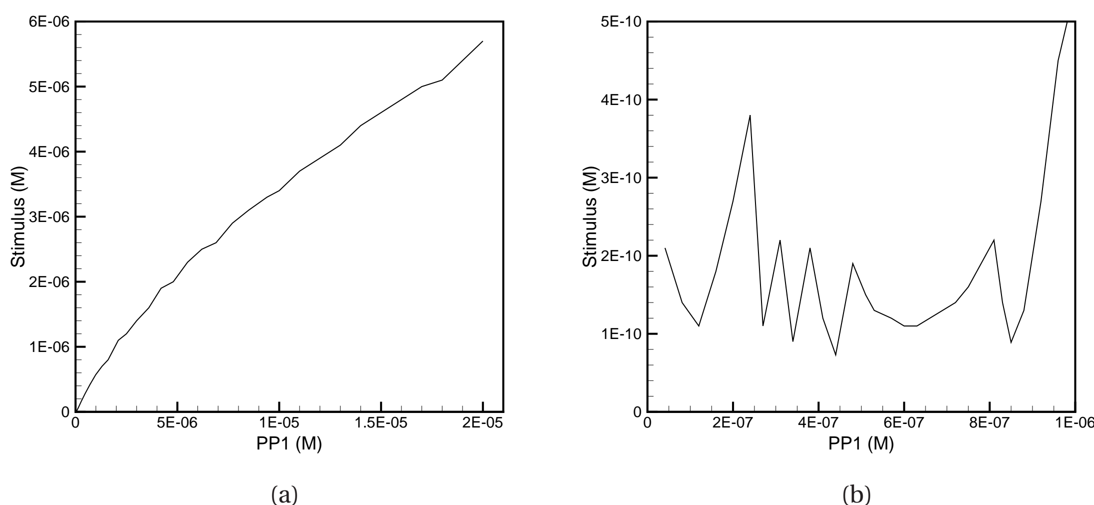


Figure 2.6: (a) Dependence of the upper limit of $PP1$ concentration on the stimulus required for autophosphorylation of $CaMKII$. The dependence is of monotonic nature as observed for $CaMKII$ and the range of stimulus is in $\mu Moles$. (b) While for the lower limit; as seen in case of $CaMKII$, the curve is of random nature and the range of stimulus is even less than $nMoles$.

2.3.3 $PP1$ dependence on autophosphorylation

The critical concentration of stimulus required for autophosphorylation of $CaMKII$ is plotted against the upper and lower limits of the concentrations of $PP1$. The variation is shown in fig. 2.6a and 2.6b. It is interesting to observe that the variation of critical concentration of stimulus required to induce autophosphorylation with the concentration of $PP1$ is almost similar as noticed in fig. 2.4a and 2.4b.

The dependence of upper and lower limits of $PP1$ on the active $CaMKII$ concentration required for direct auotphophorylation is shown in fig. 2.7a and 2.7b. From the figures it can be seen that with gradual increase in $PP1$ concentration for either upper or lower limit, initially there is a rapid increase in active $CaMKII$ concentration which stabilizes after sometime. The stabilization point of active $CaMKII$ for the upper limit of $PP1$ is lower than that of the lower limit because with increase in $PP1$ concentration in the cell, the amount of active $CaMKII$ decreases [49].

2.3.4 Correlation for calculating stimulus

It is noticed that, for a given concentration of inactive $CaMKII$, there exists an upper and a lower limits of the concentration of $PP1$ for which the switch shows bistability. And, for each combination of $PP1$ and inactive $CaMKII$ lying inside the zone of bistability there exists a critical amount of stimulus which induces autophosphorylation. Therefore, for each concentration of inactive $CaMKII$, ten equally spaced concentra-

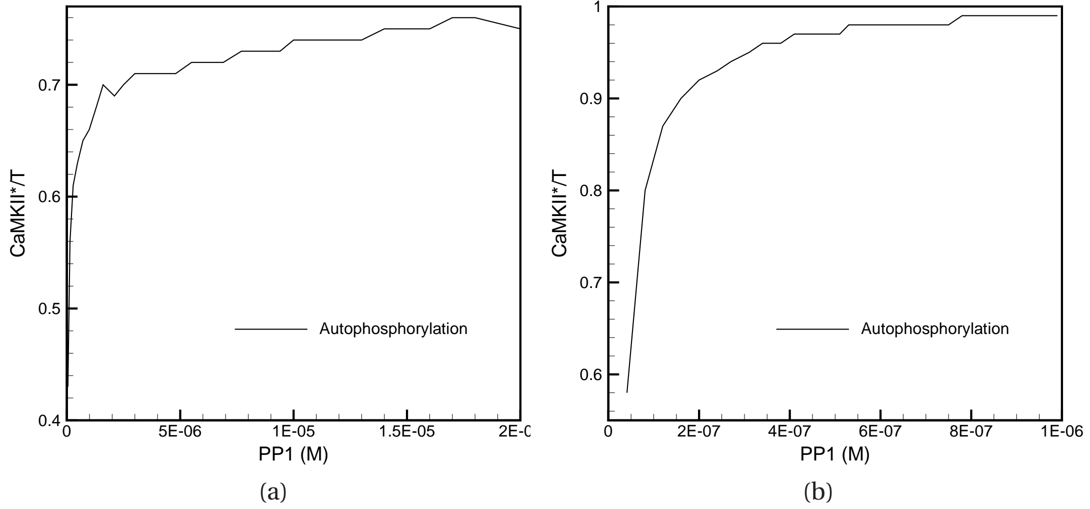


Figure 2.7: (a) Dependence of the upper limit of $PP1$ on the amount of active $CaMKII$ required for autophosphorylation. Just like $CaMKII$ dependence for $PP1$ too, the stabilization of the curve is approximately at 0.75 (b) While in case of lower limit; like $CaMKII$ the amount of active $CaMKII$ required stabilizes approximately at 0.99.

tions of $PP1$ between the lower and the upper limits of bistability zone are selected and for these combinations the critical amount of stimulus is predicted which can induce autophosphorylation. Based on the data collected using different combinations of inactive $CaMKII$ and $PP1$ inside the bistability zone, a correlation is developed for predicting the critical stimulus concentration required for autophosphorylation of $CaMKII$ for a given concentrations of $CaMKII$ and $PP1$. With the help of this correlation, the critical amount of stimulus required for the switch to participate in long term memory formation can be evaluated. First of all, the $PP1$ concentration is normalized by the following equation:

$$P_N = \frac{P_U - P}{P_U - P_L}$$

where P_N is the normalized concentration of P between 0 and 1, P_U and P_L are the upper and lower limit concentrations for a given concentration of inactive $CaMKII$ (eq. 2.3 and eq. 2.4) for which the switch shows bistability, and P represents the concentration of P such that $P_L < P < P_U$.

