Role of Lysine Acetyltransferases p300/CBP in Neurological Disorders: Implications in Therapeutics

A thesis submitted for partial fulfilment of degree of

MASTER OF SCIENCE as part of Integrated PhD program

By

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June 2017

.....To My Parents.

DECLARATION

I, hereby declare that this thesis entitled **"Role of Lysine Acetyltransferases p300/CBP in Neurological Disorders: Implications in Therapeutics",** is an authentic record of research work carried by me under the supervision of Prof. Tapas Kumar Kundu, Molecular Biology and Genetics Unit, and Dr. James Chelliah, Neuroscience Unit, Jawaharlal Nehru Center for Advanced Scientific research, Bengaluru. This work has not previously formed the basis for the award of any other diploma, degree or fellowship.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

Place: JNCASR, Bengaluru
Date : 23/06/2017

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CERTIFICATE

This is to certify that the work presented in this thesis entitled **"Role of Lysine Acetyltransferases p300/CBP in Neurological Disorders: Implications in Therapeutics"** is an authentic record of research work carried out by Mr. Akash Kumar Singh under my supervision during the time period June, 2016 to April, 2017. This work was carried out in Transcription and Disease Laboratory, Molecular Biology and Genetics Unit, and Dr. James Chelliah Laboratory, Neuroscience Unit, Jawaharlal Nehru Center for Advanced Scientific research, Bengaluru as a part of **Int. Ph.D. Master Science Programme** and has not been submitted elsewhere for the award of any other diploma, degree or fellowship.

Date: 23/06/2017

Prof. Tapas K. Kundu

ACKNOWLEGEMENTS

I would like to express my sincere gratitude to my mentor, Prof. Tapas K. Kundu for giving me the opportunity to work as a MS student in his esteemed laboratory. I am extremely grateful to him for his constant guidance, inspiration, encouragement and the kind of training he has provided me throughout this thesis work. Most importantly, his optimistic approach, scientific inputs and the aim high attitude has extracted the best out of me.

I would like to express my sincere gratitude to my co-mentor, Dr. James Chelliah for his guidance and constant supervision as well as for providing the necessary information regarding the project and his support in the completion of this project. It was undoubtedly a great learning experience for me under his guidance, for which I am deeply indebted to him.

I express my warm thanks to Ramesh sir (Dr. GR Ramesh) for teaching me the basic and important aspects of research during the MS-course work. I take this opportunity to thank MBGU faculty members Prof. Uday Kumar Ranga, chairman, MBGU, Prof. M.R.S. Rao, Prof. Anuranjan Anand, Prof. Hemlatha Balaram, Prof. Maneesha Inamdar, Prof. Namita Surolia, Dr. Kaustuv Sanyal and Dr. Ravi Manjithaya for their invaluable support.

My thanks and appreciations also go to all the current and ex members of my lab for their constant help and encouragement. Special thanks to Dr. Sarmistha Haldar and Piyush for helping me with the nanoparticle work (synthesis and conjugation).

As most of my work is focused on mouse model, I would like to thanks the JNCASR animal facility. Special thanks to Dr. Prakash for training me with the animal handling and Oral/ IP administration. Thanks to JNCASR confocal facility (Suma Ma’am and Sunil).

Staying and working in JNC has been a great fun with a group of good friends: Siddharth, Pallabi, Moumita, Pranoy, Abhik, Smitha, Aditya, Manjeet, Srikant, Raktim, Arindam, Vijay, Ipsita, Sthita, Vijaya, Aditi, Zeenat, Vikas, the lagori/pitto group (Arun, Badri, Neelakshi, Neha,

Nityanand, Manisha, Disha, Brahma, Deepak, Prabhu and others). A special thanks to my batchmates: Badri, Sarika, Jigayasa, Abhijeet, and Srilaxmi for the wonderful time we had together. Thank you all.

Finally, I would take this opportunity to express my deepest gratitude to my family for their love and encouragement. I wouldn’t have been this stronger without their constant support and motivation. This work wouldn’t have been be possible without them. They are the reasons behind my firmness, stability and robustness which always keep me going. Love you all.

List of Abbreviations:

µg	:	Microgram
µl	:	Microliter
µm	:	Micrometer
µM	:	Micro molar
µA	:	Microampere
mg	:	Milligram
ml	:	Milliliter
mV	:	Millivolts
ms	:	Milliseconds
Kg	:	Kilogram
V	:	Volts
Hz	:	Hertz
MΩ	:	Miro Ohm
°C	:	Degree Celsius
bp	:	Base pair
kb	:	Kilo base
WT	:	Wild Type
UT	:	Untreated
IC_{50}	:	Half maximal inhibitory concentration
ID	:	Internal diameter
OD	:	Outer diameter
SEM	:	Standard error of mean
EDX	:	Energy-dispersive X-ray spectroscopy
FESEM	:	Field emission scanning electron microscope
DEPC	:	Diethyl polycarbonate
rRNA	:	Ribosomal RNA

TSA : Tricostatin A

IQ : Intelligence quotient

FV : Fiber volley

fEPSP : Field excitatory post-synaptic potentiation

LTP : Long-term potentiation

E8.5 : Embryonic day 8.5

PND : Post-natal day

SynHet : *Syngap1* heterozygous

RTS : Rubinstein-tyabi syndrome

DNMTs : DNA methyltransferases

List of Contents

CHAPTER 1

Introduction

This chapter discusses about the chromatin and its dynamicity during transcription, factors that influence the status of chromatin and hence alter gene expression with especial emphasis on the role of lysine acetyltransferases p300/CBP during neural development, long term memory formation, synaptic plasticity and neurological disorder. It also describes the current status of small molecule modulators of lysine acetyltransferases as a therapeutic option for various neuro-developmental and neurological disorders. In the last section, it discusses about a mice model for Intellectual Disability, a neuro-developmental disorder.

Outline of the chapter:

1.1. Chromatin dynamics and gene regulation

1.2. KATs in chromatin function and Transcriptional regulation

1.3. p300/CBP: Role during neural development and implications in neurodevelopmental disorder

1.4. Small molecule modulators of HATs

1.5. Brain targeted drug delivery through nanoparticles

1.6. Intellectual Disability: A Syngap1 heterozygous mouse model

1.7. Rationale of this study and objectives

1. 1. Chromatin dynamics and gene regulation

1.1.1. Chromatin organization:

Deoxyribonucleic acid (DNA), which is the blueprint of life is essential for all known forms of life. In order to facilitate the packaging of 2--meter--long DNA strand in the compact nucleus of

mammalian cells roughly around 10µm in diameter, the DNA present in the nucleus of these cells associate with histones, non-histone proteins and RNA to form a highly organized and complex structure termed as chromatin. The fundamental and functional unit of Chromatin is the nucleosome, which is formed by wrapping of 146 base pairs of DNA around octamer of four core histone proteins (H2A, H2B, H3 and H4). These structures are further arranged through a series of successively higher order structures to eventually form a Chromosome which adds up both level of compactness and an extra layer of regulatory control that ensures fidelity of gene expression[1].

1.1.2. Chromatin dynamics and gene regulation:

During the process of DNA repair, replication, recombination and transcription, the DNA strand should be accessible for repair, replication, recombination and transcription machineries, but the presence of higher ordered chromatin structure acts as barrier for enzymes that unwind and copy the DNA. Thus, it is essential for cells to have certain machinery/mechanism for opening of the DNA strand from the higher ordered Chromatin structure. There are various factors/mechanism that can remove this barrier and unwind the DNA for accessibility to transcription and other cellular machineries. These factors include: A) Chromatin remodeling complexes; (B) Histone chaperones such as HIRA, NAP1, Chz1, FACT, Spt6 and others; (C) Histone variants, such as H2A.Z, CENP-A, H3.3, TH2B; (D) Nucleosome associated non-histone proteins , such as the positive coactivator4 (PC4), heterochromatin-associated protein (HP1) and poly ADP-ribose polymerase-1 (PARP1); (E) Non-coding RNAs, and (F) Chromatin modifications (fig 1.1).

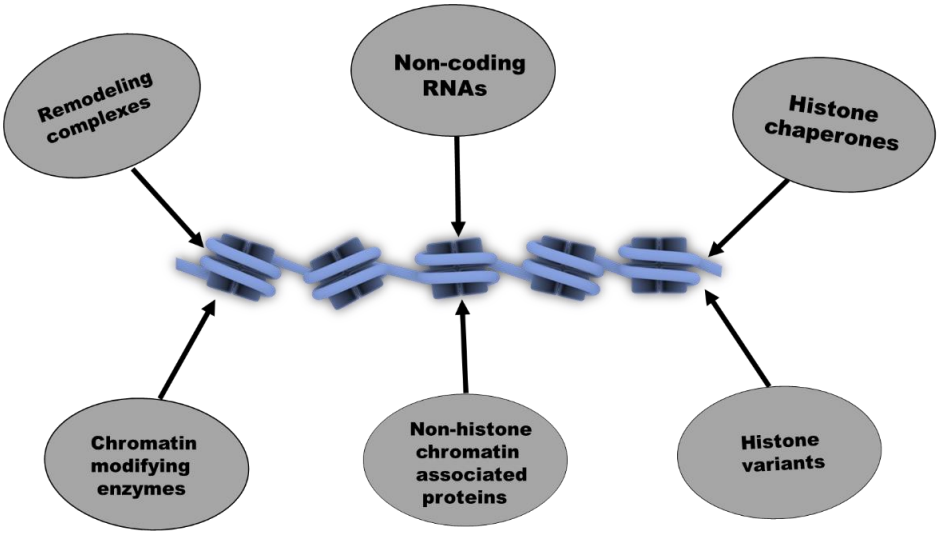


Figure 1.1. Factors involved in chromatin dynamics and regulation of gene expression.

A. Chromatin remodelers:

Chromatin remodelers usually exist in multimeric complexes and utilize energy generated from ATP hydrolysis to alter the Chromatin structure [2, 3]. Broadly, there are five families of ATP-dependent chromatin remodelers in the eukaryotes. They are: 1) ISWI complex: play important role in maintenance of higher-ordered chromatin structure and chromatin assembly after DNA replication; (2) SWI/SNF complex: disorder nucleosomes and play key roles during DNA double-strand break (DSB) repair and nucleotide-excision repair (NER); (3) NURD/CHD/Mi-2 complex: these remodelers includes histone deacetylases as one of its component, hence implicated in transcriptional repression, (4) INO80 complex: facilitates transcriptional regulation and play important role during DSB repair and NER, and (5) SWR1 complex: facilitates deposition of histone variant H2A.Z and hence play important role during DNA repair. All these chromatin remodelers share a common ATPase domain but have distinct function due to their association with different complexes. They can regulate gene expression by altering the positioning of promoter nucleosomes by sliding the nucleosomes along the DNA, eviction of nucleosomes or replacement of histones [4, 5]. Some of these remodelers contain bromodomains which recognize acetylated lysine residues on histone tails thus get recruited to the open DNA strand and mediates its ATP-dependent chromatin remodeling function to further open up the DNA strand for recruitment of transcriptional machineries [4]. In addition to this, some remodelers exist in multimeric complexes with histone deacetylases (HDACs). Thus the presence of bromodomain as well as deacetylase activity in these complexes suggest that there is a direct cross-talk between remodeling complexes and epigenetic machineries which ensures precise regulation of gene expression.

B. Histone chaperones:

Another group of factors that play an important role in chromatin dynamic are histone chaperones. They associate with histone and acts as catalyst to initiate a reaction involving transfer of histones without being part of the final product. This property is hallmark of all histone chaperones. However, all histone chaperones share the common feature of binding to histone, their structure, substrate specificity and function differs greatly during various cellular processes. The major functions carried out by these chaperones are storage and transport of histones as well as assembly and disassembly of nucleosomes. They associate with ATP-dependent Chromatin remodelers and

acts as histone sinks (histone acceptors) during histone eviction. In addition to this, they associate with histone modifiers to facilitate post-translational modifications (PTMs) of inaccessible regions of histones. Thus, they can facilitates or repress gene expression based on their association with histone modifiers. For example, Spt6 histone chaperone associate with Set2 methyltransferase which mediate H3K36me3 (trimethylation of Lysine 36 on histone H3) hence prevents histone exchange and transcription initiation from intragenic regions [6, 7]. Whereas, Anti-silencing function 1 (Asf1) histone chaperone associates with Rtt109 to mediate acetylation of H3K56 that facilitate histone exchange and hence promotes transcription initiation from intragenic region [8, 9]. Nucleophosmin1 (NPM1) is another histone chaperone that stores histones during the process of histone eviction. It interacts with core histones H2B, H3, H4 [10] and linker histone H1 [11], and disrupt the nucleosomal structure in an acetylation dependent manner eventually resulting in transcriptional activation [10].

C. Nucleosome associated non-histone proteins:

Apart from histones, there are a class of multifunctional and varied proteins collectively termed as non-histone nucleosome-associated proteins which also modulate chromatin dynamics and hence play significant role in maintenance of epigenetic state, chromatin organization, regulation of gene expression and DNA repair. One of the major component of these chromatin associated non-histone proteins are the chromatin modifying enzymes. Apart from them, there are several other proteins such as the positive coactivator4 (PC4), heterochromatin-associated protein (HP1), poly ADP-ribose polymerase-1 (PARP1), and high mobility group (HMG) proteins that associate with the chromatin and alters its dynamics. These proteins directly interact with chromatin (either histone/DNA) and always get isolated upon chromatin fractionation. These proteins undergo several post-translational modification which regulate their functions. For example, PC4 a non-histone protein strongly associates with chromatin and results in chromatin compaction leading to repression of transcription, silencing of PC4 results in chromatin decompaction and upregulation of several neuronal genes which are usually repressed in non-neuronal cells [12, 13]. Another non-histone protein that strongly associate with chromatin is HP1. HP1 is enriched at centromeres and telomeres of most of the eukaryotic chromosomes and are fundamental unit of heterochromatin packaging, hence HP1 correlates with gene repression [14].

D. Histone variants:

Apart from canonical histones there are several histone variants that are encode by separate genes and differ from canonical histones by one or a few amino acid residues. These variants are expressed at very low levels and are incorporated throughout the cell cycle. Replacement of canonical histones by these variants significantly alters the composition and organization of chromatin. This results in change in properties of the chromatin and their interaction with chromatin-associated non-histone proteins, ATP-dependent remodelers and chromatin modifying enzymes. Thus histone variants are another important factors that modulate chromatin dynamics. The impact of replacement of canonical histones with histone variants is listed in the table (1.1).

Table 1.1. A summary of replacement of canonical histones with histone variants and their impact on chromatin dynamics.

Canonical histones	Histone variants	Impact	Knockout phenotype	References
H2A	H2A.Z	Transcriptional activation, DNA repair and recombination	Embryonic lethal at E4.5-7.5	[15, 16]
	H2A.X	Transcriptional activation and chromosome segregation	Male infertility	[17, 18]
	macroH2A	Transcriptional repression	Brain malformation (Zebrafish)	[19, 20]
	H2ABBD	Transcriptional repression		[21, 22]
H3	CENPA	Kinetochore assembly	Embryonic lethal at E3.5-E8.5	[23, 24]
	H3.3	Maintains chromatin integrity and transcriptional activation	Adult infertility	[25, 26]

E. RNA:

Another factor that modulate chromatin dynamics is RNA, several long non-coding RNA molecules are known to associate with various chromatin associated proteins such as heterochromatin protein 1 (HP1), polycomb repressive complex 2 (PRC 2). They form extensive

network of ribonucleoprotein (RNP) complexes with several chromatin proteins and guide them to specific genomic loci leading to alteration in gene expression at that loci [27, 28]. In addition to these, several small non-coding RNAs such as miRNA, siRNA, and piRNA are also well-established as important regulators of gene expression. Recently, one report shows that enhancer RNA can stimulates transcription via interaction with RNA binding protein (CBP) [29].

1.1.3. Chromatin modifications and Epigenetic regulation of gene expression:

Along with the above discussed factors, chromatin modifications that are mediated by chromatin modifying enzymes play critical roles in regulation of chromatin states/dynamics and hence directly controls various cellular processes especially transcription. These modifications include methylation of DNA by DNA methyltransferases (DNMTs) and several covalent modifications of histones such as acetylation, methylation, phosphorylation and others. These all modifications are collectively termed as epigenetic mechanisms/factors for gene regulation.

The concept of epigenetics was first elaborated by British developmental biologist Conrad Waddington, who applied the concept of epigenetic landscape to explain how various phenotypes arises from identical genotypes during the process of development [30]. For example in our body all cells have the same DNA but we have different types of cells: stem cells, neurons, pancreatic cells, blood cells and many more. Thus, it is a process that regulate gene expression independent of an individual's DNA sequence [31]. It acts as a switch that determines which proteins will be transcribed under a specific stimulus/condition by turning on or off the gene expression. In mammalian cells, there are three major epigenetic mechanisms that control gene expression. Each of them act at different level: at DNA (DNA methylation), at RNA (RNA-associated gene regulation) and at protein (covalent modification of histones) (fig 1.2).

1. DNA Methylation: A chemical process that adds up methyl group at 5^{th} position of a cytosine nucleotide which is immediately adjacent to a guanine nucleotide termed as CpG site. This modification is carried out by one of three enzymes called DNA methyltransferases (DNMTs) [32, 33]. Addition of methyl groups alters the appearance and structure of DNA, leading to altered interaction of transcription machinery with DNA. Thus regulating gene expression. This modification is also involved in genetic imprinting [34], a phenomenon that is used in some genes

to differentiate which gene copy is inherited from the father and which gene copy is inherited from the mother.

2. RNA-associated gene regulation: Certain small RNA molecules like miRNA, siRNA, non-coding RNA and others can also regulate gene expression [35].