The developed correlation is given by the following equation:

$$\begin{aligned} Stimulus &= (2.7 \times 10)^{-10} + (A \times P) + B \times (P)^2 \times [1 + (C \times CaMKII) \\ &\quad + \{D \times (CaMKII)^2\}]M \end{aligned}$$

where $PP1$ and $CaMKII$ are variables representing their concentrations at a par-

ticular point and $A = -2.307 \times 10^{-7}$, $B = -1.833 \times 10^{-7} M^{-1}$, $C = -3.429 \times 10^{5} M^{-1}$ and $D = -5.160 \times 10^{9} M^{-2}$.

The above correlation is obtained using non-linear regression analysis with a goodness of fit equals to 99.74 %. A graph showing the concentration of stimulus calculated from the model and from the correlation is drawn in fig. 2.8.

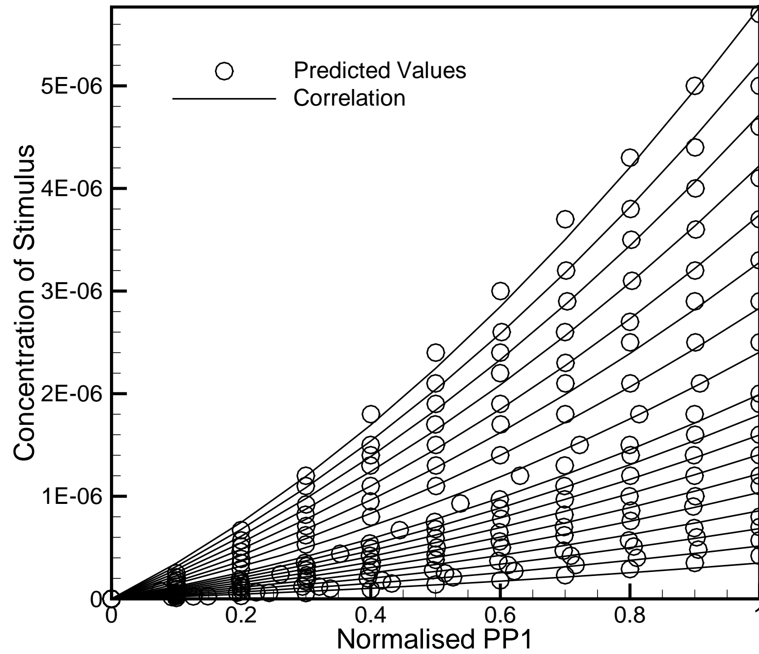


Figure 2.8: Goodness of fit of the stimulus calculated by the model (open circle) and the correlation (solid line). The stimulus is calculated after normalising the $PP1$ for each individual $CaMKII$. Each individual line denotes the $CaMKII$ concentration between 0.1 and 30 μM. The goodness of fit between the stimulus calculated from the model and the correlation is 0.9974 which means that the stimulus required to maintain the bistable nature of $CaMKII - PP1$ switch can be calculated from the given correlation when the $CaMKII$ concentration lies between 0.1 and 30 μM.

2.4 Conclusion

In the present study, a model based on $CaMKII - PP1$ switch is used to predict the dependence of $CaMKII$ and $PP1$ on the bistable nature of the switch. A range of $PP1$ concentration is identified for each individual $CaMKII$ where the switch remains bistable; the bistability of the switch leads to long term memory formation under certain circumstances. Thus, for a given concentration of $CaMKII$, between 0.1 - 30 μM, when the concentration of $PP1$ falls between the said range then the switch leads to long term memory formation. It is noticed that the critical amount of stimulus required for inducing autophosphorylation increased monotonically with the increase in the concentrations of $CaMKII$ and $PP1$ when upper limit of bistability zone is considered while

the variation is random for lower limit of bistability zone. Also, it is found that with the increase in the concentration of inactive $CaMKII$, the fraction of active $CaMKII$ increases and finally reaches a plateau with a value of 0.75 and 0.99 for upper and lower limits of $PP1$ respectively. Based on the numerically predicted data for critical amount of stimulus required for inducing autophosphorylation, a correlation formula relating critical concentration of stimulus, the concentration of inactive $CaMKII$, and the concentration of $PP1$ is proposed with a goodness of fit 99.74%. Therefore, the favourable condition for autophosphorylation can be predicted using the developed correlation which ultimately plays an important role in long term memory formation.

CHAPTER 3

Effect of morphine on LTP_{GABA}

3.1 Introduction

According to the author's best knowledge, none of the investigators have proposed the inhibition mechanism of morphine on the $sGC-NO$ pathway. In this study, we try to address this question by presenting a novel model. We show that the proposed inhibition mechanism inhibits LTP_{GABA} in the presence of morphine which in turn is responsible for addiction. The present study also demostrates the dependence of inhibition on various parameters such as morphine concentration, rate of inhibition, rate of morphine removal and rate of NO removal from the cell.

3.2 Methods

3.2.1 The model

sGC is a heterodimeric hemoprotein composed of α and β subunits and the heme moeity is the NO binding site [81]. sGC is activated by as much as 300-fold when NO binds to the heme cofactor. The activation of sGC by NO is complicated [82]. The reaction between sGC and NO is shown by a two step model as shown in Ballou et al. [83]. The binding of NO is very fast, yielding initially a 6-coordinate ferrousnitrosyl ($6C-sGC-NO$) species that would then decay to the final 5-coordinate complex ($5C-sGC-NO$) via one of the two processes; the first one NO-dependent and the second one NO-independent. The model uses NO as a catalyst in the second step such that the rate depends on the NO concentration, but NO is not consumed in this step. It is assumed that morphine activates a set of molecules X inside the cell by binding to $\mu-opioid$ receptor on the