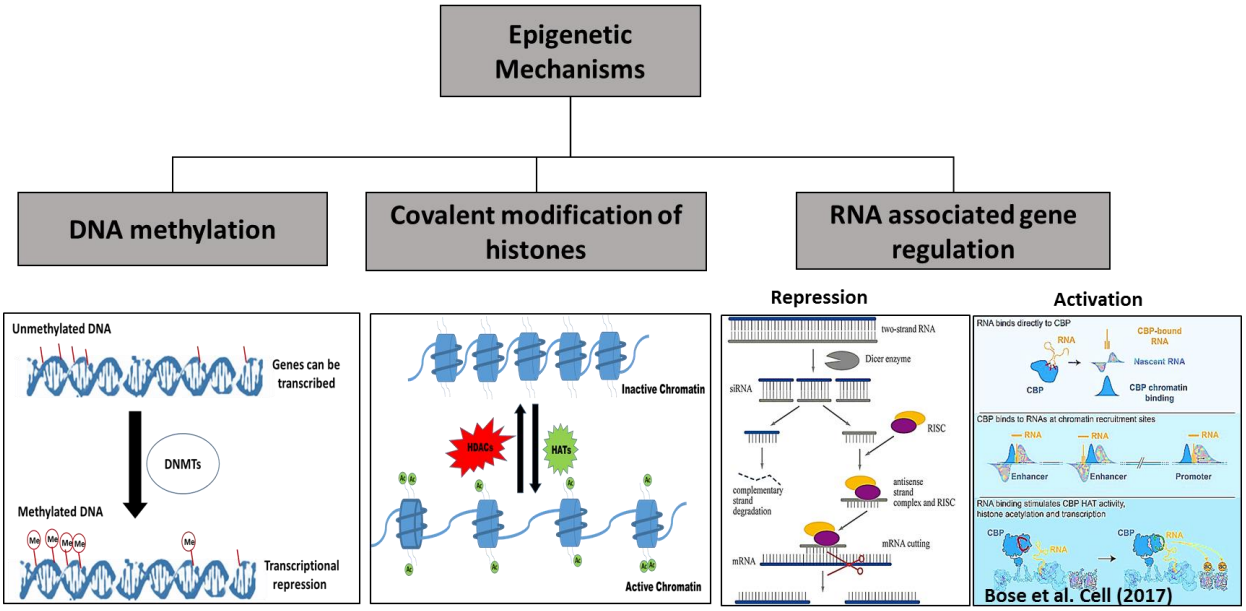


Figure 1.2. Epigenetic mechanisms for regulation of gene expression. The three major epigenetic mechanism act at three different levels: DNA methylation at DNA level, covalent modification of histones at protein level, and RNA associated gene regulation at RNA level.

3. Covalent modification of histones: Histones are positively charged protein around which negatively Charged DNA molecules are wrapped to form the basic unit of chromatin, known as nucleosome. N-terminal of these proteins are exposed to cellular machinery and undergo various modifications that alters the chromatin compaction and directly controls various cellular processes, especially transcription. The concept of Histone modifications came into existence from the pioneering's work of Vincent Allfrey's in early 1960s, when his group first time reported the phenomenon of acetylation and methylation of histones and proposed their possible role in the regulation of RNA synthesis [36]. In addition to these modifications, there are several other modifications that occur on these histones. For example, phosphorylation (the best studied one), butyrylation, citrullination, ADP-ribosylation, ubiquitination, crotonylation, hydroxyisobutyrylation, malonylation and others (reviewed in Huang et al; 2014)[37]. These

modifications are carried out by three groups of epigenetic modifiers; i) Writers: that add modifications, ii) Easers: that removes these modifications and iii) Readers: that recognize these modifications and eventually alters gene expression. One of such modifier is histone acetyltransferases and deacetylases mediating opposite functions that is reversible acetylation of histones (discussed in detail in the next section).

1.2. KATs in chromatin function and Transcriptional regulation

1.2.1. Acetylation of histones: The phenomenon of histone acetylation was first reported by Vincent Allfrey's in 1964. Reversible histone acetylation is one of the most well studied epigenetic modifications shown to be involved in diverse physiological as well as pathological cellular functions. This modification is carried out by two class of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) that catalyzes two opposite reactions that is transfer and removal of the acetyl group to and from the histones respectively. HATs mediate the transfer of an acetyl group to the ε-amino group of lysine side chains by utilizing acetyl Coenzyme A as a cofactor.

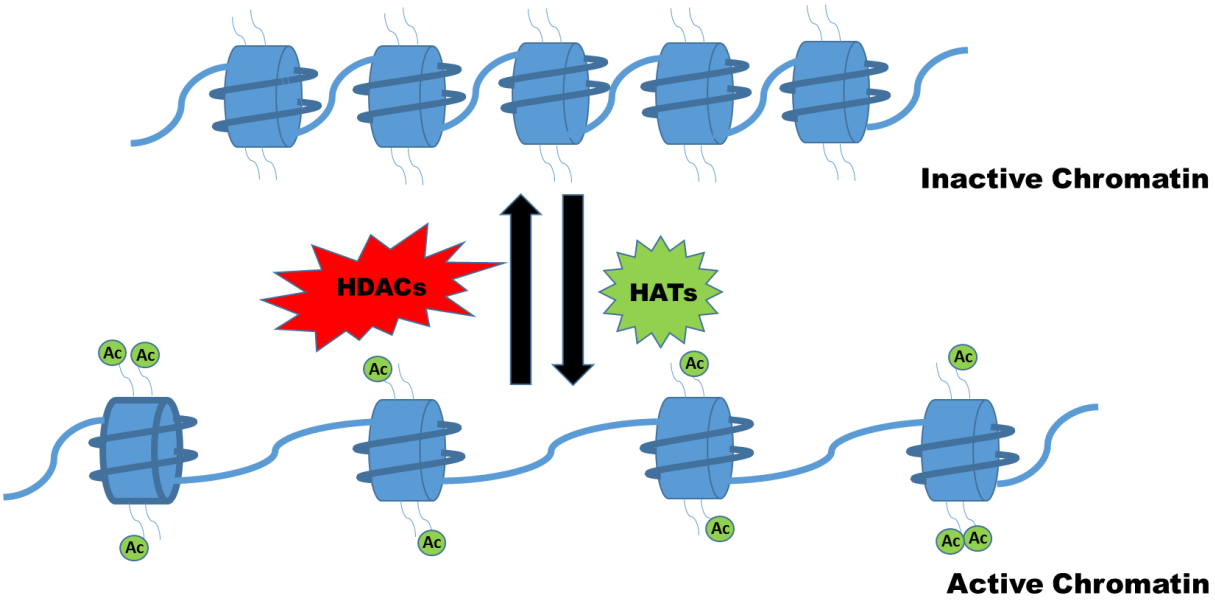


Figure 1.3. Opposite role of HATs and HDACs in chromatin dynamics and gene transcription. HATs act as writers that add acetyl group on lysine residues of histone tails and HDACs act as easers that remove these acetyl groups from histones.

Addition of this negatively charged acetyl group neutralizes the positive charge of lysine residue on histones, hence decreasing the histone-DNA interaction leading to de-compaction of the chromatin [38, 39]. Thus, this modification is linked to activation of gene expression. Several studies in the past two decades have reported several other mechanisms through which this modification can activate gene expression. First, they could be part of the "histone code" that acts as a flag to recruit various other transcription machineries [40]. Second, these enzymes are part of large remodeling complexes which upon activation initiate chromatin remodeling [1].

Types of HATs/ KATs:

Recently, the nomenclature of HATs and HDACs have been changed to KATs (Lysine acetyltransferases) and KDACs (Lysine deacetylases) [41] due to identification of acetylation/deacetylation of several non-histone proteins by these enzymes [42]. These enzymes are evolutionary conserved from yeast to humans and can be classified into two groups based on their subcellular localization. Type A KATs are nuclear in nature and regulate gene expression by acetylation of histones and non-histones proteins in context of chromatin (Weaver R (2007) *Molecular Biology*. McGraw-Hill). They possess a bromodomain which help them to recognize and bind to acetylated lysine residues on their substrates. Type B KATs are cytoplasmic in nature and predominantly acetylates cytoplasmic proteins and newly synthesized histones prior to their incorporation into nucleosomes. They lack the bromodomain because they bind to non-acetylated substrates. The more abundant nuclear KATs are further sub-divided into five major families based on their sequence homology as well as shared domain architecture and functionality [43]. These include the Gcn5-related N-acetyltransferases (GNAT) superfamily, the MYST family, the p300/CBP family, the nuclear receptor co-activator (NCOA) family and a set of transcription factor associated KATs [44, 45]. Very recently, one mitochondrial acetyltransferase, hnRNAP2 has been identified which acetylates mitochondrial target gene promoters under stress condition [46]. Till date, total of 20 KATs have been identified in mammals [47] (fig 1.4). A summary of all KATs present in mammalian cells and their grouping in the five major families is listed in the table 1.2.

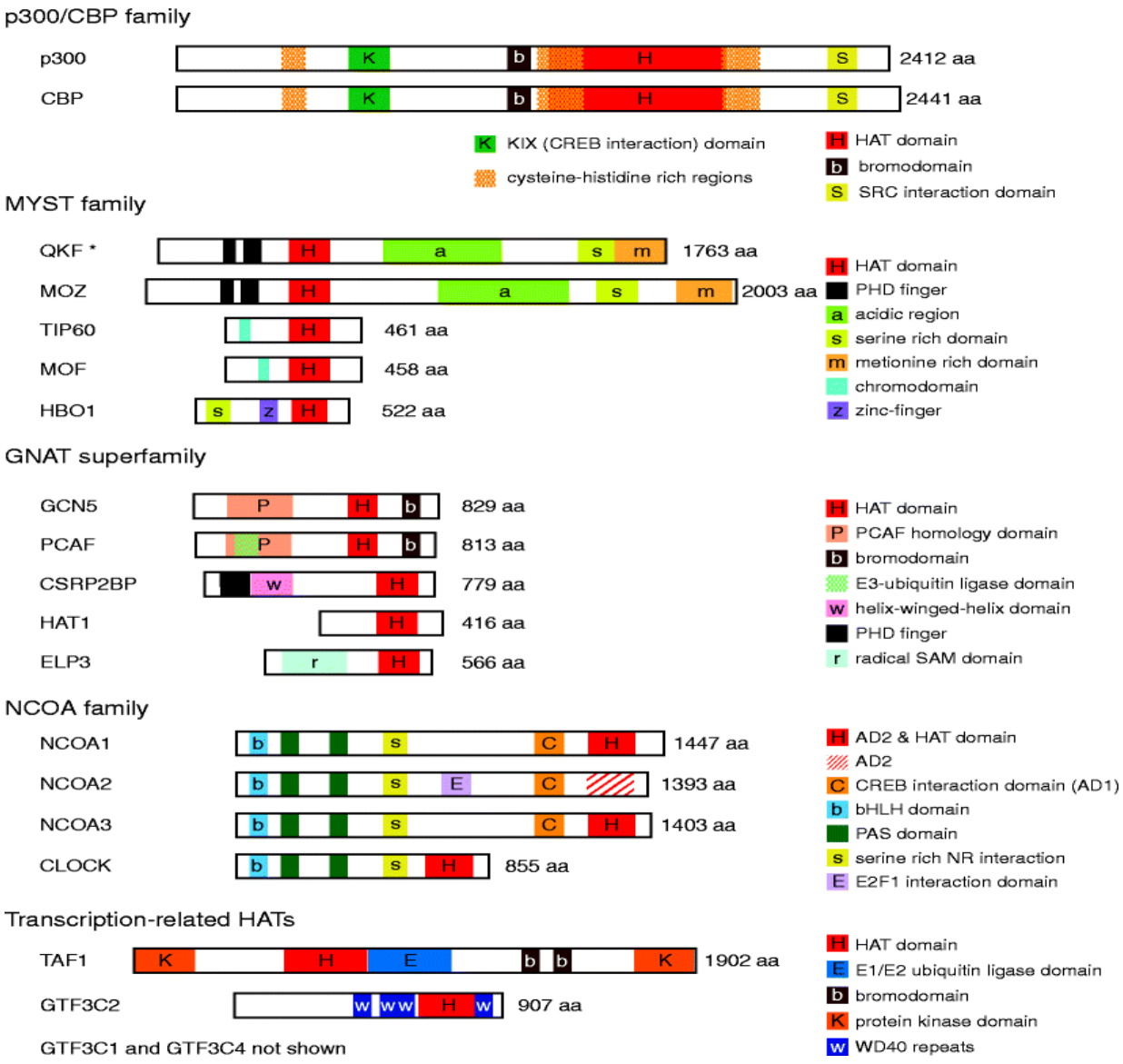


Figure 1.4. Schematic representation of HATs in mammalian system and their grouping in five families. Mammalian HATs are grouped into five families: p300/CBP family, MYST family, GNAT superfamily, NCOA family and transcription-related HATs. The protein size correspond to the size of HATs in *Mus musculus.* For further details refer the original article [47]. **(Adapted from Sheikh, B.N. Cell Tissue Res (2014) 356: 553. doi: 10.1007/s00441-014-1835-7), "With permission of Springer".** Heterogeneous Nuclear RNApolymeraseA2, HnRNPA2 (not shown here) is a novel mitochondrial acetyltransferase that acetylates mitochondrial target gene promoters during stress.

Table 1.2. Types of KATs in mammalian System.

Type	Family	Member	Complex	Function	Role during neuronal development and disease	References
A	GNAT	GCN5	STAGA, TFTC	Transcriptional co-activator	Proliferation of neuronal progenitors	[48-50]
		PCAF	PCAF	Transcriptional co-activator	----------	[50]
		ELP3	Elongator	Transcription elongation	Neuronal migration and dendritic maturation	[51]
		CSRP2BP	ATAC		Proliferation	[47, 52]
B		HAT1	--------	Acetylates non-nucleosomal histones	----------	[53-55]
A	MYST	MOZ	MSL	Leukemogenesis	DiGeorge syndrome	[56, 57]
		MORF	MSL	Leukemogenesis	Genitopatellar syndrome	[58]
		QKF	--------		NSCs self-renewal, Proliferation and differentiation of neuronal progenitors differentiation	[59, 60]
		HBO1	ORC	DNA replication	Neural patterning	[61]
		TIP60	TIP60	DNA damage repair	Axonal transport	[62, 63]
A	p300/ CBP	p300		Transcriptional co-activator	Differentiaton and maturation of neurons	[64, 65]
		CBP		Transcriptional co-activator	Differentiaton and maturation of neurons	[66, 67]

A	NCOA	NCOA1 NCOA2 NCOA3	--------	Steroid hormone-dependent transactivation	Motor impairments and delayed Purkinje cell development	[47, 68, 69]
		CLOCK	Circadian clock	Transcriptional activator	Adult hippocampal Neurogenesis	[70]
A	TFs	TAF1	TFIID	Transcriptional co-activator	X-linked Dystonia-Parkinsonism (XDP)	[71]
		GTF3C	TFIIIC	RNA polymerase III-mediated transcription	Inhibits premature maturation of neurons	[72]
----	-------	HnRNPA2	--------	Mitochondrial stress induced transcriptional co-activator	Deamentia, Myopathy and Amyotrophic lateral sclerosis (ALS)	[73]

1. GNAT superfamily: This family have five KAT members which includes the first identified acetyltransferase, general control of amino acid synthesis 5 (Gcn5); p300/CBP associated factor (PCAF); elongation protein 3 (ELP3); cysteine- and glycine-rich protein 2-binding protein (CSRP2BP) and histone acetyltransferase 1 (HAT 1). Gcn5 and PCAF are very similar to each other, both possess the same domain architecture and are almost 90% identical at amino acid level in their KAT domains. They associate with large multi-subunit complexes (SAGA, TFTC and others) and acts as transcriptional coactivators [74, 75]. Moreover, they also play key roles in DNA repair and chromatin remodeling [54].

2. MYST Family: This family is named after its founding members (MOZ, ybs2/sas3, sas2 and tip60) and contains 5 KAT members which includes querkopf (QKF), monocytic leukemia zinc finger (MOZ), the Tat interacting protein 60 (TIP60), male absent on the first (MOF) and the histone acetyltransferase bound to ORC (HBO1). This is the largest family of KATs and also the least studied. Recently, several groups have started working on members of this KAT family especially on TIP60, that suggest this particular KAT family could be critical for various cellular

processes such as DNA repair, apoptosis and chromatin remodeling [63, 76]. Dysregulation of several member of this family have been reported in various malignancies.

3. P300/CBP family: This family have only two members: p300 (KAT3B) and CREB binding protein (CBP). These two members are two of the most studied KATs. Both of them are very similar to each other and in humans share around 63% of sequence similarity, but are almost 86% identical at the amino acid level in the acetyltransferase domain [77]. Both have intrinsic acetyltransferase activity and are well-known transcriptional co-activators [78]. They have been implicated in variety of cellular processes such as embryonic development, cellular differentiation and proliferation, cell cycle regulation, apoptosis and are also implicated in variety of diseases such as cancer and neurodevelopmental disorders [47, 79, 80].

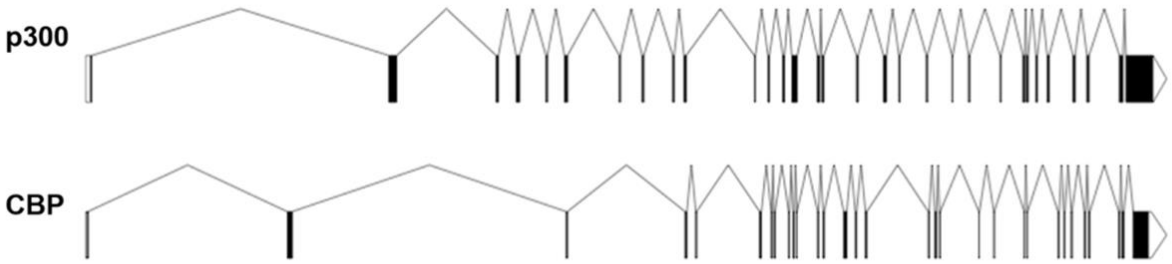
Apart from these, there are several cell signaling molecules that have acetyltransferase activity. They directly interact with various nuclear receptors and activate their transcriptional activity, hence grouped as separate KAT family called as nuclear receptor co-activator **(NCOA) family**. They includes NCOA1, NCOA2, NCOA3 and CLOCK; reviewed in [47]. There are certain general transcription factors (GTFs) such as TAF1 [81], GTF3C1, GTF3C2 and GTF3C3 [82] which also possess acetyltransferase activity and hence they constitutes the fifth family of KATs termed as **transcription factor related (TFs) KATs**.