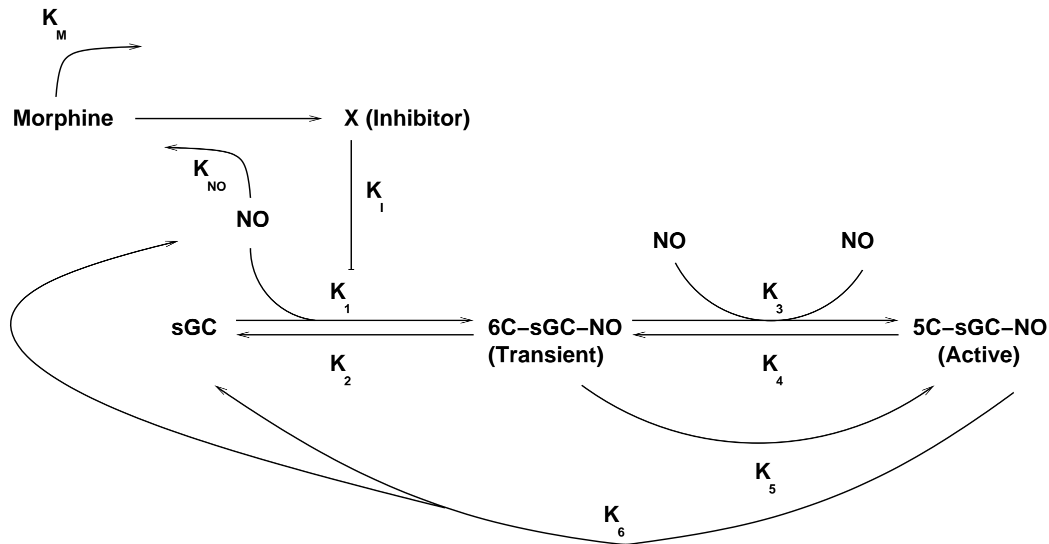


Figure 3.1: Reaction scheme of the model showing *sGC-NO* activation and morphine inhibition. The activation mechanism of *sGC-NO* complex is two step as shown in Ballou et al. [83]. Morphine is assumed to activate a set of inhibitory molecules X, which inhibit the formation of $6C-sGC-NO$ complex and thereby the activation of $5C-sGC-NO$ complex. In the second step of activation, NO is used as a catalyst such that the rate of activation depends on it but it is not consumed in the step. It is assumed that morphine dissociates from the $\mu-opioid$ receptors at zeroth order and NO is also removed from the cell at zeroth order at their respective rate constants.

neuronal cell membrane of the presynaptic GABAergic neurons. The molecule X affects the *sGC-NO* pathway by inhibiting the formation of $6C-sGC-NO$ complex as shown in Fig. 3.1.

The mode of addiction is as follows: The neuronal transmission of the postsynaptic dopamine neurons in the VTA is controlled by two opposite transmissions which leads to the regulation of reward related memories. One is excitatory transmission from the presynaptic glutamatergic neurons and other is the inhibitory transmission by presynaptic gabaergic neurons. LTP_{GABA} produced by the inhibitory gabaergic neurons counterbalances the excitatory neuronal transmission of glutamatergic neurons on the postsynaptic DA neurons and thus regulates its saturation. LTP_{GABA} is produced in the presynaptic gabaergic neurons by the $sGC-cGMP-PKG$ pahway which is activated by the retrogradely travelling NO from the postsynaptic DA neurons. NO binds to the inactive sGC and forms an active complex required for activating PKG which results in the release of GABA molecules from the presynaptic neuron. This molecules bind to $GABA_A$ receptors on the postsynaptic neuron and decrease the postsynaptic neuronal excitability of the DA neurons for a long time which results in inhibition of reward related memory formation. In the presence of morphine, active $sGC-NO$ complex formation is inhibited which stops further downstream processing and hence LTP_{GABA}.

Thus LTP_{GABA} is not produced which might have contributed in preventing the excitatory synaptic plasticity induced by morphine and may lead to reward related memory resulting in addiction.

3.2.2 Mathematical Formulation

In this model, mass action kinetics is used to solve the biochemical equations. sGC is activated when NO binds to it and forms $5C-sGC-NO$ complex by a two step method. Assuming that sGC activation follows mass action kinetics, the rate equations are expressed by Eq. 3.1 and Eq. 3.2:

$$\frac{dS}{dt} = K_1 \times S \times N - K_2 \times S_t - K_6 \times S_a \tag{3.1}$$

$$\frac{dS_a}{dt} = K_3 \times S_t \times N + K_5 \times S_t - K_4 \times S_a \times N + K_6 \times S_a \tag{3.2}$$

where S, N, S_t and S_a are the concentrations of sGC, NO, $6C-sGC-NO$ and $5C-sGC-NO$ complex. K_1 and K_2 are the rate constants of $6C-sGC-NO$ formation and degradation respectively, K_3 and K_4 are the rate constants of $5C-sGC-NO$ formation and degradation respectively by enzymatic catalysis while K_5 is the rate constant of natural $5C-sGC-NO$ formation and K_6 is the rate constant for $5C-sGC-NO$ dissociation into free sGC and NO.

The inhibition of $5C-sGC-NO$ complex formation by morphine via molecule X is described by hill's kinetics as shown in Eq. 3.3:

$$F = \frac{1}{1+(\frac{[I]}{K_i})^2} \tag{3.3}$$

where F is the fraction of sGC inhibited by inhibitor, I is the concentration of inhibitor and K_i is the logarithmic concentration of inhibitor at which the rate of $6C-sGC-NO$ formation becomes half. Following morphine inhibition, the change in the concentration of sGC is shifted as described by Eq. 3.4:

$$\frac{dS}{dt} = K_1 \times S \times N \times F - K_2 \times S_t + K_6 \times S_a \tag{3.4}$$

The hill's coefficient for the inhibition is taken as 2. The rate of dissociation of morphine from $\mu - opioid$ receptors and removal of NO from the cell is given by zeroth order reactions as shown in Eq. 3.5 and Eq. 3.6:

$$D_M = -K_M \tag{3.5}$$

and

$$R_{NO} = -K_{NO} \tag{3.6}$$

where D_M is the rate of dissociation of morphine from $\mu - opioid$ receptors and R_{NO} is the rate of removal of NO from the cell while K_M and K_{NO} are the rate constants of their dissociation and removal respectively.

The numerical simulations were carried out by implicit method. Parameters for the standard system are given in Table 3.1. In the following figures where one parameter varies, all others are fixed according to the table, unless otherwise stated. For those parameters in the table without experimental references, we chose values that were in a reasonable range as provided in the literature for similar chemical reactions.