1.2.2. p300/CBP:

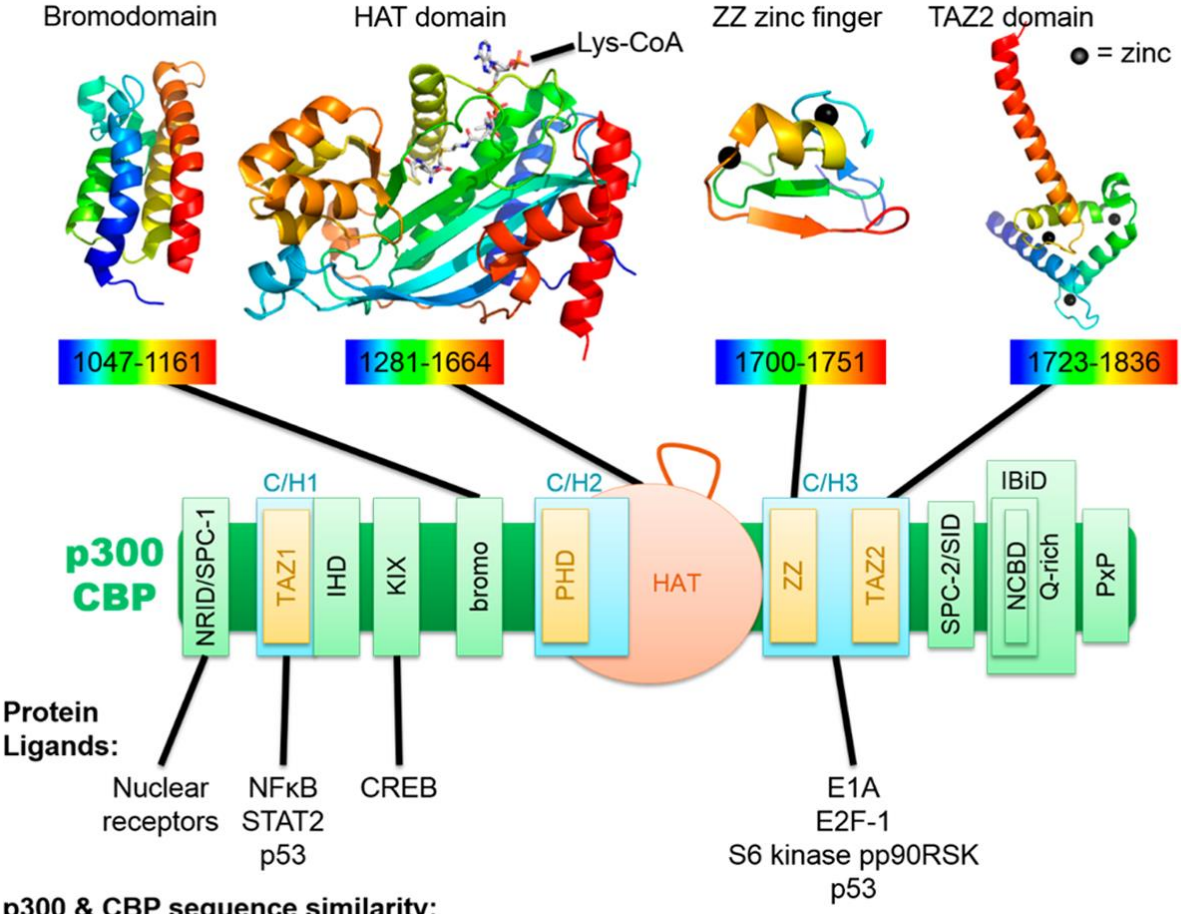
p300 was initially identified in 1994 through its interaction with the adenoviral transforming protein E1A [83]. Similarly, CBP was initially identified in 1993 through its interaction with phosphorylated form of cAMP response element binding (CREB) transcription factor [84]. These two paralogs share an identical domain architecture and are almost 86% identical at amino acid level in their KAT and KIX domain. Furthermore, other domains of p300/CBP also show significant sequence homology including several protein-protein interaction motifs (figure 1.5) [85]. Both are very large proteins and interacts with over 400 proteins sharing a set of around 40 similar transcription factors, thus acting as a transcriptional co-activator regulating precise expression of numerous genes in temporal and tissue-specific manner [77, 79]. Since these two proteins have a large cohort of interactors/substrates, they play a crucial role in numerous cellular

processes (described above). As a transcriptional co-activator, they have various modes of action. Due to their large structure, they may acts as a bridging protein to bring two or more proteins in close proximity to each other. For example they can interacts with various sequence-specific transcription factors and bring them in close proximity to transcription machinery. They may also acts as a scaffold to form large multimeric transcriptional regulatory complexes [86]. Moreover, they have intrinsic acetyltransferase activity through which they acts as a chromatin remodeler to control gene expression by altering the chromatin structure and dynamics. p300/CBP utilizes acetyl co-enzyme A as cofactor to catalyze transfer of acetyl group on conserved lysine residues of their substrates. It acetylates all the four core histones present in the nucleosome [87]. Addition of acetyl groups imparts negative charge on histones, weakening their interactions with positively charged DNA strands leading to opening of chromatin for binding of numerous transcriptional factors. Hence, hyper-acetylation of histones is directly correlated with transcriptional activation or increased gene expression. Besides histones, p300/CBP have several non-histone substrates which includes component of basal transcriptional machinery such as TFIIE and TFIIF as well as some transcription factors such as c-MYB, CREB, NPM1, MYOD, p53, STAT, E2F-1/2/3 and GATA 1 [77] . In most of the cases, acetylation by p300/CBP increases expression of their target genes by increasing DNA-binding activity of these transcription factors.

Gene structures:



p300/CBP protein domains:



p300 & CBP sequence similarity:

p300 purification:

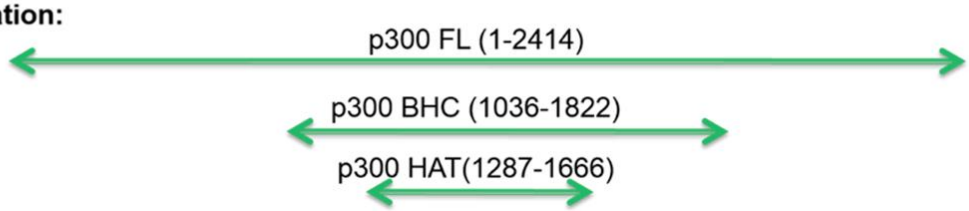


Figure 1.5. Domain structure of p300/CBP. Exon−intron gene diagrams are shown for p300 and CBP (top). Below are example protein structures for the bromodomain (PDB 3I3J, 2.33 Å), catalytic HAT domain (PDB 3BIY, 1.7 Å), ZZ zinc finger (PDB 1TOT), and TAZ2 domain (PDB 3IO2, 2.5 Å). All structures were produced using purified p300, except the ZZ zinc finger, which used purified CBP. p300/CBP proteins are colored with a rainbow, with blue at the N-terminus and red at the C-terminus, and residues included in the structure are listed below each. Zinc ions are black spheres. All structures are based on X-ray crystallography, except the ZZ zinc finger structures from solution NMR. The p300 bromodomain structure shown here is remarkably similar to an independently generated CBP bromodomain structure (not shown, PDB 3DWY, 1.98 Å). Below is a model for full-length p300/CBP with all domains shown, and is a compilation based on several recent analyses [88, 89]: three cysteine/histidine-rich (C/H) domains are shown in turquoise, three zinc fingers are shown in yellow, and the catalytic acetyltransferase domain is shown in orange, with its autoacetylated regulatory loop drawn above, which corresponds to residues 1523−1554. A few examples of proteins that bind p300/CBP are listed below the protein model, with the particular domain involved in binding indicated with a black line. Below that, amino acid similarity is indicated, for comparing p300 and CBP sequences within either the catalytic BHC region (from the bromodomain to the C/H3 domain) or the entire protein. At the bottom, commonly purified active p300 variants are indicated, including p300 acetyltransferase/HAT domain, BHC enzyme (bromodomain-HAT-C/H3), and full-length protein. It should be noted that p300 HAT has a deletion in residues 1529−1560. **(Adapted from Dancy, B.M. and P.A. Cole, *Protein lysine acetylation by p300/CBP.* Chem Rev, 2015. 115(6): p. 2419-52).**

1.2.3. Regulation of p300/CBP activity:

p300 is one of the master regulator of gene expression which interacts with more than 400 proteins including histones and non-histone proteins. P300/CBP are implicated in several critical cellular processes such as development, differentiation, cell-cycle regulation and in several diseases especially in variety of cancers and neurological disorders. Thus, the precise regulation of p300/CBP activity is essential. This regulation is achieved mainly by two mechanisms: i) interaction with several of its binding partners and ii) covalent modifications of its amino acid residues. These two mechanisms are not mutually exclusive and work in parallel to each other, where covalent modification of certain amino acid residues enhanced binding of its partner and some of these binding partners inturn mediate covalent modification of amino acid residues in different domains of p300/CBP. Phosphorylation of serine and threonine residues is one of the well-studied and characterized PTM. There are several kinases which includes PKA, CaMKIV, MAPKs, AKT, cyclin E/CDK-2 and PKC that have been reported to phosphorylate p300 and CBP at various sites [90]. Depending on the kinase and its site of modification, phosphorylation may positively or negatively regulate their acetyltransferase activity, stability and protein-protein interactions. P300/CBP also undergo some other modifications such as methylation, sumoylation, citrullination and ubiquitination. Methylation by CARM1 inhibits its interaction with CREB

inhibiting CREB signaling and activating apoptotic response [91]. Sumoylation occurs on three lysine residues in the KAT domain repressing its KAT activity leading to transcriptional repression [92]. p300/CBP undergo citrullination at arginine (R-2142) by PADI4 which impairs its methylation by CARM1 [91]. p300 also undergoes ubiquitin mediated proteasomal degradation in lung cancer through cAMP signaling pathway via Epac and p38 MAPK [93]. Besides these modifications, work from the past decades had shown that p300 controls its own activity by autoacetylation of its own residues. This phenomenon of autoacetylation of p300 is first identified by Hamamori et. al, when they found out that p300 could also acetylate itself in addition to its histone substrates in a gel based acetylation assay [94]. Mass spectrometric analysis of p300 KAT domain by Thompson et al [95] identified 13 lysine residues to be acetylated in this domain and most of these residues are present in a proteolytically sensitive loop which resides in the substrate binding pocket of p300 acting as an inhibitory loop by interfering with efficient substrate binding. Several structural and NMR studies show that acetylation of lysine residues present in this loop brings about structural changes that displaces the this loop from the substrate binding site making it free for efficient binding of its substrates for acetylation [86]. Therefore, autoacetylation of p300 increases its KAT activity. Further studies show that this autoacetylation process is trans in nature where two p300 molecules interacts/come in close proximity to acetylate each other [95]. Recently one study shows that RNA can also regulate CBP activity [29]. In this study, Berger and group had shown that RNA which are transcribed in proximity to CBP-chromatin binding sites directly interact with the RNA binding region (RBR) located within the catalytic HAT domain of CBP and stimulates its HAT activity leading to increase histone acetylation and transcription of target genes.

1.2.4. Factors affecting autoacetylation of p300:

The process of autoacetylation principally involves acetylation of a p300 molecule by other p300 molecules promoting its acetyltransferase ability, resulting in enhanced acetylation of its substrate proteins. The activity of p300 thus depends on its acetylation status. This phenomenon of p300 autoacetylation can be modulated by cellular factors as well as acellular factors.

A. Cellular factors: This includes metabolites and proteins that are already present inside the cells and are involve in various processes. Since these KAT enzymes utilize acetyl coA as cofactors for their acetyltransferase activity, the status/level of this cellular metabolite greatly regulates their

KAT activity [96, 97]. In general, high energy status that corresponds to high level of acetyl coA facilitates KAT activity. Whereas, low energy status that corresponds to low levels of acetyl coA and high levels of nicotinamide adenine dinucleotide (NAD)- a cofactor utilized by sirtuin deacetylase enzymes facilitates deacetylation . In terms of protein factors, work from the past one decade shows that several substrates of p300 has the capability to induce the phenomenon of p300 autoacetylation. These substrate includes adenoviral transforming protein E1A [98], anaphase promoting complex/cyclosome (APC/C) [99], MAML1 [100], GAPDH [101] and NPM1 [102]. The first factor that induce p300 autoacetylation was identified through the work of Turner and group in 2005, where they found that two components of APC/C; APC/5 and APC/7 directly interacts with the co-activators p300 and CBP, and this interaction stimulates their autoacetylation and potentiates CBP/p300 dependent transcription. Recently MAML1, GAPDH and NPM1 was also found to induce the phenomenon of p300/CBP autoacteylation leading to potentiation of p300/CBP dependent transcriptional activation. The early gene1A (E1A), the first gene to be expressed upon adenoviral infection has recently found to induce p300 autoacteylation through interaction with its N-terminal domain. In the same study, it was found that E1A mediated p300 autoacteylation results in reduce H3K18 acetylation implicating its role in transcriptional repression [98].

B. Acellular factors: These includes synthetic and natural compounds that can directly bind to p300/CBP. Immense efforts from several groups had led to identification and characterization of various small molecules modulators of p300/CBP KAT activity. These molecules falls into two categories depending on their mode of action on the KAT activity, they may act as inhibitors or as activators (discussed in section 1.4).

1.3. p300/CBP: Role during neural development and implications in neurodevelopmental disorders

There are around 170 billion cells (approximately 86 billion neurons and almost similar amount of non-neuronal cells) with specific functions in the human/mice brain, making it a highly complex and specialized organ [103] . Interestingly, all cell types are originated from a common neural-lineage restricted stem cells (NLRSCs) which possess the same genetic content [104]. Thus it is

quite evident that epigenetic modifications play a key role during neural development by precisely controlling gene expression in temporal, spatial and cell-type specific manner. Reversible histone acetylation is one of the most studied and well characterized epigenetic modification that have been implicated in precisely controlling gene expression in these manners. Studies by several groups have dissected out the role of individual KATs during various stages of neural development (reviewed in [47]). A summary of the role of individual HATs during neural development is shown below (fig 1.6).

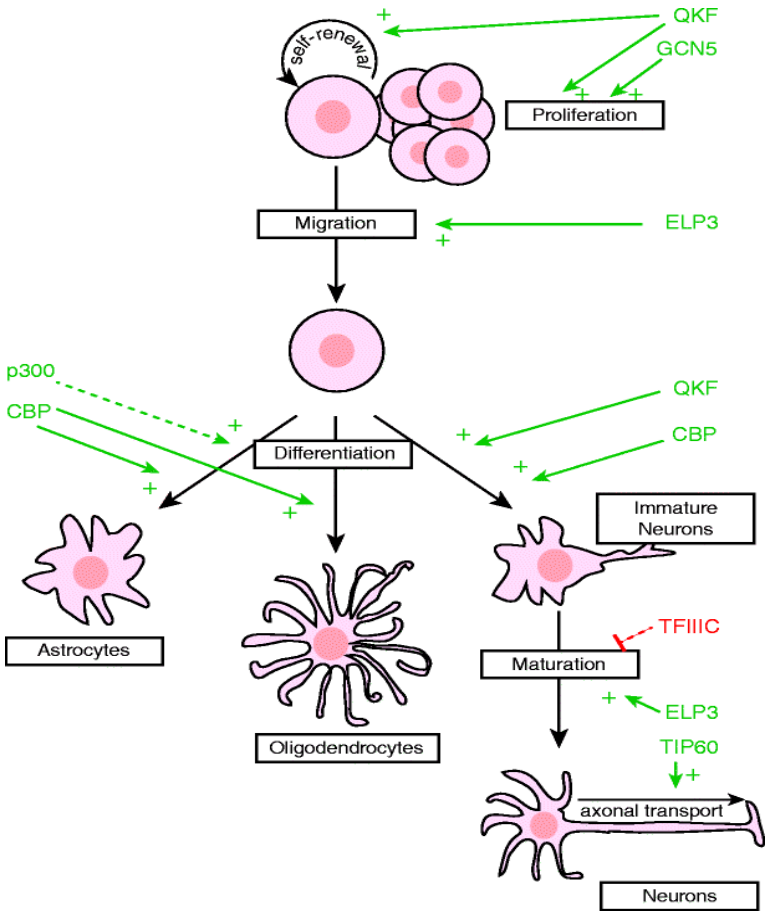


Figure 1.6. Role of individual HATs during neuronal development. QKF maintains the self-renewal and multipotency of adult NSCs, promotes proliferation of neural progenitors and differentiation into the neuronal lineage during embryonic development. GCN5 promotes proliferation of neuronal progenitors during embryonic development. CBP/p300 is required during the differentiation of progenitors into astrocytes, oligodendrocytes and neurons. ELP3 is needed for the timely migration of neuronal precursors and for the maturation of neurites, while TFIIIC appears to inhibit premature neuron maturation. Tip60 mediates axonal transport and is likely to be required for neuronal survival (in drosophila model). **(Adapted from Sheikh, B.N. Cell Tissue Res (2014) 356: 553. doi: 10.1007/s00441-014-1835-7), "With permission of Springer**".

1.3.1. p300/CBP during neural development: Among the KATs, role of p300/CBP is extensively studied and well-characterized in neural development using various drosophila and mice models. These studies show that p300/CBP play an important role in the process of neural differentiation [66, 105, 106]. p300/CBP associates with different binding partner/ transcription factors to give rise to specific neuronal lineage from the NSCs (fig 1.7).

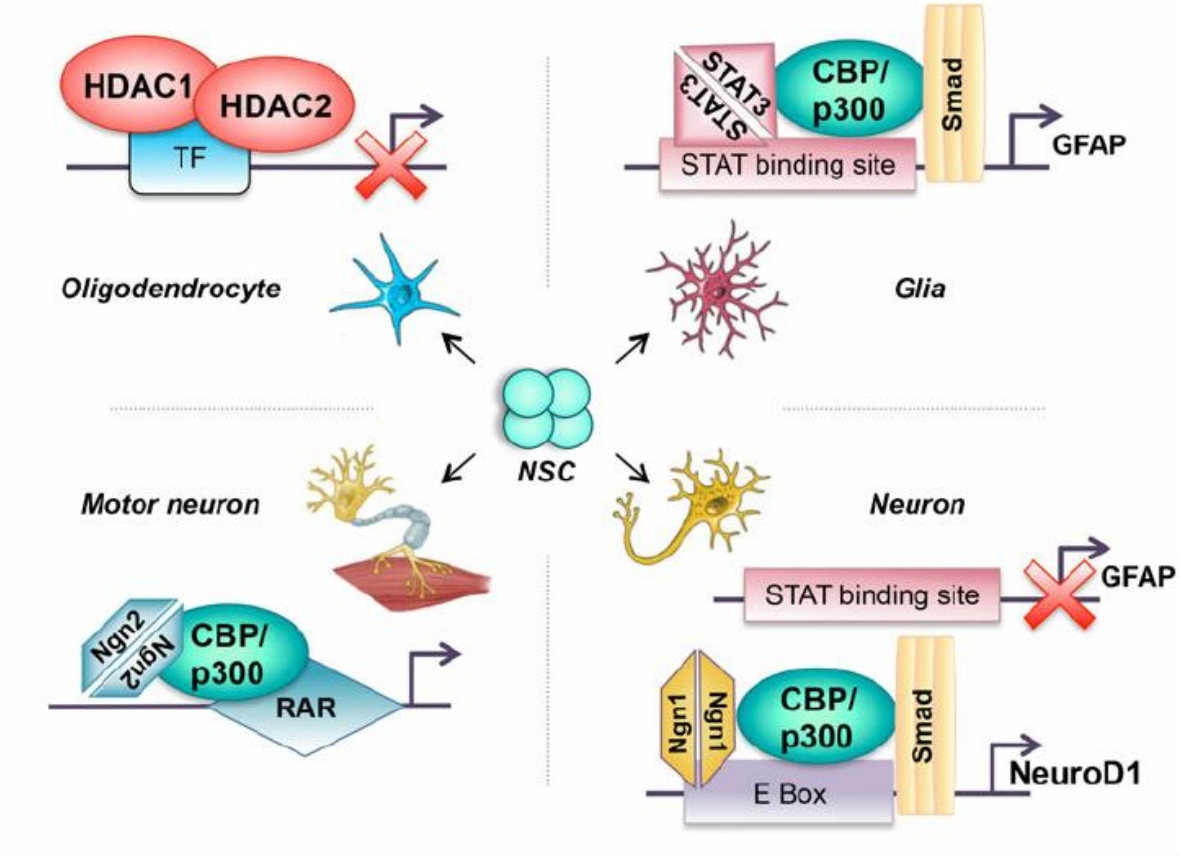


Figure 1.7. Role of p300/CBP during Neural differentiation. p300/CBP associates with different binding partner/ transcription factors to give rise to specific neuronal lineage from the NSCs. Interaction with STAT and SMAD promotes differentiation into glial lineage. Increased expression of neurogenin (Ngn1) dissociates STAT from the p300/CBP-SMAD complex and the new Ngn1-p300/CBP-SMAD complex promotes differentiation into neurons. Association with neurogenin 2 (Ngn2) and retinoic acid receptor (RAR) results in differentiation into motor neurons. HDAC1 and HDAC2 prevent transcription from neuronal genes leading to differentiation into oligodendrocytes. **(Adapted from Schneider, A., Chatterjee, S., Bousiges, O. et al. Neurotherapeutics (2013) 10: 568. doi: 10.1007/s13311-013-0204-7), "with permission from springer".**

Both proteins are highly expressed at E8.5 along the neural tube and dysregulation of either of these proteins by mutations or some other means results in several neurodevelopmental disorders [107-110]. Transgenic mice having mutations in CBP or p300, defective KAT domain or harbouring only one allele of either of these two genes exhibits severe growth arrest and neural tube closure between E9.5 and E11.5, molecular mechanism of which is still unknown. Moving to humans, reduced level of histone acetylation due to heterozygous mutations either in p300 and CBP results in Rubinstein-Tyabi syndrome (RTS) [106, 111]. RTS is one type of intellectual disability and patients suffering from this disorder have severe mental retardation and several craniofacial abnormalities.

1.3.2. Role in memory and cognitive processes: The role of transcription and protein synthesis in formation of long-term memory has been known for a long period [112, 113], and was supported by ex-vivo models of synaptic plasticity (Long Term Potentiation/LTP, a persistence increase in the strength of synaptic connections in response to recent patterns of activity which leads to long term storage of information/memory) [114]. Work from several groups show that the cAMP response element-binding protein (CREB) and NF-kB family of transcription factors regulate transcription of genes that are involved in synaptic plasticity and long term memory formation [115]. With the advent of several new techniques in molecular biology and genetics, recently it has been observed that reversible histone acetylation is one of the major process that regulate transcription of these genes during memory formation reviewed in [116]. The availability of various mouse models and work from several groups in the last 15 years directly link the role of p300 and CBP in memory and cognitive processes. Transgenic mice harbouring an inhibitory form or sub-region specific conditional knockout of p300 exhibit impairment in long-term memory formation [117, 118]. However, several studies suggest that mice harbouring mutation in p300 have mild impairment in memory and cognitive processes as compared to mutation in CBP [119]. In 2004, Korzus and group showed that CBP HAT activity is a critical component for stabilization of short term memory into long term memory [120]. They observed impairment of memory consolidation in transgenic mice expressing HAT deficient CBP, and this impairment was rescued by both suppression of the transgene expression or by use of HDAC inhibitor (TSA). This observation was further supported by studies from several groups, where they observed similar phenotypes using various transgenic mouse models of CBP [121, 122]. More recently, Barrett and colleagues used viral vectors to specifically delete CBP in hippocampal region and they observed

impairment in LTP and long term memory for object recognition and contextual fear [123]. It seems that the two homologs can act synergistically in various cellular processes, but they individually could not completely compensate the loss of function for each other in long-term memory and synaptic plasticity. This is evident by the observation that both these homologs have slightly different roles during the learning process. It has been observed that during motor learning CREB-CBP interaction is required as mutation in KIX domain of CBP results in deficits in motor learning, whereas mutation in p300 KIX domain did not [124]. Thus these data suggest that both p300 and CBP play important role in memory and synaptic plasticity. However, the molecular mechanism underlying the role of histone acetylation by p300 /CBP in memory and synaptic plasticity is still not well defined. There are some studies that suggest a link between histone acetylation and its downstream targets that could be involved in these processes. For example, it has been observed that after learning and synaptic plasticity there is increase in histone acetylation levels. This increase in acetylation level results in increased expression of BDNF, a brain derived neurotrophic factor and neuroD1 a neuronal differentiation1 transcription factor at mRNA level [65]. Both play crucial role during neural maturation and differentiation, as neuroD1 is required for maturation and differentiation of terminal neurons and bdnf is required for survival and proliferation of new born neurons. Chromatin immunoprecipitation (CHIP) studies show that the promoters of these two genes are hyperacetylated after fear conditioning. However, there is lack of studies for other candidate genes that might be involved in these processes.

1.3.3. Dysregulation of p300/CBP/KAT3B proteins in neurodevelopmental and neurological disorders: current therapeutic approaches: p300/CBP interacts with over 400 proteins and play a critical role in variety of cellular processes such as embryonic development, growth, differentiation and proliferation. As previously discussed, p300/CBP play an important role during neural development especially in neural differentiation and proliferation, as well as long-term memory and synaptic plasticity. Therefore, dysregulation of these proteins leads to several neurodevelopmental disorders. Mutations in KAT domains of these proteins give rise to RTS (a form of intellectual disability). Several recent studies have reported dysregulation of KAT3B proteins (p300/CBP) in several neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's and others (reviewed in [44, 45, 125]). Current therapeutic approaches that have been taken for treatment of these disorders are based on use of small molecule modulators (either

activators or inhibitors) targeting these proteins. A summary of current therapeutic approaches taken for these disorders is shown below (fig1.8).

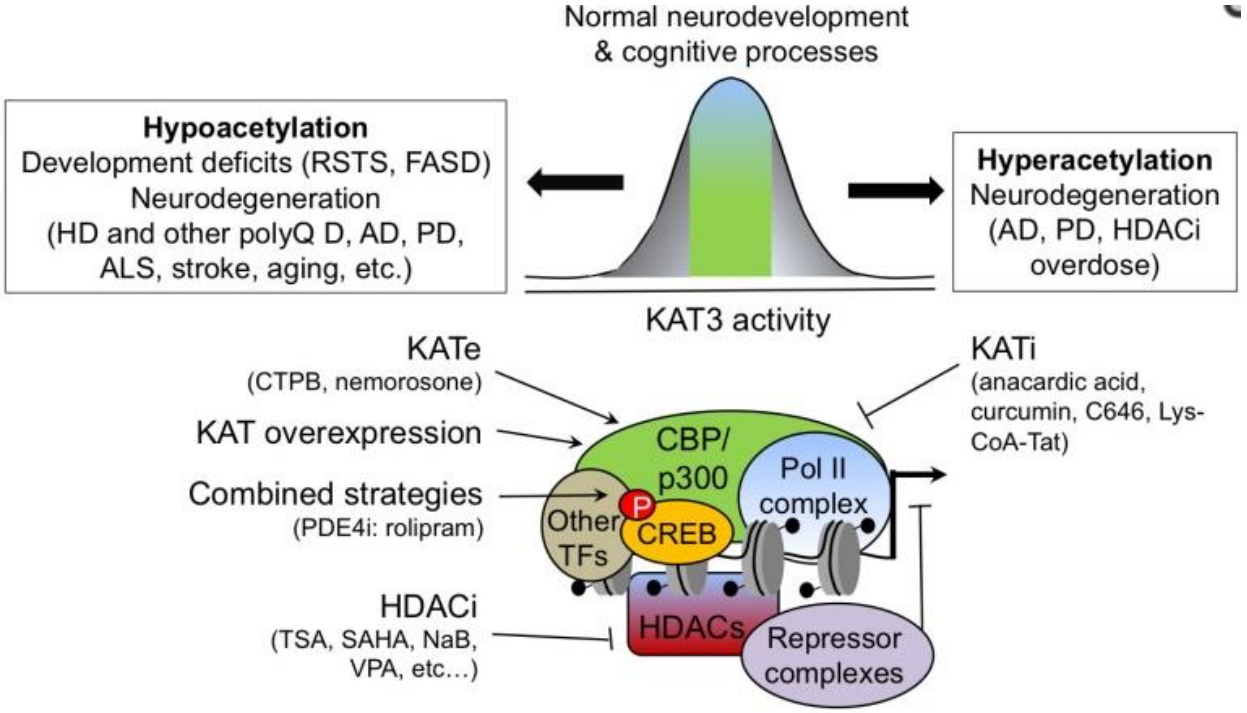


Figure. 1.8. Summary of neuropathologies related to KAT3 proteins and therapeutic approaches targeted to these proteins. The balance between KATs and HDACs define the optimal level of chromatin acetylation required for developmental and cognitive functions. The alteration of this balance, favoring either histone hyperacetylation (right box) or hypoacetylation (left box), may result in cognitive impairment and/or deleterious effects. This is the case of well-known pathological conditions like the Rubinstein-Taybi syndrome (RSTS), Fetal Alcohol Spectrum Disorder (FASD), Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and other neurological disorders. Therapeutic approaches aimed at restoring the balance, including HDAC inhibitors (HDACis), KAT enhancers (KATes) and inhibitors (KATis) and genetic overexpression of KAT3 genes, are indicated under the corresponding boxes. [Adapted from **M Valor, Luis, et al. Current pharmaceutical design 19.28 (2013): 5051-5064].**

Till date, due to lack of cell-permeable KAT activators most approaches were based on use of HDACs inhibitors (HDACis) to enhance histone acetylation. The major problem with HDACis is their pleiotropic effects and limited specificity. Hence, there is a need to identify/design molecules that specifically target KAT activity. Recently some KAT activators have been identified (discussed in the next section) that show promising results at cellular level.

1.4. Small molecule modulators of HATs/KATs

As discussed in the previous section, dysregulation of KATs have been implicated in various neurodevelopmental and neurological disorders. Hence, modulating the acetyltransferase activity of these KATs could give us a lead for potential therapeutic options for some of these disorders. These molecules can be designed or chemically synthesized to target different domains of these KATs, which play crucial roles in various cellular processes. Depending on the context of their usage/applications, they could be an activator or an inhibitor of the acetyltransferase activity. Thus, identification of small molecules that modulate the Lysine acetyltransferase activity of these KATs is an emerging field of research. Therefore, several groups have focused their research on identification of natural compounds and designing of synthetic and natural chemical probes that target the activity of these KATs. In the past two decades, several small molecules have been reported by these groups that modulate KAT activity and interestingly some of these molecules have shown to act as a therapeutic drug for some of the neurological disorders in Mice models (reviewed in [45, 125]). Most of these small molecules are KAT inhibitors, very few are KAT activators.

A. Small molecule KAT inhibitors: The initial step for identification and screening of KAT inhibitors were based on designing molecules that mimics the ternary complex of Lysine substrate and acetyl-coA that forms within the active site of these enzymes during the process of acetylation. This was achieved by covalently conjugating Acetyl-coA cofactor to lysine residue of a histone peptide substrate. Hence, these molecules are known as bisubstrate inhibitors of KATs. The first KAT inhibitor, Lys-coA was identified by Philip Cole and group in 2000 [126]. This compound act as a potent inhibitor of p300 KAT activity with IC_{50} value of 0.5 μM. Efforts from several group have led to identification of several bisubstrate inhibitors for various KAT enzymes such as H3-CoA-20 inhibits PCAF at IC_{50} of 0.3 μM, Boc-C5-CoA inhibits p300 at IC_{50} of 0.07 μM and Spd-CoA inhibitor of spermine/spermidine acetyltransferase (SSAT). In the last two decades there is surge for screening of large number of natural products from fruits, plants and microbial extract for their KAT modulating activity. This led to identification of several natural compounds that possess KAT inhibitory activities. One common factor of these natural KAT inhibitors is presence of similar structural scaffolds such as polyphenols. Most of these compounds lack specificity and show pleiotropic effects in cellular context, which impedes further usage of these compound.

Therefore several chemical and structural based approached have been taken to optimize the properties of these compound for their specific activity. One such natural compound is anarcardic acid, isolated from cashew nut shells. Initially it was found to be a nonspecific inhibitor of p300/CBP and PCAF [127], later on it was found to target Tip60 also [128]. To bring specificity to this compound, several groups started on synthesizing several derivatives of this compound by substituting various functional groups. This led to identification of a novel KAT activator that specifically targets p300/CBP and activates transcription (discussed in next section). The second natural compound is curcumin, a major curcuminoid of turmeric (curcuma longa). This compound is an inhibitor of p300 with IC_{50} value of 25 μM, but has no effect on PCAF activity [129]. Later studies showed that this molecule has pleiotropic targets such as DNMT1, KDACs and several other non-chromatin proteins [130]. The third compound that possess KAT inhibitory activity is Garcinol. It is a polyisoprenylated benzophenone isolated from Garcinia indica (pomegranate) fruit. It inhibits both p300 and PCAF KAT activity [131]. One chemical derivative of this compound LTK-14 is found to be a specific inhibitor of p300 KAT activity and attenuate T-cell cytotoxicity [132]. Fourth natural compound is Embelin isolated from Embelia ribes (black pepper). It targets PCAF KAT activity and is reported to block differentiation in C2C12 cells via inhibition of PCAF mediated MyoD acetylation [133]. Plumbagin is another p300 specific KAT inhibitor, isolated from Plumbago rosea [134]. Recently one more compound has been identified to be a novel KAT inhibitor, Oridonin. It is a diterpenoid isolated from medicinal herbs (Rabdosia rubescens). It shows potent anti-tumor activity but targets multiple of KATs such as GCN5, PCAF, p300 and Tip 60 [135].

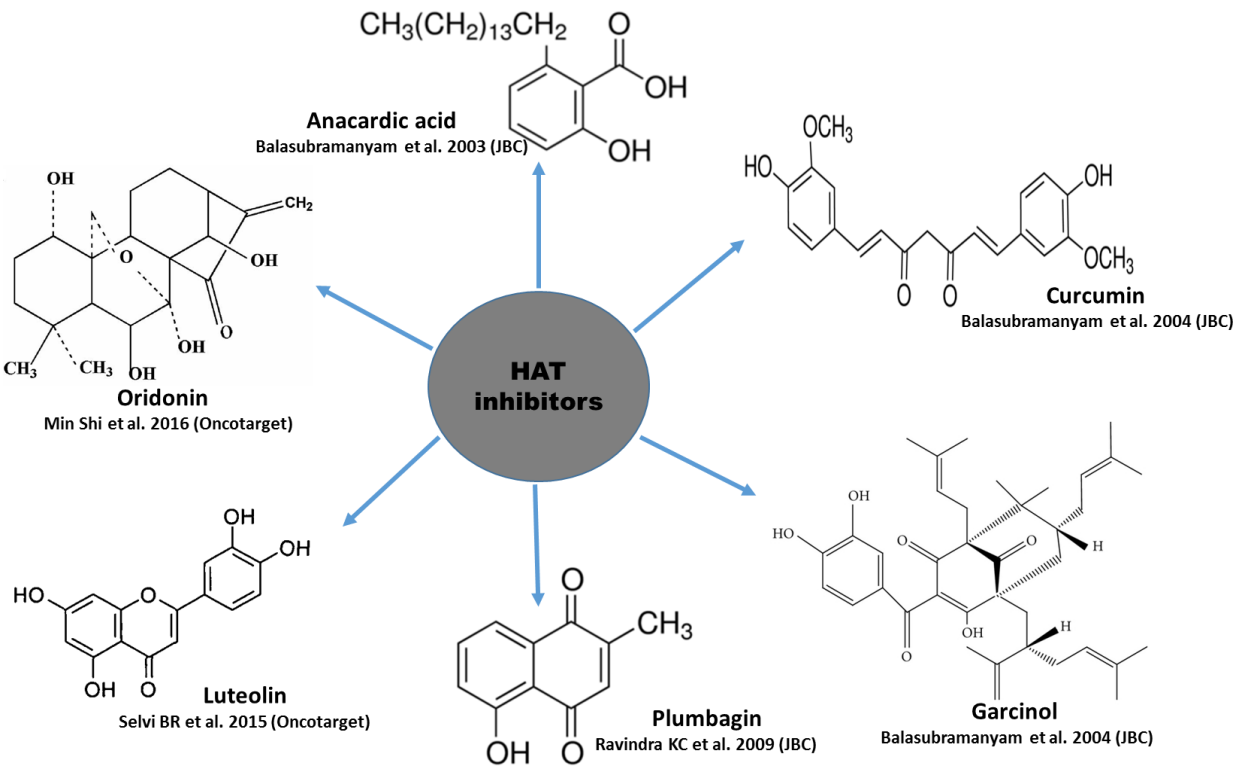


Figure 1.9. Some of the known natural HAT/KAT inhibitors.