3.3 Results and Discussion

3.3.1 Morphine blocks the production of LTP_{GABA}

Previous finding suggests that 24 hours after in vivo exposure to morphine, LTP_{GABA} is completely blocked [74], but the mechanism of morphine inhibition is still unknown. A more detailed understanding of the process by which morphine alters LTP_{GABA} could provide valuable information about the effects of morphine on the VTA and the brain reward circuit. We, therefore, propose a mechanism of morphine inhibition which is able to block LTP_{GABA}. The simulations are carried out in the presence of morphine. In the model, sGC and NO bind together and form an active complex which plays a major role in generating LTP_{GABA}. It is observed that in the presence of morphine, sGC is unable to bind with NO and, hence, it does not form an intermediate $6C - sGC - NO$ complex. Hence, the formation of active $5C - sGC - NO$ complex is also inhibited. As shown in fig. 3.2, a lag phase is observed before sGC starts binding with NO to form an intermediate $6C - sGC - NO$ complex. Due to this, a delay in the formation of $5C - sGC - NO$ complex is observed. As a result, further downstream, activation of $cGMP$ and PKG is also paused, and hence the generation of LTP_{GABA} is blocked in the presence of morphine [78, 84]. Thus, administration of morphine completely blocks the ability of the GABAergic synapses to undergo LTP_{GABA}. A single administration of morphine is, therefore,

Table 3.1: Parameters used in the model:

Parameter	Symbol	Value	Unit	Reference
Total concentration of sGC	S	0.5	μM	[83]
Initial concentration of NO	N	0.75	μM	[83]
Initial concentration of Morphine	MO	$0.1 - 1.0*$	μM	This paper
Rate constant for $6C-sGC-NO$ formation	K_1	1.55×10^8	$M^{-1}s^{-1}$	[83]
Rate constant for $6C-sGC-NO$ degradation	K_2	1.0×10^{-2}	s^{-1}	[83]
Rate constant for enzymatic $6C-sGC-NO$ turnover	K_3	3.3×10^5	$M^{-1}s^{-1}$	[83]
Rate constant for $5C-sGC-NO$ deactivation	K_4	1.0×10^{-3}	s^{-1}	[83]
Rate constant for natural $6C-sGC-NO$ turnover	K_5	5.0×10^{-4}	s^{-1}	[83]
Rate constant for $5C-sGC-NO$ dissociation	K_6	1.0×10^{-3}	s^{-1}	[83]
Rate constant for morphine dissociation	K_M	$3.0 \times 10^{-10} - 1.0 \times 10^{-9}*$	s^{-1}	This paper
Rate constant for NO removal	K_{NO}	$7.5 \times 10^{-11} - 2.5 \times 10^{-10}*$	s^{-1}	This paper
Logarithmic concentration of inhibitor when the velocity of $6C-sGC-NO$ formation becomes half	K_i	$1.0 \times 10^{-10} - 5.0 \times 10^{-10}*$	M	This paper

* Specific values are given in figure legends.

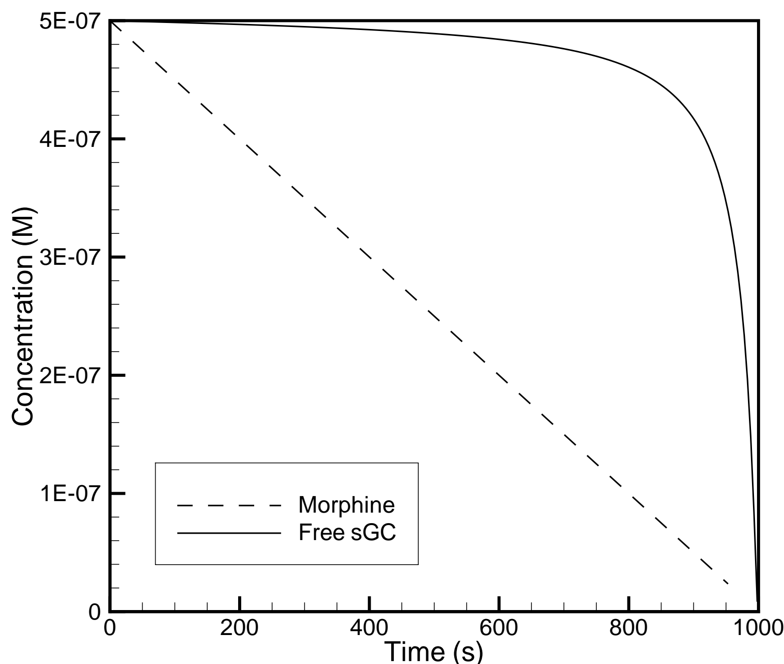


Figure 3.2: Inhibition of $sGC - NO$ complex formation by morphine. When morphine is present then there is inhibition of the $sGC - NO$ complex formation and as soon as morphine is completely dissociated from $\mu - opioid$ receptor, sGC becomes completely saturated with NO to form $sGC - NO$ complex which results in sGC activation and hence LTP_{GABA} generation. Initial morphine concentration, $MO = 5.0 \times 10^{-7} M$ and initial sGC concentration, $S = 5.0 \times 10^{-7} M$.

able to potentiate excitatory synaptic transmission [66], while at the same time it prevents a complementary increase in inhibitory transmission that normally would have counterbalanced the increased excitation. Thus, blockade of LTP_{GABA} by morphine prevents DA neuron inhibition that might be able to reverse or prevent synaptic plasticity at excitatory terminals induced by drugs of abuse, and hence contribute to the development of addiction.

3.3.2 Sustainence of LTP_{GABA} independent of NO

Sustained activity of protein kinases such as protein kinase C (PKC) and Ca^{2+}/CaM kinase type II ($CaMKII$) have been proposed to be involved in maintenance and expression of LTP [49, 50]. We also tested, here, whether constitutive release of NO is necessary to sustain LTP_{GABA} or, instead, is a brief exposure sufficient to persistently enhance GABA release? From fig. 3.3, it is observed that sGC remains active for a long period of time even after NO has depleted from the presynaptic GABAergic neuron. As a result, it is able to activate further downstream molecules, i.e. $cGMP$ and PKG which