B. KAT activators: The first reported synthetic molecule to induce KAT activation in cultured cells is CTPB which is an anacardic acid derivative. This compound is initially identified as KAT activator in a screening of compounds derived from anacardic acid as KAT inhibitors [127]. Anarcardic acid is a potent inhibitor of p300 and PCAF KAT activity, whereas CTPB acts as a potent KAT activator [127, 136]. It is specific to p300/CBP KAT activity but does not alters PCAF KAT activity. However, its therapeutic usage is limited by its inability to penetrate inside the cell (cell-impermeability). More recently, two other small compounds were found as KAT activators which are nemorosone and CSP-TTK21. Nemorosone, a polyprenylated benzophenone (a major constituent of floral resin) was identified through screening of several natural compounds. In-vitro and culture based studies show that this compound has high affinity for p300/CBP, modulate histone acetylation, and penetrate cell-membrane but mice studies are still missing [44, 125]. In search for specific and potent KAT activators, previously in our lab several derivatives of CTPB have been synthesized and assayed for its function as KAT activator through a low-throughput enzyme assay. Upon screening the 21st derivative TTK21 was found to be a potent activator of p300 and CBP KAT activity. In cell based assay, TTK21 was also found to be cell-impermeable

but when it was conjugated to a glucose derived carbon based nanospheres (CSP) it efficiently goes inside the cells and induce histone acetylation [65]. Previous work from our lab show that upon intra-peritoneal (IP) administration of CSP-TTK21 in adult wild type mice, it crosses the Blood-Brain Barrier (BBB) without apparent toxicity and efficiently induces histone acetylation (H2B and H3) in dorsal hippocampus and frontal cortex [65]. Some of the natural known HAT modulators are listed below (Table 1.3.)

Table 1.3. Some of the known HAT modulators.

Types	Small molecules	Specificity	IC_{50}	References
HAT inhibitors	Anacardic acid	p300, PCAF and Tip60	5-10 μM	[94, 95]
	Curcumin	P300 but has pleiotropic targets (DNMT1 and several chromatin associated proteins)	25 μM	[129]
	Garcinol	p300 and PCAF	5-7 μM	[131]
	Embelin	PCAF	7.2 ± 1.5 μM	[133]
	Plumbagin	P300	20-25 μM	[134]
	Oridonin	Multiple KATs; PCAF, p300, GCN5 and Tip60	5-25 μM	[135]
HAT activators	CTPB	p300/CBP		[127, 136]
	Nemorosone	p300		[44, 125]
	CSP-TTK21	p300/CBP		[65]

1.5. Brain targeted drug delivery by nanoparticles:

The brain is the most complex and delicate organ in mammals, and hence has got several layers of protection which maintains the integrity of brain internal environment that is required for optimal functioning of the integrative neuronal networks. Among these layers, the Blood Brain Barrier (BBB) is the most substantial and exclusive one. This barrier is formed of large number of tightly connected brain capillary endothelial cells and a dispersed layer of pericytes which allow passage of small molecules like water, some gases and hydrophobic molecules by passive diffusion, but

excludes large and hydrophilic molecules from the outside environment such as circulating blood cells, neurotoxic molecules and evading pathogens. These cells bear a large number of transporter proteins on their membrane which selectively transport molecules which are crucial for neural functions such as glucose and amino acids. Thus, the BBB protect the brain from external harmful pathogens and neurotoxic compounds hence acts as a shield. During brain/neural injury or disease the same shield acts as a major barrier for delivery of drugs making the treatment of Neurological disorders/diseases quite challenging. In last few decades various strategies have been developed for efficient delivery of drug molecules in the brain. Some of these are non-invasive strategies which are based on the use of prodrugs and delivery systems such as liposomes, polymeric nanoparticles (NPs), and antibodies, whereas others are invasive strategies that involves intracerebral implants, intraventricular infusion and sometimes BBB disruption. Among these, targeted delivery by systemic administration of nanoparticles (NPs) is emerging as a promising field with higher efficiency of drug delivery to the brain.

Nanoparticles are made up of biodegradable and biocompatible polymeric materials (either synthetic or natural) and ranges in diameter from 1-1000nm. They are used as drug delivery system where the drug is either dissolved, adsorbed, encapsulated, or chemically conjugated to the surface of these particles. The drug molecules loaded on these nanoparticles are usually release in the body from the surface of these particles by diffusion, erosion or chemical degradation. As a drug delivery system nanoparticles have several advantages over other drug delivery systems: they have high stability, high drug loading capacity, increased retention and adsorption in brain blood capillaries, can be endocytosed by the epithelial cells of blood capillaries. Moreover, these systems also provide additional advantages such as attainability of incorporation of hydrophobic as well as hydrophilic drugs, extended or controlled release of drugs, feasibility of other routes of drug administration's such as oral, intraperitoneal and nasal delivery, and protection from chemical and enzymatic degradation before reaching the targeted site leading to decreased dose with lesser or no side effects. Nanoparticles are efficiently and extensively used as drug carriers for spatial and temporal delivery of drugs in several diseases especially in cancer cells, Doxil (Doxorubicin Liposome formulation) - the first FDA approved nano-drug is a well example of this. In the recent years, nanoparticles have shown promising results in delivering drugs to several brain related disorders and diseases such as Alzheimer's, Parkinson's, and Brain tumors. Some of the

Nanovehichles that are currently being used for brain targeted drug delivery for various brain disorders are listed in table 1.4.

Table 1.4. List of nanoparticles used for brain targeted drug delivery.

Nanoparticles	Drug/peptide	Target/use	Remarks	References
PLGA-PCL (copolymer)	Etoposide	Several cancers	Enhanced bioavalaibilty and reduced toxcitity	[138]
PLGA (copolymer)	Coenzyme-Q10	APP/PS1	Improved uptake to the brain, improve behaviour in AD mice model	[139]
PLA (aliphatic polyster)	Imatinib Mesylate	Chemotherapy Drug	Improved uptake to the brain	
	Dopamine	Parkinson's disease	Increased dopamine levels and reduced dopamine-associated toxicity.	[140]
	Urocortin	Parkinson's disease	Attenuate 6-hydroxydopamine induce striatum lessions and improves dopamine content	[141]
Chitosan (polysaccharide)	Venlafaxine	Serotonin-norepinephrine reuptake inhibitor	Increased efficacy in treatment of depression	[142]
	Z-DEVD-FMK	Caspase 3	Decrease infarct volume, neural deficits and caspase 3 activity	[143]
	Z-DEVD-FMK and bFGF	Caspase 3	Enhanced transport to the brain, improves motor activity	[144, 145]
BCA (cyanoacrylate ester)	Amyloid affinity drug (125)I-clioquinol	Amyloid plaques	Enhanced transport to the brain.	[145]

PBCA (poly cyanoacrylate ester)	Doxorubicin	Glioblastomas	Enhanced permeability through the BBB	[146]
	NGF	Parkinson's disease	Reversed amnesia and improves recognition and memory	[147]
CBSA-PEG (PEGlygated)	Tanshinone IIA	Ischemic brain	Reduced infarct volume and improves neuroprotection	[148]
PAMAM (dendrimers)	hGDNF	Parkinson's disease	Reduce dopamine neuronal loss, improves monoamine transmission and behavioral activities	[149]
PLA-PEG (PEGlygated)	Amphotericin B	Antifungal infection and leishmanias	Enhanced transport across BBB with improved therapeutic activity and low toxicity to liver and kidney	[150]
CSP (carbon sphere)	CTPB	p300/CBP	Induce hyperacetylation of histones in brain	[137]
	TTK21	p300/CBP	Promotes adult neurogenesis and extends long-term spatial memory	[65]
Iron oxides	Fibrin γ377-395	Tau tangles	Inhibition of microglial cells	[151]
GNS (Gold spheres)	CLPFFD-THR	β-amyloid aggregates	Increased permeability through the BBB	[152]
	KRGD-PEGSH	Brain cancer	Photoacoustic detection of glioma	[153]
	Insulin	Diabetes	Improve brain delivery of gold nanoparticles	[154]
	EGFR-GNS-Pc 4	Glioblastomas	Increased drug delivery to brain tumors	[155]

PEG-GNS (PEGlygated Gold sphere)	NCAM L1 fragment	Drug delivery platform for neurons	Promote neurite outgrowth and survival of neurons	[156]

Expansion of the abbreviated nanomaterials: PLA: poly(lactic acid), **PLGA:** poly(lactic-co-glycolic acid), **PCL:** polycaprolactone BCA: n-butyl-2-cyanoacrylate, **PBCA:** poly(n-butyl cyanoacrylate), **CBSA:** cationic bovine serum albumin, **PAMAM:** poly(amidoamine) **PEG:** poly(ethane glycol), **CSP:** carbon nanosphere, **CTPB:** ((N-(4-chloro-3-trifluoromethylphenyl)-2-ethoxybenzamide) **TTK21:** N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide, **GNS:** gold nanospheres, **DEVD-FMK:** n-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl kitone, **bFGF:** basic fibroblast growth factor, **NGF:** nerve growth factor, **hGDNF:** human glial cell derived neurotropic factor, **THR: KRGD-PEGSH:** Lys-Arg-Gly-Asp peptide-PEG, **EGFR:** epidermal growth factor receptor, **NCAM L1:** neural cell adhesion molecule L1.

Among these nanoparticles, glucose-derived carbon nanospheres (CSPs) prepared by hydrothermal treatment of glucose are emerging as a potent intracellular delivery system. It has intrinsic fluorescence property which helps intracellular tracking without any requirement for other tags. Their surfaces are fully functionalized and did not require any further modifications. Work from our laboratory shows that CSPs could efficiently cross the BBB without any apparent toxicity and are efficiently removed from tissues over time. Further, we have shown that these nanospheres can be used as an efficient nuclear drug delivery system by delivering a cell-impermeable histone acetyltransferase (HAT) activator, CTPB ((N-(4-chloro-3-trifluoromethylphenyl)-2-ethoxybenzamide) into several mammalian cells as well as inside the mice brain. Biochemical and cellular assays shows that ATP driven clathrin mediated endocytosis process is one of the major mode through which CSPs are entering inside the mammalian cells. In addition, CSPs show high preference for cells possessing large number of glucose receptors/transporters such as Glial cells. These all results together show that CSPs has an immense potential as a brain targeted drug delivery system. However, the mechanism by which it crosses the blood-brain barrier is yet to be elucidated.

1.6. Intellectual Disability: A Syngap1 heterozygous mouse model

1.6.1. Intellectual disability (ID):

ID is a common neurodevelopmental disorder that occur before adulthood (age of 18) and is characterized by reduced ability to learn and understand new and complex things which impairs most of the daily routine works (AAIDA, 2013) [179]. Patients suffering from this disability show

significant limitations in both cognitive and adaptive behaviors and they have intelligence quotient (IQ) below 70. Based on the severity/IQ level, ID is divided into 4 types: mild (IQ is 50-70), moderate (IQ is 35-49), severe (IQ is 20-34) and profound (IQ is <20) [157, 158]. In addition to this classification, depending on the presence of morphological abnormalities (such as) or co-morbidities it can be of two types: Non-syndromic (i.e., without presence of clinical features and co-morbidities) and Syndromic (presence of multiple abnormalities and co-morbidities) [158]. ID can be caused either by genetic factors (such as chromosomal abnormalities, translocation, gene mutations, etc.) or environmental factors especially at the time of birth (such as exposure to adverse conditions, metabolic abnormalities, childhood diseases, etc.). The major cause of ID is still unknown for most of the ID cases. Only one-third of ID cases have been linked to occur through genetic aberrations and over 450 genes have been implicated in ID [157, 158]. One of such gene is *SYNGAP1* which encodes a protein SYNGAP1, a Ras-GTPase activating protein that is present in the post synaptic-density complex and has been reported to play important role in formation of neuronal connections and plasticity [159, 160]. Mutations in *SYNGAP1* have been implicated in 2-3% of non-syndromic ID (NSID) patients [161, 162]. The first report of *SYNGAP1* mutation in NSID patients came in 2009 [162] after that there is huge increase in number of children with *SYNGAP1* related NSIDs. Thus, pathogenic mutations in *SYNGAP1* is one of the major causes of ID.

1.6.2. Mice model for intellectual disability (ID):

SYNGAP1 is essential for proper brain function and development. It plays a very important role during neuronal development [163], and is critical for the survival of the animal during early development, as homozygous knockout mice mutants die in the first week after birth [163]**.** Transgenic mice expressing only one copy of *Syngap1* gene show imbalance in excitatory/inhibitory neuronal activity where excitation precedes inhibition which results from accelerated maturation of dendritic spine synapses. This lead to premature critical period closure by 1 week [164], which results in failure of cognitive and behavioural maturation (fig 1.9).

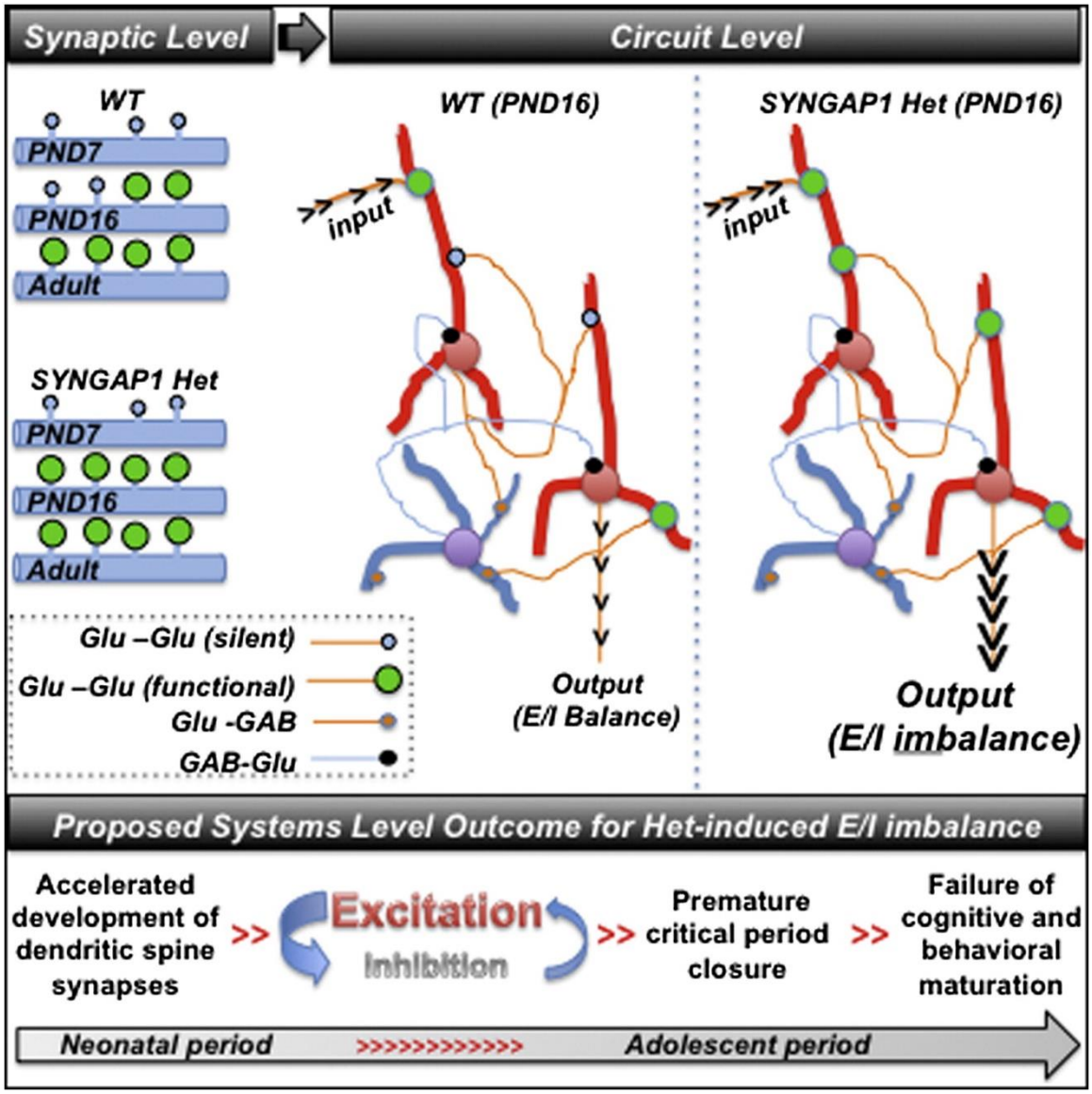


Figure 1.9. Failure of cognitive and behavioural maturation in *Syngap1* heterozygous mice. Mice expressing only one copy of *Syngap1* show imbalance in excitatory and inhibitory neuronal activity due to accelerated maturation of dendritic spine synapses, where excitation precedes inhibition. This enhanced excitation leads to premature closure of critical period of development resulting in failure of cognitive and behavioural maturation. **(Adapted from Clement, J.P., et al. Cell, 2012. 151(4): p. 709-23) "With permission from Elsevier".**

Reduced expression of this protein leads to several behavioural abnormalities such as working memory-deficits, lack of social memory and abnormal response to emotional cues and these mice model symptoms of ID, Schizophrenia and autism [165] (fig 1.10). In addition to these changes, these mice also show deficits in LTP. Recently, *Syngap1* heterozygous knock-out (SynHet) mice

have emerged as a robust model for understanding the molecular mechanisms that leads to reduced cognition, abnormal behavior and social isolation in ID patients.

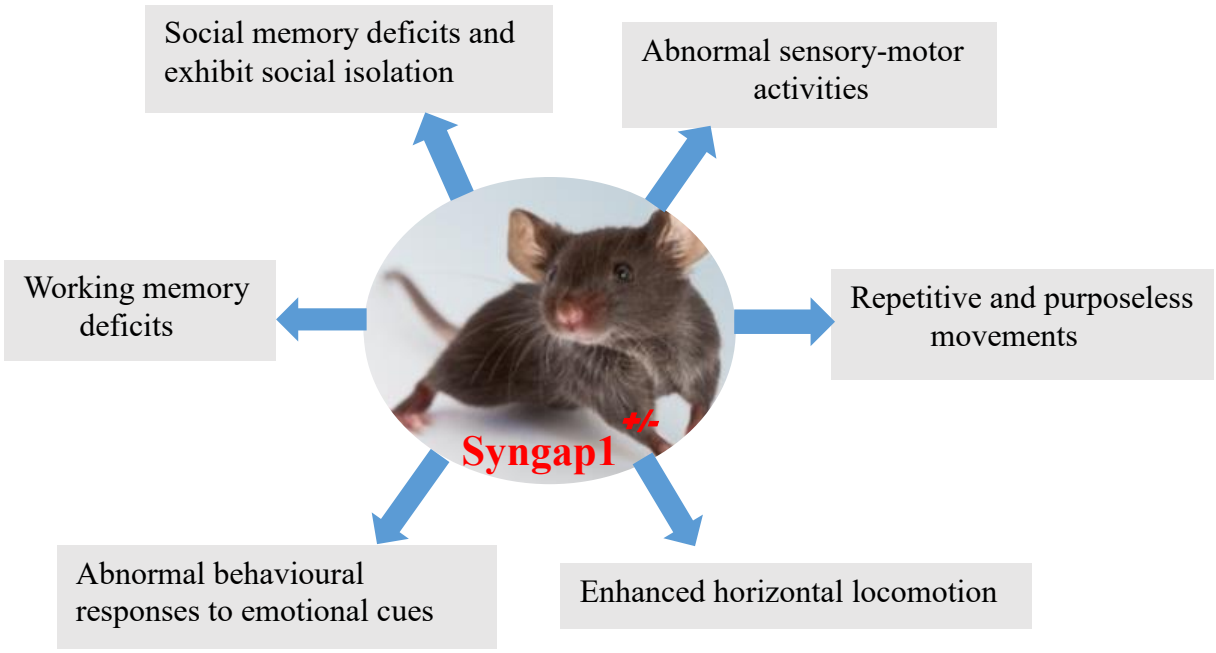


Figure 1.10. Summary of behavioural abnormalities associated with Syngap1 heterozygous mouse.