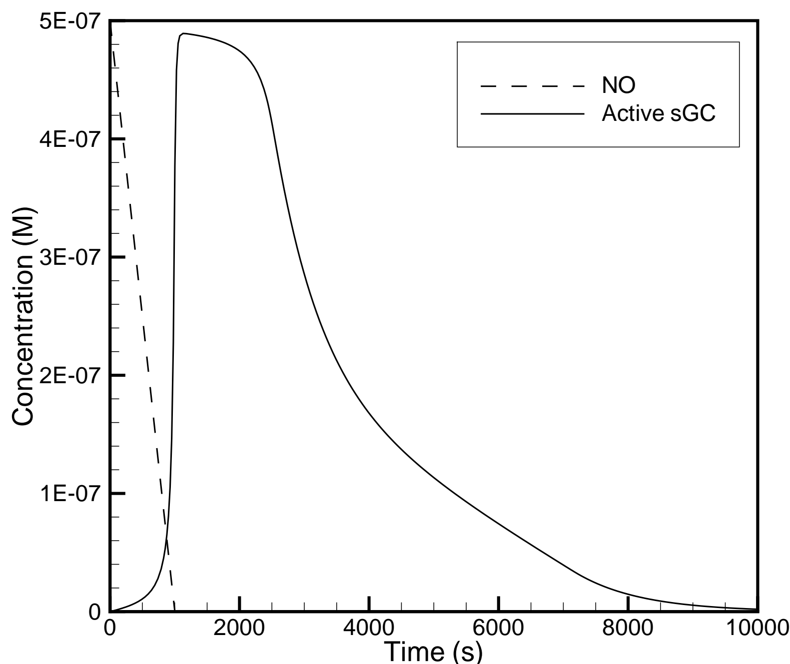


Figure 3.3: sGC remains activated even after NO is depleted from the cell. Although NO is completely removed from the cell, it is observed that activated sGC i.e. $5C - sGC - NO$ takes a long time to come back to its inactive form i.e. sGC. It assists in activating further downstream molecules such as $cGMP$ and PKG which results in LTP_{GABA}. Thus it is shown that NO is not required to sustain LTP_{GABA}. Initial NO concentration, $N = 5.0 \times 10^{-7} M$.

are necessary for generating LTP_{GABA}. Thus, even a brief exposure of NO in the presynaptic GABAegic neurons is able to induce long lasting LTP_{GABA} which counterbalances the excitatory synaptic potentiation induced by presynaptic glutamatergic neurons on the postsynaptic DA neurons. Therefore, when morphine inhibits NO to bind with sGC to form an active complex and blocks LTP_{GABA}, the excitatory potentiation is not reversed and so the behavioural responses towards the drugs of abuse are modified in the mesolimbic dopamine system which could lead to the development of addiction. These results are similar to the one obtained by Nugent et al. [84], where an NO scavenger, PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, 300 μM) was added after the NO donor elicited synaptic potentiation, but the potentiation did not decay back to control values.

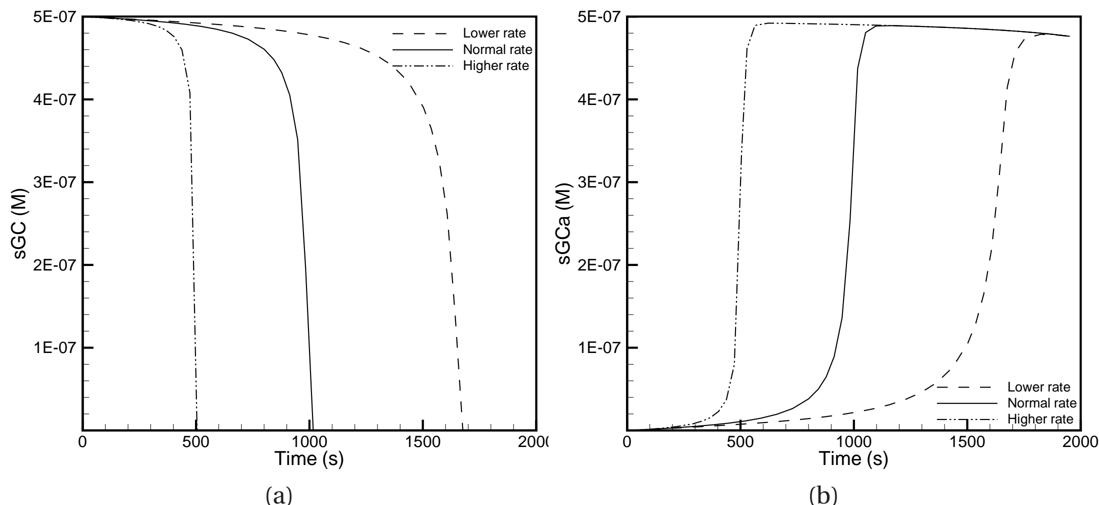


Figure 3.4: (a) Initiation of sGC complex formation depending on the value of K_M. Depending on the value of K_M, a lag phase is observed before the $sGC - NO$ complex formation is initiated. At lower values of K_M, a longer lag phase is observed and as the value of K_M increases, the lag phase decreases and free sGC is quickly saturated into its activated state. (b) Dependence of sGC activation on the rate of morphine dissociation. With increase in the value of K_M, morphine dissociates quickly and hence the inhibition by morphine reduces which results in reduced lag phase. Thus sGC is quickly converted to its active form by forming complex with NO. Lower rate= 3.0×10^{-10}, normal rate= 5.0×10^{-10} and higher rate= 1.0×10^{-9}.

3.3.3 K_M and MO affect the initiation of $6C - sGC - NO$ complex formation

It is found that in vivo morphine administration entirely blocked LTP_{GABA} [74]. $GABA_A$ synapses in VTA slices from rats that had received morphine 24 hours earlier did not exhibit LTP, but after 5 days the effect of morphine was nonexistent. So, we examined whether the changes in morphine concentration affects the inhibition of LTP_{GABA}. The morphine concentration at any given time depends on K_M, the rate of dissociation of morphine from the $\mu - opioid$ receptor and MO, the initial concentration of morphine bound to the $\mu - opioid$ receptors present on the cell membrane of presynaptic GABAergic cells. As shown in fig. 3.4a, it is observed that when the value of K_M decreases then the rate of morphine dissociation also decreases which results in a longer lag phase for initiating $6C - sGC - NO$ complex formation. As a result, a delay is observed in the activation of $5C - sGC - NO$ complex which accounts for the reduced rate of $cGMP$ and PKG activation. So, a prolonged inhibition of LTP_{GABA} is observed due to which there is a decrease in inhibitory transmission to the postsynaptic DA neurons, which in turn increases the chances of addiction. But when the value of K_M is increased, morphine dissociates from the $\mu - opioid$ receptors at a faster rate and hence the inhi-

bition on sGC also vanishes rapidly. The initiation of $6C-sGC-NO$ complex formation is started quickly which results in early formation of $5C-sGC-NO$ complex and thereby decreased inhibition on LTP_{GABA}. Therefore, the inhibitory transmission generated from the presynaptic GABAergic cells could counteract the excitatory transmission on the postsynaptic DA neurons and reduce the chances of addiction. As $5C-sGC-NO$ is the active form of enzyme, sGC which is used for subsequent downstream pathways [81], we have also studied the effect of K_M on the rate of formation of $5C-sGC-NO$ complex. From fig. 3.4b, it is observed that with decrease in the value of K_M, morphine binds to the $\mu-opioid$ receptors for a longer duration of time which results in increased inhibition of sGC. So, the initiation of sGC activation takes more time and a longer lag phase is observed. As a result, $cGMP$ and PKG activation slows down and LTP_{GABA} is inhibited for a prolonged duration. This results in increased probability of addiction because of the absence of inhibitory synaptic transmission which could counteract the excitatory synaptic transmission. Another interesting observation made from fig. 3.4b is that with decrease in the value of K_M, the maximum amount of $5C-sGC-NO$ complex that can be activated also decreases. This happens because as the value of K_M decreases, morphine remains bound to the $\mu-opioid$ receptors for a long time. As a result, sGC is unable to bind NO for a prolonged time. So, the maximum concentration of $5C-sGC-NO$ decreases with a decrease in K_M. This shows that along with the duration of inhibition of sGC, K_M also influences the maximum amount of $5C-sGC-NO$ complex that can be formed. Thus, it can be inferred that K_M is able to control the duration and amount of LTP_{GABA} inhibition.

A recent study by Kitumura et al. [85] revealed that there is a marked change in behavioural sensitization at the mesolimbic dopamine system between the investigator-administered drug (passive administration) and self-administered drug (active administration). These changes occur due to time course over which drugs are administered and their amount of administration [75]. Therefore, we studied here the effect of different concentrations of initial morphine bound to the $\mu-opioid$ receptors i.e. MO on the inhibition of LTP_{GABA} because the amount of drug also plays a very important role in development of addictive behaviour. It is observed from fig. 3.5a that the effect of MO is almost similar to that of K_M. When the morphine concentration increases, complete withdrawal of morphine from the $\mu-opioid$ receptors takes more time. So, morphine is able to inhibit the binding of sGC with NO for a longer duration and a longer lag phase for initiating $6C-sGC-NO$ complex formation is observed. As a result, the active complex of sGC, i.e. $5C-sGC-NO$, is generated slowly; due to which the activation of $cGMP$ and PKG also slows down. This implies that there is an increased inhibition of LTP_{GABA} which can play an important role in developing addiction. As shown in fig. 3.5b, it is observed that with the increase in morphine concentration, there is an increase in $5C-sGC-NO$ inhibition by morphine and so the amount of time taken for sGC to

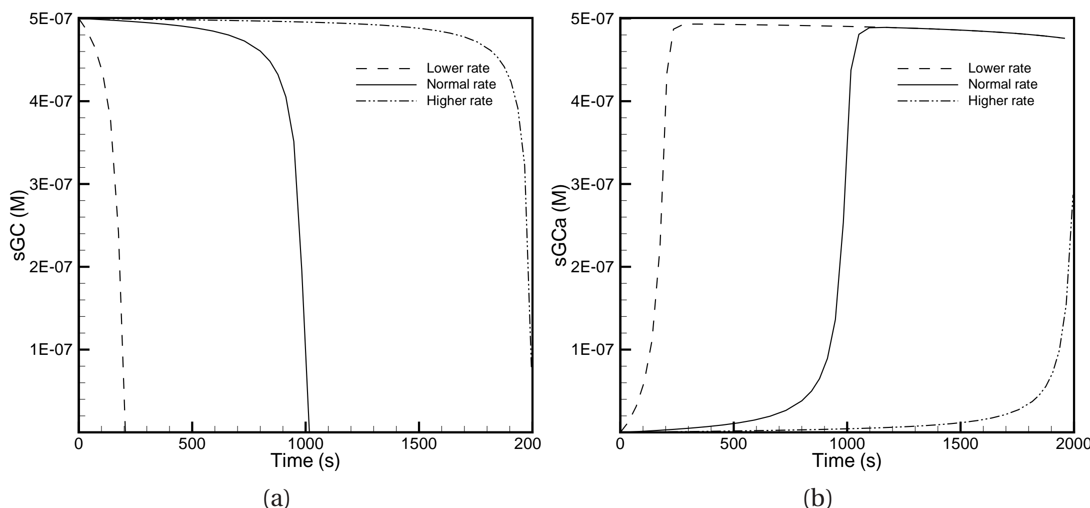


Figure 3.5: (a) Dependence of initial morphine concentration on the initiation of $sGC-NO$ complex formation. As the initial concentration of morphine is decreased, the initiation of complex formation started rapidly because of decreased inhibition. Thus, a smaller lag phase is observed which increases with the increase in initial morphine concentration. (b) The activation of sGC is dependent on the initial concentration of morphine. When the initial concentration of morphine is increased, the inhibition persists for a longer duration and therefore sGC becomes active after some time. Thus, a longer lag phase is observed in case of sGC activation which decreases with the decrease in initial morphine concentration. Lower rate= 3.0×10^{-10}, normal rate= 5.0×10^{-10} and higher rate= 1.0×10^{-9}.

get activated also increases. As a result, further downstream pathway for LTP_{GABA} generation is blocked for a prolonged duration. So, LTP_{GABA} is also inhibited for a long duration of time with an increase in morphine concentration which usually results in increased chances of addiction.

3.3.4 Regaining initial sGC concentration, after $5C-sGC-NO$ dissociation, depends on K_{NO}

Addiction is not triggered instantaneously upon acute exposure to drugs of abuse. It involves complex neural adaptations that develop with repeated drug exposure at different time intervals ranging from hours to days to months. Research work to date suggests that during exposure to opiates, LTP_{GABA} is blocked by the opiate via inhibition of sGC to bind with retrogradely travelling NO and form an active complex [74]. To induce addiction by repeated drug exposure, sGC has to be present in free form in the presynaptic GABAergic neurons. Here, we have studied the effect of K_{NO} on the model because it affects the time taken for free sGC to regain its initial concentration after it has formed an active complex, i.e. $5C-sGC-NO$. Depending on the rate of NO removal from the