Recently, *Syngap1* heterozygous knock-out (SynHet) mice have emerged as a robust model for understanding the molecular mechanisms that leads to reduced cognition, abnormal behavior and social isolation in ID patients. Adult restoration of *Syngap1* expression in these mice completely rescue the LTP deficits but does not show any improvement in behavioural, social and cognitive deficits associated with these mice [166].

1.7. Rationale of this study and objectives

Lysine acetyltransferases p300/CBP play crucial roles during neural development, long term memory formation and synaptic plasticity. And dysregulation of p300/CBP has been well-established in variety of neuro-developmental and neurological disorders. Hence targeting the activity of these acetyltransferases have been the major focus of research for development of therapeutic drugs for these disorders. Identification and characterization of small molecule

modulators of acetyltransferase activity is an emerging field. Previous work from our laboratory have reported a small-molecule (TTK21) activator of the CBP/p300 KAT activity, which, when conjugated to glucose based carbon nanospheres (CSP), could be efficiently delivered in mice brain with no apparent toxicity [137]. The activator could induce histone acetylation, promote adult neurogenesis and extend long-term memory retention upon the intraperitoneal (IP) administration in wild type mice [65]. Thus, we think that this molecule could acts a drug for treatment of neuro-cognitive disorders. In this aspect, the two major objectives of my thesis project is listed below:

1. **Exploring the possibility of CSP-TTK21 oral delivery and its effect in mice brain**: Since oral delivery is more common and easier than other routes of administrations in humans. Here we wanted to check whether Oral delivery of the drug is possible or not. The following questions were studied using oral gavaging: a). Does it cross the BBB? b). Is it active in the brain? And c). Is it potent as IP administration?

2. **Exploring the therapeutic potential of this molecule on *Syngap1* heterozygous (SynHet) mice model for intellectual disability**: Since CSP-TTK21 could induce adult hippocampal neurogenesis and extend long-term spatial memory. Here we wanted to check its effect/action on a mice model for intellectual disability (ID), that have severe long-term memory deficits and behavioural abnormalities. And one such mice model is *Syngap1* heterozygous mice that phenocopy most of the behavioural abnormalities and cognitive deficits of ID [165].

CHAPTER 2

Materials and Methods

This chapter describes about the materials and methodology used for experimental purpose during this thesis work.

Outline of the chapter:

2.1. General methods

2.2. Treatment with CSP and CSP-TTK21

2.3. Extra-cellular field recordings

2.4. Statistical analysis and significance test

2.1. General Methods

2.1.1. Breeders

Since *Syngap1* null mutation is lethal, *Syngap1* heterozygous mice were used as a model. *Syngap1* heterozygous mice were created by inserting a neomycin resistance cassette by homologous recombination [159]. *Syngap1* wildtype mice were crossed with *Syngap1* heterozygous mice. All animals were kept in standard mouse cage under conventional laboratory conditions (12h/12h light-dark cycle, temperature: 22±2°C, humidity: 55±5%) with *ad libitum* access to food and water. 4-8 weeks old mice were used for each experiment. All the experiments were performed in accordance with Institutional Animal Ethics Committee, and Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA).

Mice Genotyping: To determine the genotype of the pups born to these parents, Polymerase Chain Reaction (PCR) were performed. Three primers were used for genotyping- primers 1, 2 and 3. Primer3 is designed to anneal with wild type sequence only, while Primer1 can anneal with the sequence of inserted cassette only. Primer2 can anneal with both wild type and cassette sequence mice. Thus, Primers 2 and 3 will give a 500bp band for wild type sequence and Primers 1 and 2

will give a 300 bp band for the cassette sequence. Hence, The PCR gives a single 500bp amplicon for wild type mice and both 500 bp and 300 bp amplicons for heterozygous knockout mice (fig 2.1).

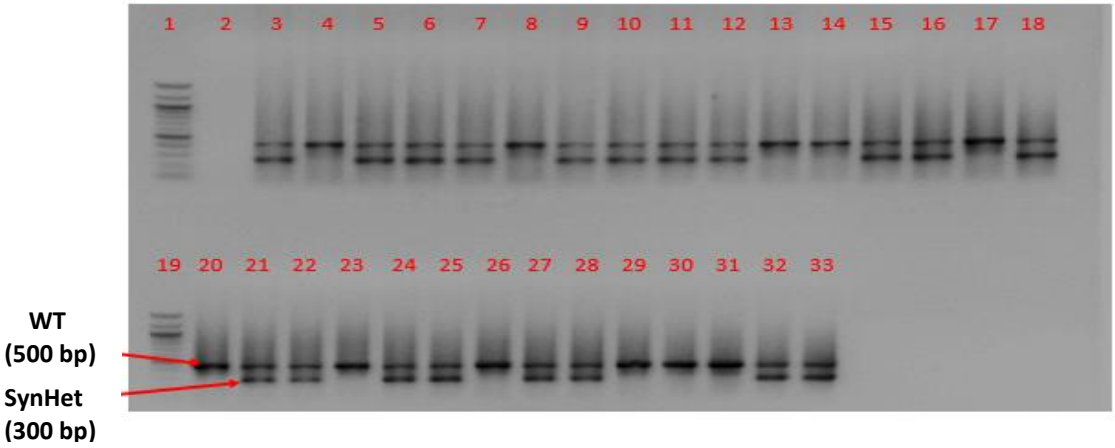


Figure 2.1. Genotyping of Syngap animals. Electrophoretogram showing PCR bands when electrophoresed through 1% agarose gel. Lanes 1, 19: Molecular Weight Ladder (100bp); Lane 2: No Template (negative) control; lanes 18, 33: Positive Control (SynHet genotype); Lanes 3, 5-7, 9-12, 15, 16, 21, 22, 24, 25, 27, 28, 32: mice having SynHet genotype; and Lanes 4, 8, 13, 14, 17, 20, 23, 26, 29-31: mice having Syngap1 WT genotype.

A. DNA Isolation: 2-3mm of tail was cut from each mouse and chopped into smaller pieces with a scalpel blade. 180µl of 5M NaCl was added to lyse the cells. The mixture was kept at 95°C for 10 min and vortexed. Then 20µl of Tris-Cl (pH 8.0) was added to dissolve the DNA present in the cell lysate. The lysate was then centrifuged at 12000rpm for 10 min at room temperature. Supernatant was collected and DNA concentration was measured by Nano-drop.

B. Polymerase Chain Reaction (PCR): Reaction was set-up as follows: Taq buffer (final conc.1X); Template (75ng); dNTP (1 mM); Forward Primer (1µM); Reverse Primer (1µM); IPC primer (1µM); Taq Polymerase (1U/µl) and volume was made up to 20µl with AMQ water. PCR Program was set up as follows: Initial Denaturation for 3 mins at 95°C, Denaturation for 30 sec at 95°C, Annealing for 45 sec at 61.9°C, Extension for 35sec at 72°C, this was done for 35 cycles, then Final Extension was done for 2 min at 72°C, and Held on for 10 min at 4°C. After PCR, 10µl of each PCR product (20µl reaction) was mixed with 6X loading dye to obtain a final concentration

of 1X, and then loaded onto agarose gel for checking amplification. Electrophoresis was carried out at the required voltage. DNA bands were visualized using a gel documentation system.

2.1.2. Mammalian cell culture: U87 glioblastoma cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% serum and appropriate antibiotics in 5% CO_2 incubator.

A. Stock preparation: Approximately 2 million cells were suspended in 1ml Medium (40% incomplete media, 50% FBS and 10% DMSO). For long term storage, temperature was gradually brought down to -80°C in Iso-propanol with the help of thermos cooler, and then the stock cells were stored in liquid nitrogen.

B. Cell revival: Cells were revived by keeping the stock vials in 37°C water bath for 2-3 min followed by removal of DMSO by washing the cells with 10 ml of incomplete media. Cells were seeded in 25mm flasks and kept at 37°C in 5% CO_2 incubator for growth. Once the desired confluency was attained cells were trypsinized with 0.05% Trypsin-EDTA solution at 37°C for 2 min, followed by immediate neutralization with complete media. Then the cells were centrifuged at 1000rpm at 4°C for 3 min, supernatant was discarded and pellet was re-suspended in fresh complete media. Finally cells were seeded in 25mm flasks and 6 well-plates for maintenance and experimentation, respectively.

2.1.3. IP administration and Oral Gavaging:

A. IP administration: 25 gauge 25mm (1 inch) needles were used for IP injections. While injection mice were manually restrained in such a way that the abdominal side was up and the head was pointed down. The needle was kept bevel-side up and angled at 25-35 degree at the time of abdominal wall penetration. It was ensured that the needle just penetrate the abdominal wall and not the abdominal organs such as colon or bladder. The solution was injected slowly and at constant rate.

B. Oral Gavaging: Stainless steel curved mice feeding tube with a ball tip was used for gavaging. While gavaging, the mice were scruff in such a way that the fore legs were extended on sides and the head was extended back (in same plane). The gavage tube was advanced to the esophagus

through the pharynx solely with gravitational pull. The solution was gavaged slowly and after gavazing the tube was pulled back at the same angle as used during insertion. Mice were monitored for 5-10 mins after gavaging for potential complications.

2.1.4. Lysate preparation:

A. Brain Lysate: Mice were sacrificed by cervical dislocation; brain was rapidly removed (in less than 1min) and thoroughly washed with 1X PBS to remove the blood samples. Various parts of brain were dissected out, and homogenized in lysis buffer (either RIPA or Laemmli) containing protease inhibitors & NaBu. Homogenized samples were centrifuged at 12,000rpm at 4°C for 20 min. The supernatant was collected and aliquoted (20µl) in fresh microfuge tubes and stored at -80°C for further usage.

B. Cell Lysate: Cells were harvested by scrapping followed by centrifugation at 2000rpm at 4°C for 5 min. The cells were washed with chilled 1X PBS followed by centrifugation. Pellet was re-suspended in 10 times volume of obtained cell pellet in 1X Laemmli buffer (62.5 mm Tris-HCl pH6.8, 25% glycerol and 2% SDS). Cell suspension was homogenized with pipette tips and the homogenate was kept at room temperature (RT) for 30 min followed by heating at 90°C for 10 min and cooling at RT for 10 min. This heating and cooling steps were repeated twice. The homogenate was sonicated at 35 amplitude (2sec on and 3 sec off) for 15 sec for uniform lysis. The Lysate were cleared by centrifugation at 12,000rpm at 4°C for 10 min, supernatant was collected and stored at -20°C till further usage. Lysate protein concentration was determined by Bradford method using Bio-Rad's protein assay and RC-DC kit.

2.1.5. SDS – PAGE: It was performed to separate the proteins according to their molecular weight to check their expression level through western-blotting. Resolving gels were made in various percentages of acrylamide (Stock: 30%, Acrylamide: Bis-acrylamide – 29:1) along with 0.375 M Tris pH 8.8, 0.1% SDS, 0.1% APS and 8 % TEMED. Stacking gels were made with 5% acrylamide along with 0.375M Tris pH 6.8, 0.1% SDS, 0.1% APS and 8% TEMED above the separating gel. Protein samples were made in 1X sample buffer (50mM Tris-HCl pH 6.8, 100mM DTT, 0.1% bromophenol blue, 10% glycerol) boiled at 90°C for 5 mins before loading on to the gel. Gels were

electrophoresed using Tris-Glycine buffer (25mM Tris, 250mM Glycine pH 8.3, 0.1% SDS). Gels were visualized by staining with coomassie (45% methanol, 10% acetic acid, 0.25% Brilliant blue) followed by destaining (30% methanol, 10% acetic acid in H2O).

2.1.6. Western Blotting: For tissue lysates; 100µg of each protein sample was heated at 95°C for 10 minutes with sample buffer. The samples were electrophoresed through an SDS-PAGE gel (5% stacking gel and 15% resolving gel) for 1 hour at 150V. The separated proteins were transferred to a PVDF membrane at 80V for 1.5 hours (for wet transfer) and 25 mins (for dry transfer). The blot was stained with Ponceau S stain to view protein bands and to check transfer efficiency. After destaining, the blot was blocked for 1 hour using 5% skim milk (in 1X PBS), and probed with rabbit primary antibodies against H3 (1:5000), H3K14Ac(1;10000) and mouse β-tubulin (1;2000) separately overnight (at 4ºC) and secondary antibody Goat anti-rabbit (1:10000) and Goat anti-mouse (1;10000) for 1 hour at RT on rocker. The blot was developed using the Luminol chemiluminescent method in Syngene Gel-documentation system.

2.1.7. RNA Isolation: Mice were sacrificed after 84 hours of CSP/CSP-TTK21 treatment, the dorsal hippocampus was dissected out (within 2-3 min) and chopped finely with a fresh razor blade. All reagents and instruments (tips, surgical items, and microfuge tubes) used for RNA isolation were treated with DEPC the previous day. The tissue pieces were homogenized in Trizol reagent (Invitrogen). RNA was isolated by the traditional phenol: chloroform method. After phenol chloroform treatment, aqueous phase was collected and ethanol precipitated using absolute ethanol. The pellets were washed twice with 70% ethanol and air dried. RNA pellets were resuspended in RNase-free water and concentration was determined by Nano-drop. RNA quality was checked by running 1µl of each isolated RNA sample onto agarose gel followed by visualization using gel documentation system (fig 2.2). 8µg of individual RNA samples was used for DNase treatment. After 30 min of DNase treatment, RNA was ethanol precipitated, washed twice with 70% ethanol and resuspended in RNase-free water. Again RNA concentration were determined by Nano-drop.

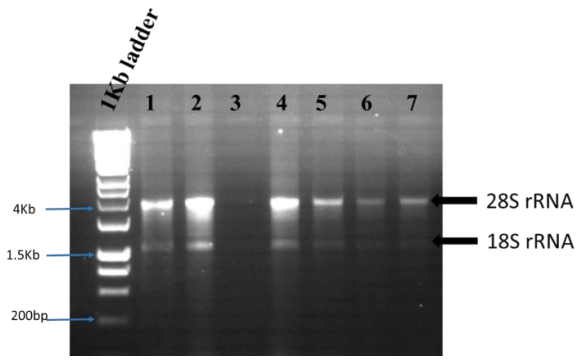


Figure 2.2. Total RNA extraction from dorsal hippocampus of mice brain. RNA was isolated from 7 mice (1: WT-saline; 2, 3, 4: SynHet-CSP treated; 5, 6 and 7:SynHet-CSP-TTK21 treated). 0.8% agarose gel stained with EtBr showing the quality of the purified RNA samples.

2.1.7. cDNA Synthesis: 0.5µg of total RNA was used for synthesis of cDNA. 0.5µg of RNA was taken with 10mM dNTP mix, and denatured at 70ºC for 10 min followed by reverse-transcription (RT) by MMLV RT. The qRT-PCR was carried out with gene specific primers and the run cycle was set as follow: Initial Denaturation for 10 mins at 95°C, Denaturation for 15 sec at 95°C, Annealing and extension for 30 sec at 60°C, this was done for 40 cycles. Melting curve analysis was done as follow: 15 sec at 90°C, 1 min at 60°C and again 15 sec at 95°C.