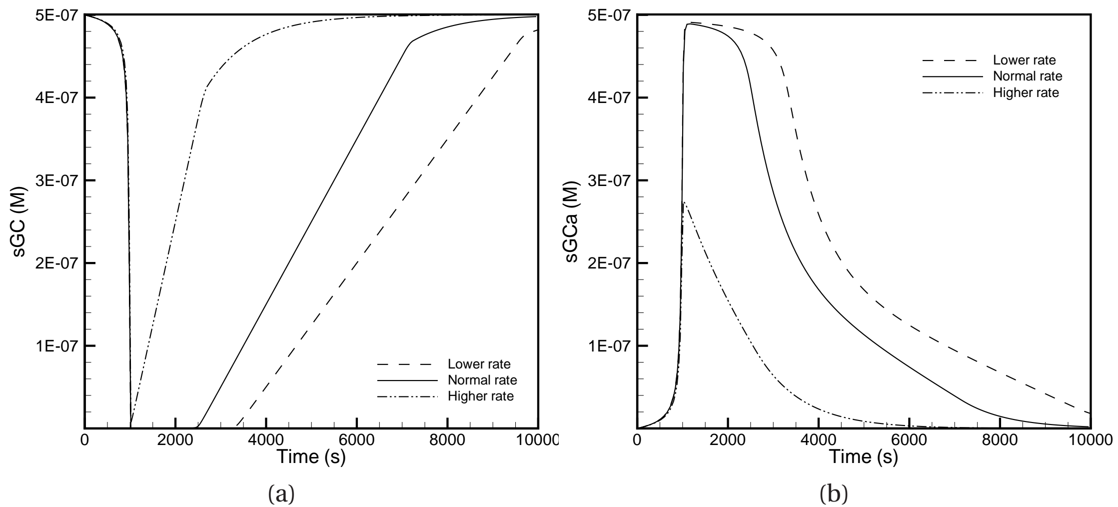


Figure 3.6: (a) The rate of free sGC restoraton to its initial concentration depends on the rate of NO removal. With a decrease in the value of K_{NO}, NO remains in the cell for a longer amount of time and sGC is able to bind with it and form complex. Therefore, sGC is not available in free form and it reaches its initial value after a prolonged time. (b) Formation of active sGC i.e. $5C - sGC - NO$ depends on the rate of NO removal from the cell. With a increase in the value of K_{NO}, the period of time for which sGC remains in active form decreases because the dissociated NO is quickly removed from the cell before it could again complex with free sGC to become activated; and after a critical increase in K_{NO}, the maximum amount of sGC found in active form also decreases. Lower rate= 7.5×10^{-11}, normal rate= 1.0×10^{-10} and higher rate= 2.5×10^{-10}.

cell, the complex between sGC and NO is broken down and free sGC regains its initial concentration in the neuron. This free sGC, again, does not form complex because of nonavailability of free NO in the GABAergic neuron. From fig. 3.6a, it is observed that when the value of K_{NO} decreases then sGC remains in its active form for a long period of time. During this time, if morphine is re-exposed then it is not able to inhibit LTP_{GABA} generation because it only blocks free sGC and NO complex formation; but here sGC is not available in free form. Therefore, with reduction in the value of K_{NO}, the primary LTP_{GABA} generated counterbalances the excitatory synaptic transmission of the presynaptic glutamatergic neurons developed because of drug exposure and hence reduces the chances of addiction. So, it can be concluded that as the value of K_{NO} decreases, it takes a long time for free sGC to shoot up to its initial value and hence upon re-exposure to morphine, sGC is not available in free form to get inhibited again. Thus, morphine is not able to block LTP_{GABA} again and this reduces the chances of addiction. An interesting observation is made from fig. 3.6b, which shows that with increase in the value of K_{NO}, the maximum amount of sGC found in active form decreases. As the value of K_{NO} increases, the rate of NO removal also increases and the concentration of active sGC complex decreases from the cell at a faster rate. This happens because, with increase in

the rate of removal of NO, i.e. K_{NO}, the rate of formation of active sGC decreases as free NO is not present in the cell to bind with free sGC. Therefore, sGC remains in its inactive form; as a result, the maximum amount of sGC found in active form at any given time in the neuron decreases. At the same time, this inactive form of sGC is ready to be inhibited after morphine re-exposure which in turn results in LTP_{GABA} blockade. This blockade serves to enhance the chances of addiction in the mesolimbic cortical system. But when the value of K_{NO} decreases, sGC remains in its active form for a prolonged time, because NO takes longer time to deplete from the cell. Therefore, upon re-exposure of morphine, there is unavailability of free sGC in the neuron which results in nonblockade of LTP_{GABA} and eventually decreases the chances of addiction. It is also observed that the amount of sGC that is converted to active form also depends on the value of K_{NO}. After a certain increase in its value, the maximum amount of sGC, that has been converted into active form, decreases because the rate of removal of NO exceeds the rate of formation of $5C - sGC - NO$ and, before the dissociated NO from $5C - sGC - NO$ is again able to form complex with free sGC, it is removed from the cell and hence sGC is not converted back to its active form.

3.3.5 K_i changes the nature of morphine inhibition

Although morphine inhibits sGC to bind with retrogradely travelling NO and form an active complex but the type of inhibition, i.e. complete inhibition or partial inhibition, is dependent on the rate of inhibition. In the case of complete inhibition, sGC will not form complex with NO, even when morphine is present in very small amount. While in the case of partial inhibition, sGC will partially bind with NO to form an active complex but at reduced concentrations. Thus, during complete inhibition, even though initial morphine concentration bound to the $\mu - opioid$ receptors is low, it will lead to increased inhibition; on the other hand, during partial inhibition, even in the presence of excess morphine, sGC would initiate binding with NO. We studied here the effect of the rate of inhibition on the nature of morphine inhibition. This rate of inhibition (K_I) is inversely proportional to the logarithmic concentration of inhibitor when the rate of $6C - sGC - NO$ formation is halved (K_i). It is observed from fig. 3.7a that when the value of K_i decreases then the rate of inhibition increases and the reaction between sGC and NO is not initiated for a long duration of time due to which downstream molecules such as $cGMP$ and PKG also take a long time to get activated. So, LTP_{GABA} is generated after a long gap. Thus, excitatory synaptic transmission from the presynaptic glutamatergic neurons is not counterbalanced which could result in increased chances of addiction. But when the value of K_i increases then the reaction between sGC and NO is initiated quickly and so LTP_{GABA} is quickly produced which is able to nullify the effect of excitatory transmission on the postsynaptic DA neurons. As a result, the chances of addiction