2.2. Treatment with CSP and CSP-TTK21

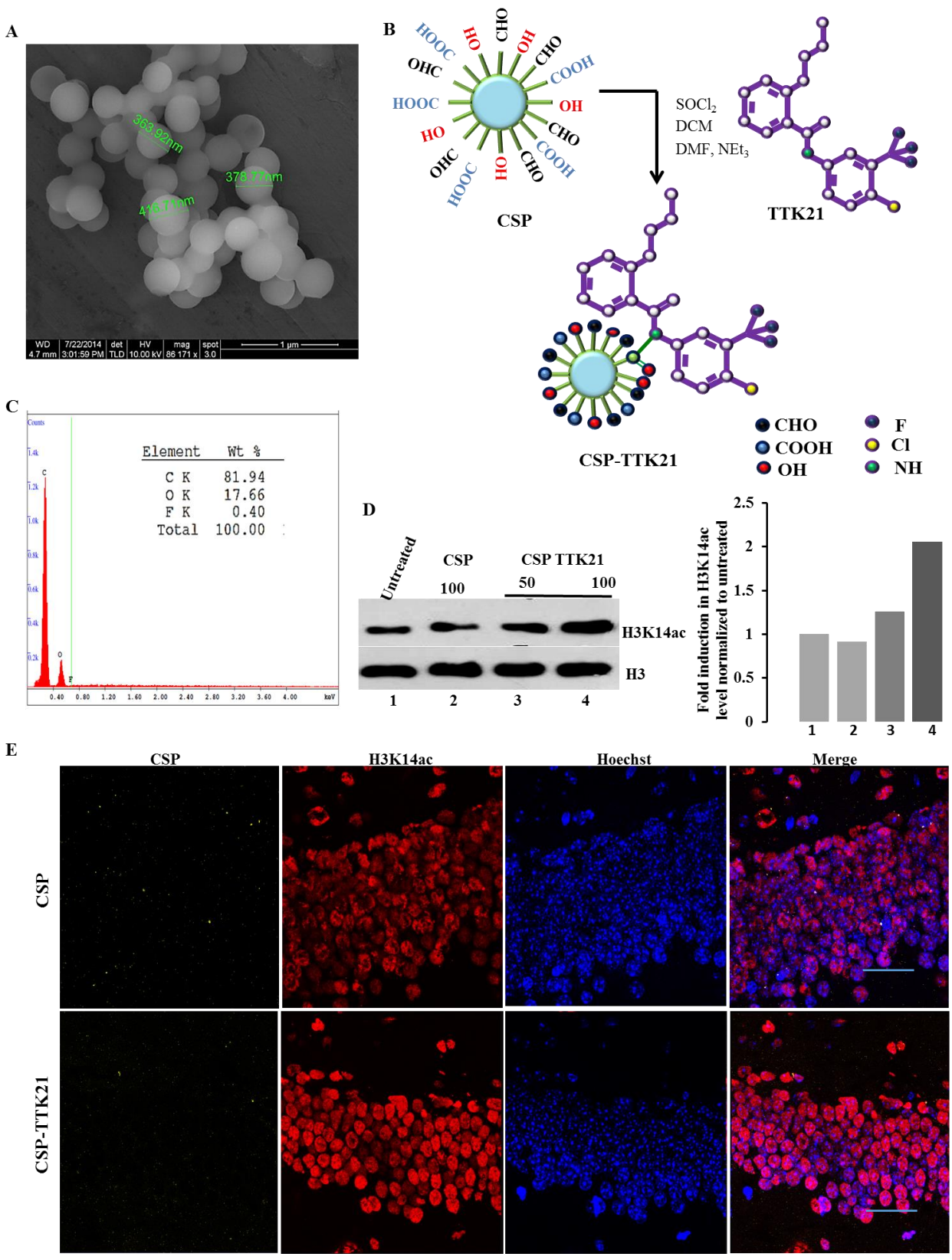
2.2.1. Synthesis and characterization of CSP and CSP-TTK21:

The glucose derived carbon nanospheres (CSP) was prepared freshly by hydrothermal process: 5g glucose in 50 ml deionized water at 180°C for nearlt 12 hr, the product were isolated by centrifugation at 16000rpm, purified by repeatedly washing with water and ethanol and dried for 4 hr at 80°C. From field emission scanning electron microscopy (FESEM) imaging, we observed that the final product were spheres with average diameter of 400nm (fig 2.3.A). TTK21 Synthesis and conjugation with CSP was done as described previously (figure 2.3.B) [Chatterjee et. al, 2013]. Energy Dispersive X-ray Spectroscopy (EDX) analysis showed presence of Fluorine (wt%-0.4) which belongs to TTK21, indicating the covalent conjugation of CSP-TTK21 (fig 2.3.C). Characterization of CSP-TTK21 activity was done by confocal imaging and western-blotting from

mice and u-87 cells treated with CSP/CSP-TTK21 respectively (fig 2.3.D and E). The same batch of CSP or CSP-TTK21 was used for the entire work. Stocks were kept at -20ºC. For treatment, both were resuspended in autoclaved miliQ (AMQ) water at a final concentration of 1.5mg/ml and sonicated for 180 sec for proper suspension.

A. Mammalian cells: 10^6 cells were seeded in each well of 6-well-plates and kept for growth at 37°C. After 12 hours of growth cells were treated with different doses of CSP (50 µg and 100 µg) and CSP-TTK21 (50, 100 and 150 µg) for 12 hours.

B. Mice: Mice were injected orally and intraperitoneal with CSP and CSP-TTK21 at the dose of 20 mg/kg of their body weight. For oral and IP comparison wild type (WT) mice were used and for Intellectual Disability Syngap1 heterozygous and WT mice were used. After defined periods (84 hours), mice were deeply anesthetized with ether and brains were then rapidly removed from the skull and post-fixed for 12 h in 4% paraformaldehyde (PFA) fixative at 4°C. Fixed brains were then kept in 20% sucrose for 24 h and in 30% sucrose for 6 h at 4°C.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DCM
DMF, NEt3
TTK21
CSP-TTK21
CHO
COOH
OH
F
Cl
NH
C
Element Wt %
C K 81.94
O K 17.66
F K 0.40
Total 100.00
D
Untreated
CSP
CSP TTK21
100
50
100
H3K14ac
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1 2 3 4
Fold induction in H3K14ac level normalized to untreated
2.5
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CSP
H3K14ac
Hoechst
Merge
CSP
CSP-TTK21

Figure 2.3. Synthesis and characterization of CSP-TTK21. (A) FESEM image showing CSP particles, (B) Schematic shows protocol for CSP and TTK21 conjugation, (C) The presence of fluorine in EDX analysis of CSP-TTK21 confirms the conjugation of TTK21 with CSP. (D) Increased H3K14 acetylation upon western-blotting of CSP and CSP-TTK21 treated U-87 cells shows CSP-TTK21 is active and (E) Confocal imaging from mice dorsal hippocampus region shows presence of fluorescence CSP particles and induction of histone acetylation in CSP-TTK21 treated mice. Scale bar 50μM.

2.2.2. Immunofluorescence and Immunohistochemistry for animal tissue:

Freezing of the brains were performed in optimal cutting temperature (OCT-Leica) medium for 20 min at -20°C. Coronal sections, 20 μm in thickness, were made through the dorsal hippocampus using a Cryotome (Leica). The tissue sections were permeabilised in 1X PBS/2% Triton X-100 for 15 min. Nonspecific labelling was blocked by 1X PBS/0.1% Triton X-100/5% horse serum for 30 min at 37°C. The sections were then incubated overnight with the indicated antibodies (rabbit H3K9Ac, H3K14Ac and H4K12Ac) separately. After 3 washes, sections were incubated with the appropriate secondary antibody. For immunofluorescence, sections were incubated with goat anti-rabbit conjugated with fluorescent dye (1 h at room temperature), followed by 3 washes with 1X PBS/0.1% Triton X-100, and the nuclei were stained with Hoechst (1:1000 dilutions) for 5 min. Then sections were mounted on slides. Fluorescence images were taken through confocal scanning laser microscope LSM510 META and Zen LSM810. For immunohistochemistry, after primary antibody sections were further incubated with anti-rabbit horseradish peroxidase-conjugated antibody (#sc2004; Santa Cruz Biotechnology) for 1 h. After three washes with 1X PBS/0.1% Triton X-100, 0.05% DAB (with 0.04 M Tris, pH 7.5, 0.03% H_2O_2) was added and sections were mounted with a Roti Histokit II (Roth). For immunofluorescence, images were acquired with a Zeiss LSM510 and LSM810 confocal scanning laser microscope and images were processed using Zen black software.

2.3. Extra-cellular field recordings

2.3.1. Acute hippocampal slice preparation:

Adult mice (>3months) of desired age groups were sacrificed by cervical dislocation, and brains were rapidly removed and kept in chilled carbogenated (95% O_2 and 5% CO_2) sucrose solution (124 mM Sucrose, 3 mM KCl, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.25 mM $NaH2PO_4$, 1 mM $MgSO_4$, and 10 mM D-Glucose) for 1 min. Then, cerebellum was removed, and brain was fixed on vibratome platform, and 350µm thick hippocampal slices were prepared in ice-cold sucrose cutting solution with continuous carbogen supply. Slices were kept for an hour at 37°C water bath in a chamber on a nylon mesh containing artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 3 mM KCl, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.25 mM $NaH2PO_4$, 1 mM $MgSO_4$, and 10 mM D-Glucose) with continuous carbogen supply. After 1 hour at 37°C, the slices were kept at room temperature with continuous carbogen supply till further use. Prior to recordings, one slice was transferred to the recording chamber continuously perfused at the rate of 400-550 µl/min with warmed (34°C) carbogenated ACSF. The rest of slices were maintained at room temperature during recordings from one slice.

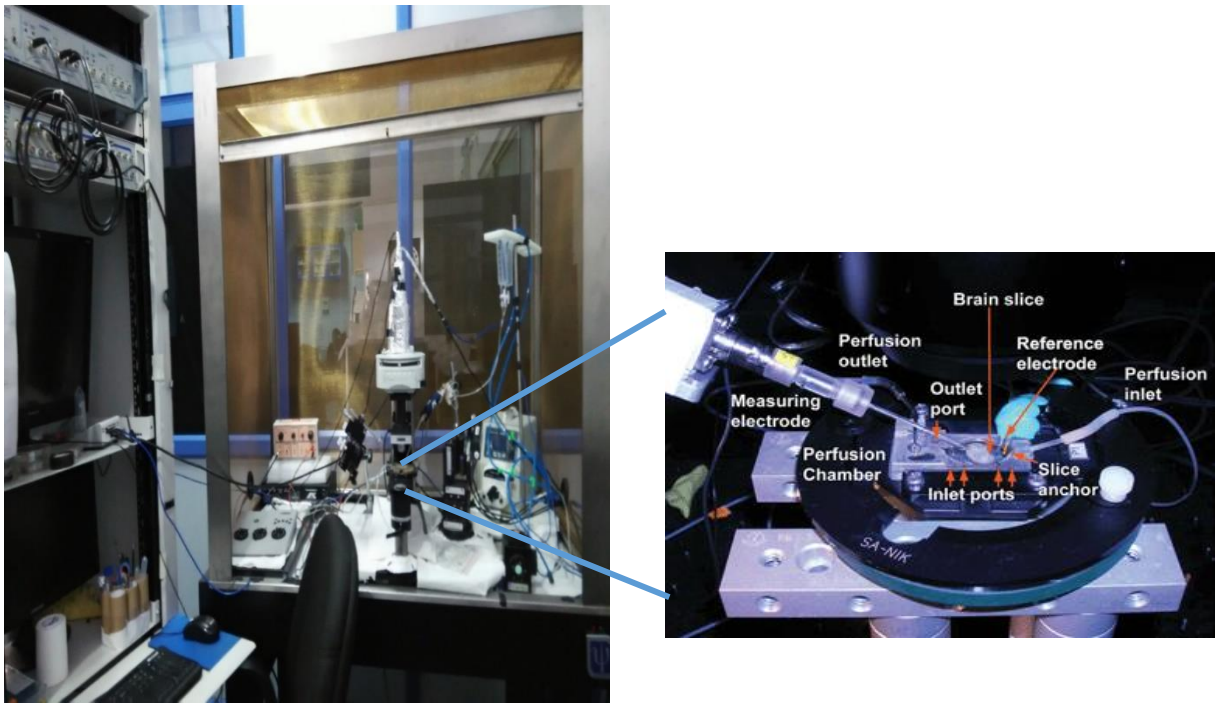

Figure 2.4. Electrophysiology Rig. A schematic showing the rig used for recordings. A zoomed image of the slide holder showing the orientation of electrodes (reference and recording), perfusion chamber, slice anchor and Brain slice.

2.3.2. Extracellular Field recordings from Hippocampal slices:

A. Input-output Curves: Input-output (I/O) measures the post-synaptic response to a given presynaptic stimuli. I/O measures the change in neuronal response (output) with varying stimulus intensity (input). I/O is performed to study the basal synaptic transmission. In addition, it is used to establish the appropriate stimulus intensity for extracellular recordings.

B. Paired pulse ratios: Paired-pulse ratio (PPR) is performed at an increment of quarter log unit stimulus interval. It measures the facilitation of excitatory post-synaptic response when two pre-synaptic spikes are evoked at different intervals. This suggests any change in the probability of neurotransmitters release from pre-synapse Therefore, any change in PPR is directly linked to pre-synaptic effect.

C. LTP: A persistence increase in the strength of synaptic connections in response to high frequency stimulation (theta burst or 100 Hz), which leads to long term storage of information (memory). Hence, it is a direct cellular correlate of long term memory.

Extracellular potentials were recorded in CA region (fEPSPs) with low-resistance (3–5 MΩ) glass electrodes pulled from borosilicate glass capillaries (ID: 0.6mm, OD:1.2 mm, Harvard Apparatus) using a horizontal micropipette puller (Flaming-Brown P-97, Sutter instruments Co., USA) filled with aCSF (pH 7.3). All stimulating and recording electrodes were mounted on a micromanipulator, which could move in three axes to allow accurate placement of electrodes on the slices. The stimulating and recording electrodes were placed in the slice approximately 45° to the vertical. Stimulation frequency was set to 0.1 Hz. Input-output curves were generated by setting a stimulation range of 20–40 μs and by adjusting the stimulus intensity by 20 μA per sweep with increments from 0–160 μA. Prior to baseline setup, Paired-pulse facilitation (PPF) were assessed using a succession of paired pulses separated by intervals of quarter log units, with the intervals ranging from 10 to 1000. Recordings were discarded if baseline was not stable. Baseline activity was recorded for 15-20 min and then the desired protocol was applied to induce long term potentiation (LTP). LTP was induced by 1 train of 100Hz stimuli for 1 sec and the corresponding activity was recorded for 55-60 min. The digitized data for the recordings were captured at sampling frequencies 20-50 kHz through Clampex 10 software (Axon instruments) and stored on a computer for off-line analysis.

2.3.3. Analysis of stimulus induced responses

Clampfit 10 software was used for analysis. To eliminate the possible contamination of the response from population spike, instead of peak amplitude, the slope of the rising phase of the fEPSP was used as a measure of the size of the fEPSP. Input-output curves were generated by plotting a graph between the measured fEPSP slope and stimulus intensity. For paired pulse, the degree of facilitation was measured by calculating the ratio of the fEPSP slope of second response relative to first in each pair.

2.4. Statistical analysis and significance test

All the statistics were carried out in Microsoft Excel 2013 and GraphPad Prism 7.02. All the graphs were made in Microsoft Excel 2013. Data are presented as the mean ± standard error of the mean (S.E.M.) and n refers to the number of times an experiment was repeated, each in a different slice. Paired or unpaired student's *t*-test and Two-way Anova with repeated measures were used to determine the significance.

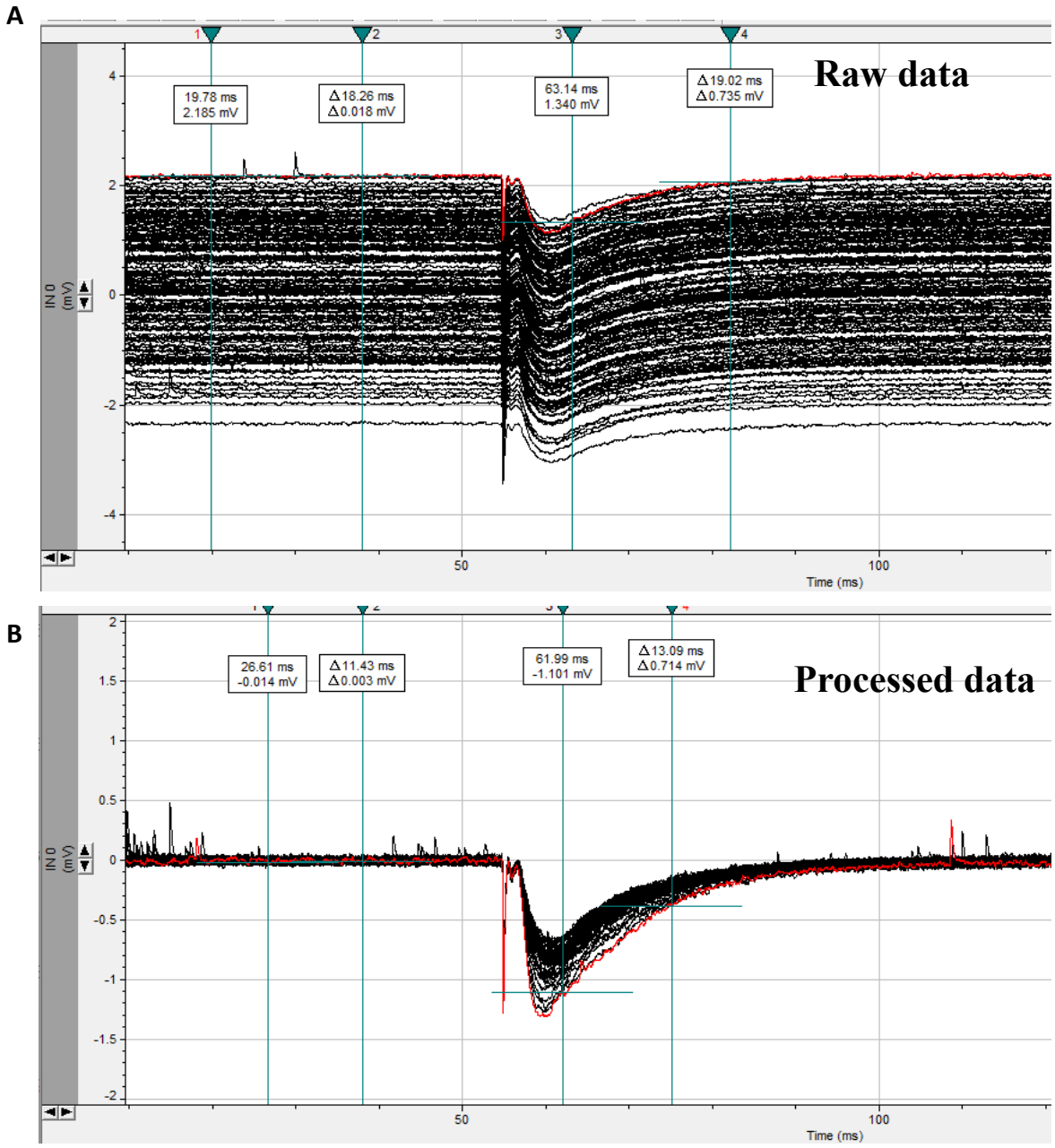

Figure 2.5. Raw and processed data. Clampfit software was used for data processing and analysis. A file (A) showing the raw data where region 1 (between cursors 1 and 2) show the region selected for baseline adjustment, and region 2 (between cursors 3 and 4) show the region used for fEPSP slope measurement. A few traces having noise signal in these regions were deleted. File (B) show the processed data after baseline adjustments.

CHAPTER 3

Oral Gavaging of CSP-TTK21: comparison with Intra-peritoneal administration (IP)

This chapter briefly discusses about a small molecule activator of p300/CBP acetyltransferase activity, its role in adult neurogenesis and retention of long term spatial memory upon IP administration. Here in this chapter, by using a series of molecular biology, microscopy and electrophysiological techniques we have addressed the aspect of Oral Gavaging of CSP and CSP-TTK21 in wild type mice and compared its functional activity with IP administration. Our results show that this molecule induces long term potentiation in hippocampus and does not alters basal synaptic transmission. In addition to this, we observed oral delivery of CSP-TTK21 is as efficient as IP administration.