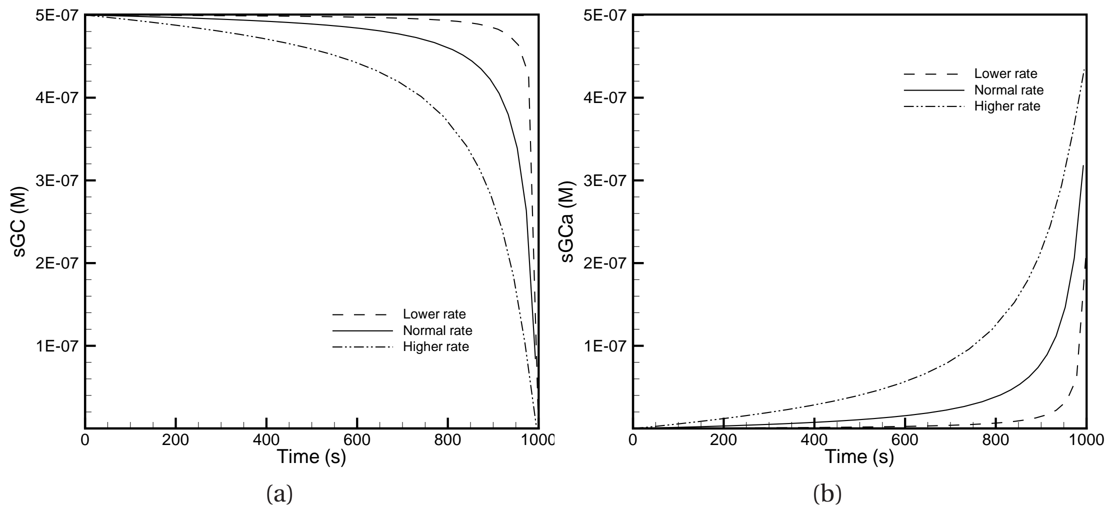


Figure 3.7: (a) Dependence of the rate of morphine inhibition on the $sGC - NO$ complex formation. K_i is inversely proportional to the rate of inhibition and as its value increases, the rate of morphine inhibition decreases and sGC quickly initiates active complex formation with NO. It is observed that K_i does not affect the time taken for sGC to reach saturation because for the same concentration of morphine and its rate of dissociation, K_i only affects the type of inhibition whether sGC will quickly initiate complex formation with NO or will remain in free form for a longer duration. (b) Dependence on the type of sGC activation on the rate of morphine inhibition. As K_i is inversely proportional to the rate of morphine inhibition, when its value increases the rate of sGC activation also increases. It only affects the type of activation and not the time in which $5C-sGC-NO$ would reach saturation because of the same amount of morphine and its dissociation rate. Lower rate= 1.0×10^{-10}, normal rate= 2.5×10^{-10} and higher rate= 5.0×10^{-10}.

are reduced. It is interesting to note that unlike K_M, K_i does not change the time taken for the reaction to reach complete saturation as seen in fig. 3.7a, which signifies that either K_i or K_I only affects the amount of time sGC would be inhibited to react with NO and form an active complex but not the rate at which they both will reach saturation. As shown in fig. 3.7b, as the value of K_i increases, sGC becomes active rapidly because K_I decreases and the rate of inhibition decreases which results in rapid activation of $cGMP$ and PKG. Therefore, LTP_{GABA} is generated at the earliest which decreases the chances of addiction. It is also observed that there is no change in the time taken for active sGC to reach its maximum value. This happens because K_i only affects the initial time when sGC starts forming the complex with NO and get converted into active sGC i.e. $5C - sGC - NO$.

3.4 Conclusion

In this study, a novel mechanism of morphine inhibition on LTP_{GABA} is proposed. It is observed that sGC is inhibited to form complex with NO in the presence of morphine which ultimately results in LTP_{GABA} inhibition. Here, it is shown that morphine is able to activate an intracellular molecule X which binds to the NO binding site on sGC and thus prevents $sGC-NO$ complex formation. This inhibition is shown to be dependent on the initial morphine concentration (MO), dissociation rate of morphine from the $\mu-opioid$ receptors (K_{MO}), rate of NO removal from the cell (K_{NO}) and the rate of morphine inhibition (K_I). K_M and MO regulate the amount of time sGC is inhibited by X and unable to bind to NO and form an active complex, thereby activating $cGMP$ and PKG which induces LTP_{GABA}. K_{NO} controls the rate at which free sGC reaches its initial concentration. Depending on the rate of NO removal, NO is available for the dissociated sGC to form an active complex again and produce LTP_{GABA}. K_i is only responsible for the nature of morphine inhibition. It only regulates the amount of time sGC remains in its free form at the start but does not play any role in the time taken for $5C-sGC-NO$ to reach saturation. Although an inhibition mechanism of morphine is proposed, we remain unclear about the molecular processes taking place behind this mechanism. Advances in this direction would enhance our understanding of the interplay of molecular and network properties in determining the basic mechanism behind morphine addiction.

CHAPTER 4

Summary

This research broadly consists of two parts — (i) Model development and theoretical studies of the $CaMKII-PP1$ bistable switch and (ii) Proposing a novel mechanism of morphine inhibition on LTP_{GABA} and studying its effects on morphine addiction.

$CaMKII-PP1$ bistable switch

Based on the results of the $CaMKII-PP1$ model in chapter 2, the range of $CaMKII$ and $PP1$ are defined wherein the switch maintains bistability and takes part in long term memory formation. For each individual concentration of $CaMKII$, a upper and lower limit of $PP1$ is calculated for maintaining bistability. With increase in $CaMKII$, the range between upper and lower limit of $PP1$ increases which demonstrates the preference for high level of $CaMKII$ inside the cytosol. As autophosphorylation of $CaMKII$ is very important for further downstream processing of the pathway for a long duration of time which leads to long term memory formation. So, the critical stimulus intensity required for $CaMKII$ to initiate autophosphorylation is also calculated from the model. A correlation is also developed for calculating the critical stimulus concentration based on the concentrations of both $CaMKII$ and $PP1$.

Morphine inhibition of LTP_{GABA} and addiction

It is shown in chapter 3 that morphine blocks the binding of sGC with NO to form an active complex which leads to blockade of LTP_{GABA}. Morphine regulates its activity by binding to the $\mu-opioid$ receptors and thereby activating secondary messengers which block sGC binding. The dependence of inhibition on various parameters such as initial morphine concentration, dissociation rate of morphine from the $\mu-opioid$ receptors,

rate of NO removal from the cell and the rate of morphine inhibition is also studied. The results show that these parameters play a major role in morphine inhibition and thereby controlling addiction.

4.1 Limitations and Recommendations

In the model developed to study the $CaMKII - PP1$ bistable switch, $CaMKII$ is considered to be made up of two subunits for the simplification of the numerical analysis. A more complex model can be developed based on the heteromeric complex made up of 12 subunits.

Due to lack of knowledge about the secondary molecules taking part in sGC inhibition by morphine, a direct approach is used here to show the effect of morphine inhibition on LTP_{GABA}. Advances in this direction would help us understand the molecular interplay taking place behind morphine addiction.

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