Outline of the Chapter:

3.1. CSP-TTK21 a small molecule activator of p300/CBP acetyltransferase activity

3.2. CSP-TTK21 crosses the BBB upon oral gavaging and is active

3.3. Oral Gavaging is as effective as IP injections

3.4. CSP-TTK21 does not alters basal synaptic transmission

3.5. CSP-TTK21 induces LTP in mice hippocampal slices

3.1. CSP-TTK21 a small molecule activator of p300/CBP acetyltransferase activity:

The ability of an individual to encode, store and recall information whenever required (almost every time for normal day-to-day activity) is termed as memory. It is a complex phenomenon that requires dynamic changes in gene expression that alters the structure and function of neuronal synapses. Depending upon the mode of action whether an information is stored, recalled or erased, there are extensive modulation of dendritic spines and neuronal synapses. This phenomenon is termed as synaptic plasticity, and is a very dynamic process. However, the underlying molecular mechanism that governs synaptic plasticity and its role in memory and cognitive processes are not well-known. Epigenetic modifications such as DNA methylation and histone acetylation play an

important role in memory and cognitive processes [167, 168]. Increased histone acetylation by utilizing KDAC inhibitors (KDACi) have been reported to enhance learning and cognitive properties in mouse models. Recently several studies have shown that KDACi could be used as a therapeutic drug for several neurodegenerative disorders [125, 169]. However, due to their pleiotropic effect and limited specificity these molecules lack human trials. Thus, an alternative use of KDACi is KAT activators. One such molecule is CSP-TTK21, a novel KAT activator that specifically activates p300/CBP KAT activity in vitro as well as in-vivo. Previous work from our lab show that upon intraperitoneal injections in mice, this molecule could cross the blood-brain barrier (BBB) and induce histone acetylation in dorsal hippocampus and frontal cortex region of the brain. In addition to this, it also promotes adult neurogenesis and significantly extends retention of long term-spatial memory [65]. However, electrophysiological studies are missing that could tell us about the effect of this molecule at synaptic level, whether this drug modulates synaptic transmission or long term potentiation (LTP). Therefore, here we have done some electrophysiological studies to see the effect of CSP-TTK21 on LTP and synaptic transmission. CSP-TTK21 possess tremendous potential as therapeutic molecule/molecular conjugate. Oral delivery of CSP-TTK21 should further increase its probability as a drug molecule. We investigated here the possible oral delivery of CSP-TTK21 using mice as a model system.

3.2. CSP-TTK21 crosses the BBB upon oral gavaging and is active:

CSP and CSP-TTK21 was synthesized, conjugated and characterized for this study (fig 2.3). The same batch was used for the entire study. Initially, we started with a pilot experiment to check whether oral delivery of this molecule is possible or not. Upon immunohistochemistry (IHC) of mice treated with CSP or CSP-TTK21, we find that the molecule is present in the mice brain (fig 3.1A) and is active as seen by induction of H4K12ac mark in dorsal hippocampus (fig.3.1B). We found that maximum number of nanospheres are present in the brain at day 4 upon Oral Gavaging, instead of day 3 as observed previously upon IP administration. The presence of lesser number of fluorescence CSP particles in the brain upon oral gavaging as compared to IP administration could explained by the fact that IP injections results in delivery of molecules/compound directly into the peritoneal cavity from where they gets efficiently absorbed into the blood stream and delivered to various tissues, this takes lesser time. Whereas during oral delivery the molecules/compounds have

to pass through the esophagus, stomach and gastro-intestinal (GI) tract before getting absorbed into the blood stream, which takes longer time. Since there are several proteases and digestive enzymes present in the stomach and GI tract, the observation that upon oral gavaging CSP-TTK21 is actively inducing histone acetylation in the brain especially in the hippocampal region shows that the molecules remains intact after passing through the entire route. However, we could not rule out the possibility of CSP particles getting digested upon oral gavazing as it is a glucose based carbon derived nanospheres. Since, our major objective was to see the effect of this molecule in brain upon oral delivery, this experiment gave us a clue that this molecule could be delivered orally and could mediate its normal function.

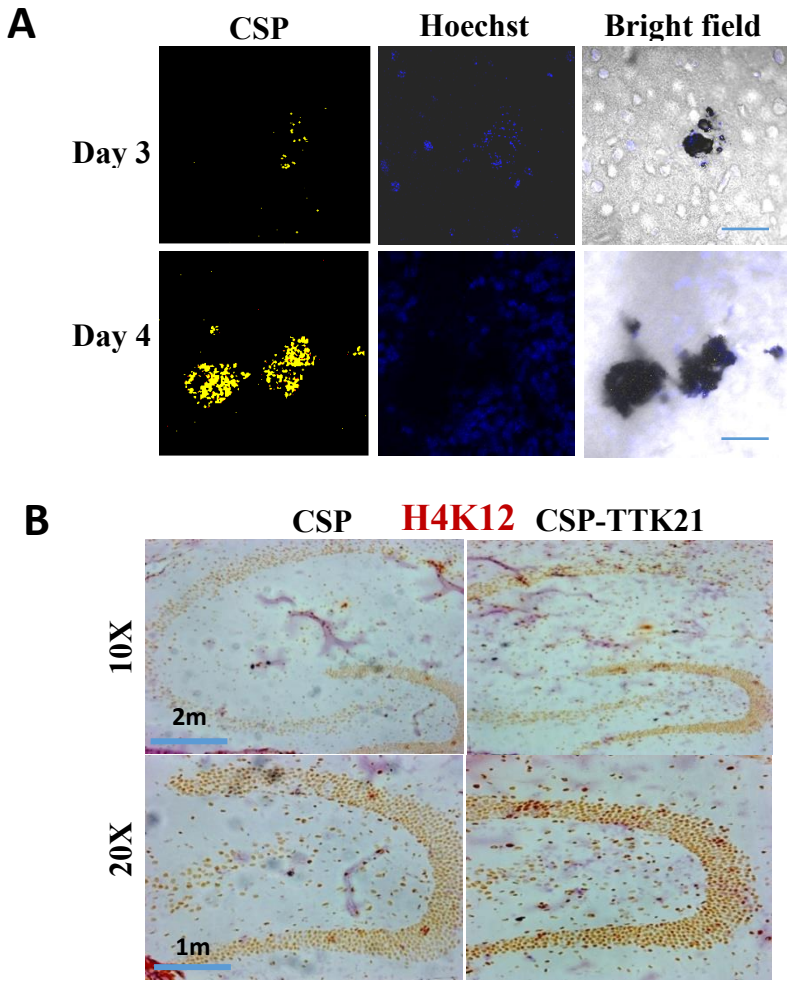

Figure 3.1. Oral Gavaging of CSP and CSP-TTK21 in mice. (A) Confocal image showing presence of auto-fluorescence CSP particles in mouse hippocampal brain. Scale bar 50 µM. (B) DAB staining with H4K12 acetylation showing induction of Histone acetylation in CSP-TTK21 treated mice as compared to CSP control (10x and 20x magnification).

3.3. Oral Gavaging is as effective as IP injections:

Next we wanted to compare the efficiency of both route of administration in terms of HAT activation function, so that we can choose one route of delivery for our further studies. Since we observed maximum number of particles at day 4 instead of day 3 as observed previously upon IP injections, for comparison purpose we choose a middle time point that is at 84 hours (3 ½ days). A single dose of CSP and CSP-TTK21 was administered in two groups of mice, in one group by oral gavaging and in the other group by IP injections. After 84 hours of treatment, the mice were sacrificed and each hemisphere of the brain was processed for confocal imaging as well as electrophysiological studies (fig 3.2). Upon immunohistochemistry we found that whether the route of administration is oral or IP, there is significant increase in histone H3K14 and H4K12 (not shown here) acetylation level in the dorsal hippocampus in mice treated with CSP-TTK21 as compared to CSP control mice (fig 3.3 and 3.4). Moreover, when we compared the increased acetylation marks in both conditions we did not find any significant difference. Hence we think that oral delivery of CSP-TTK21 is as efficient as IP injections. This thought was further supported by electrophysiological experiment where we got comparable results from LTP experiments (discussed in the next section).

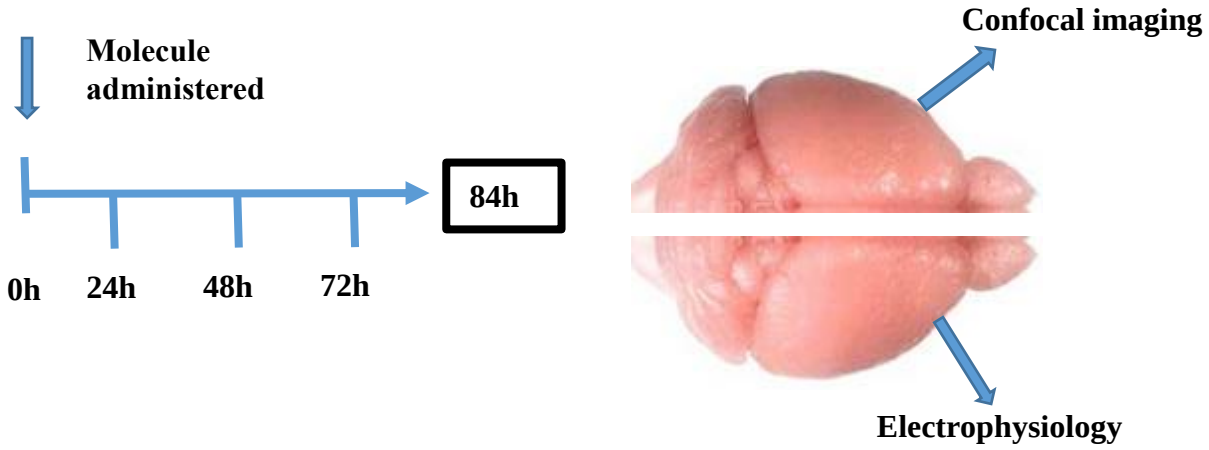

Figure 3.2. Schematic showing the protocol used for comparison of oral and IP administration. 84hrs after drug treatment mice were sacrificed followed by immunohistochemistry and electrophysiological studies.

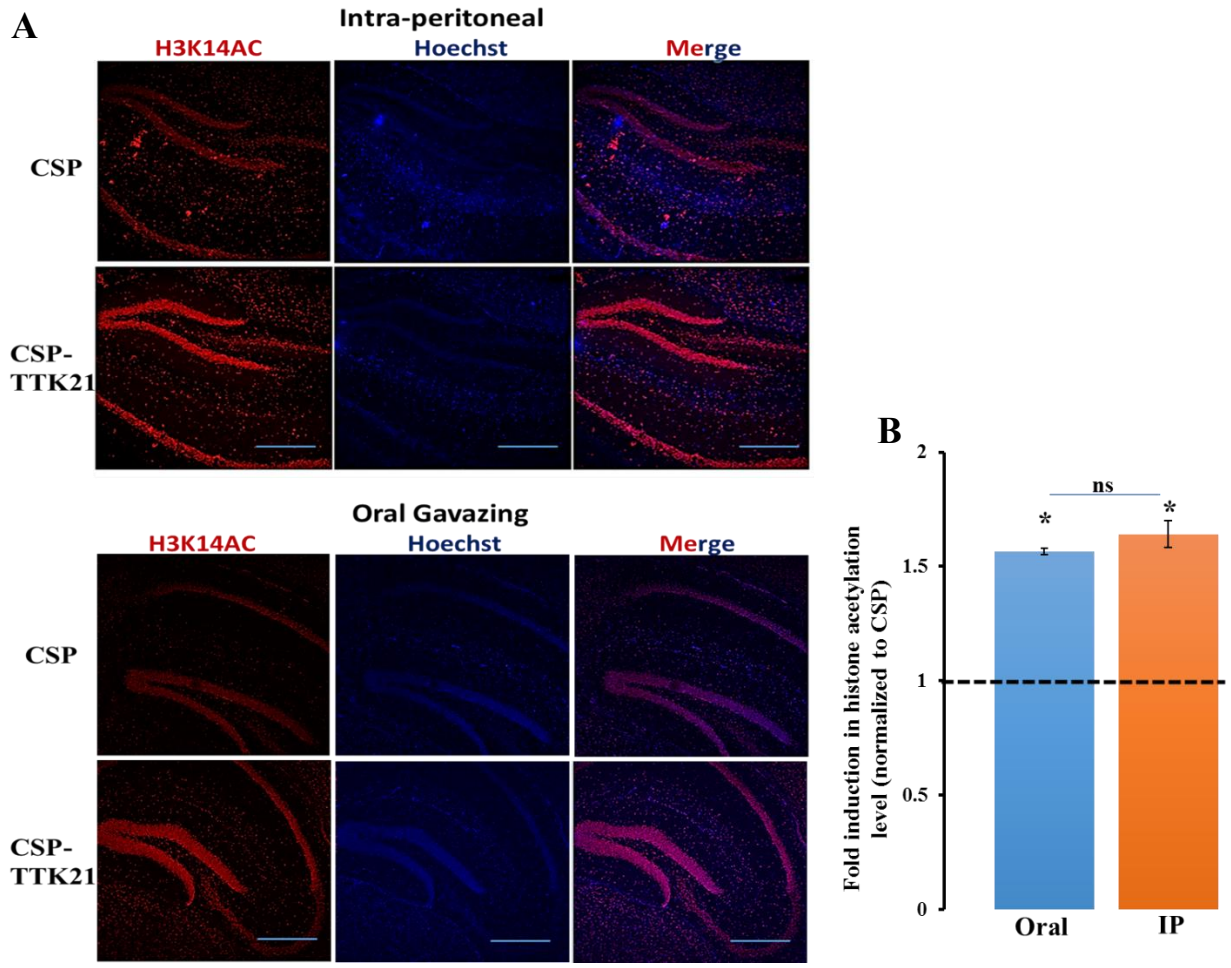

Figure 3.3. CSP-TTK21 induces histone acetylation upon IP injection and Oral Gavaging in dorsal hippocampus of mice. (A) Immunohistochemistry was performed on 20µm thick sections with an anti-acetylated H3K14 antibody. In both cases IP and Oral administration, acetylation was increased in the hippocampal region as compared to CSP-control (n=4). Scale bar 200µm. (B) Quantitation of fold induction in histone acetylation level upon Oral and IP administration of CSP-TTK21 (normalized to CSP). Error bars represent standard error of mean (SEM). Student's t-test was done for significance test and the p-value obtained, *$p<0.05$.

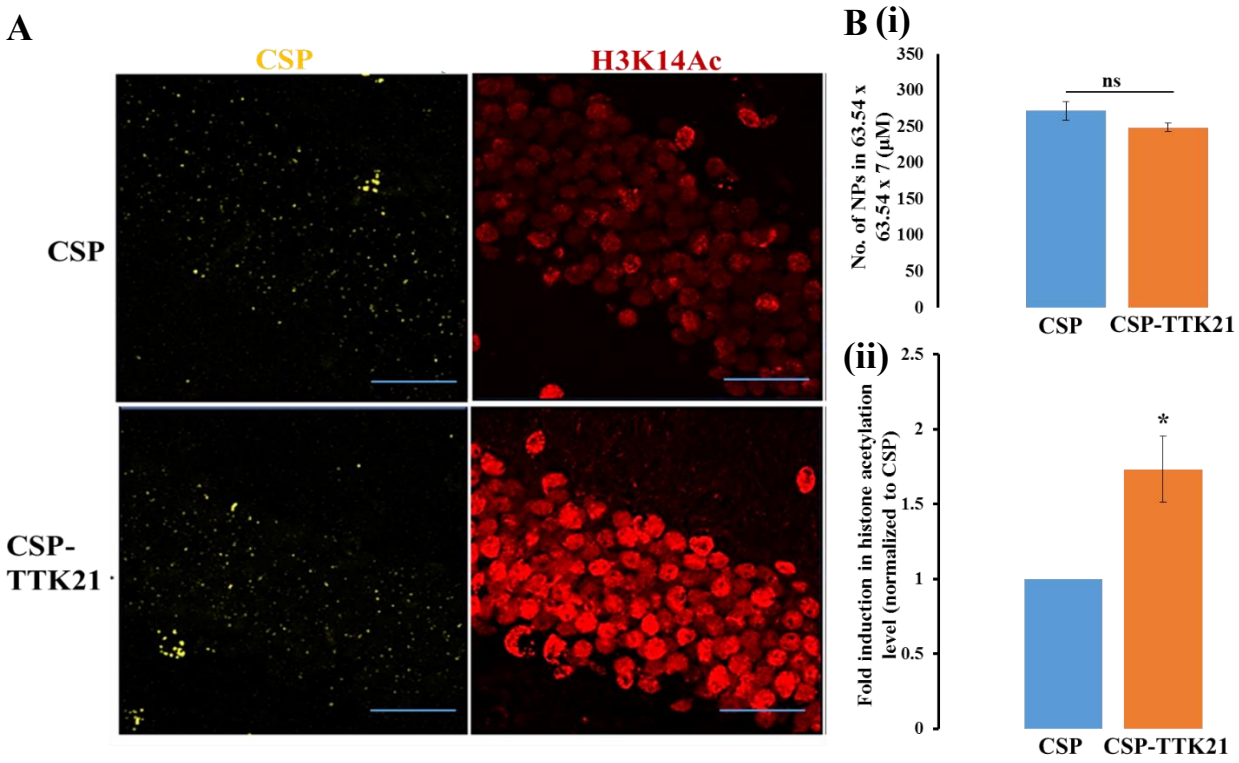

Figure 3.4. Presence of fluorescence CSP particles in Dentate Gyrus region. A 63X zoomed image of a specific region of dentate gyrus, depicting presence of fluorescence CSP particles and increase histone acetylation upon oral gavaging (A). (B) Quantitation showing significant increase in H3K9 acetylation level in CSP-TTK21 treated mice as compared to CSP control (ii), with no significance difference in the number of CSP particles in CSP and CSP-TTK21 treated mice (i). Scale bar 50µm. Error bars represent SEM. Student's t-test was done for significance test and the p-value obtained, ***p<0.05**.

3.4. CSP-TTK21 does not alters basal synaptic transmission:

This specific modification (acetylation) of lysine14 residue on histone H3 is previously been reported to occur during activation of some neuronal receptors such as dopamine receptor, muscarinic-acetylcholine receptor and glutamate receptor [170]. Thus, we went on to check the effect of this molecule on basal synaptic transmission, and long-term potentiation (LTP) in these mice by extracellular field recordings from hippocampal region (Schaffer collateral pathway). By input-output analysis(for a given action potential an equivalent response from post-synapse), we found that this molecule did not alters basal synaptic transmission in CSP-TTK21 treated mice with respect to CSP control as can be seen from the FV vs fEPSP plot (fig 3.5D). This suggests that the molecule did not affect the basal synaptic transmission.

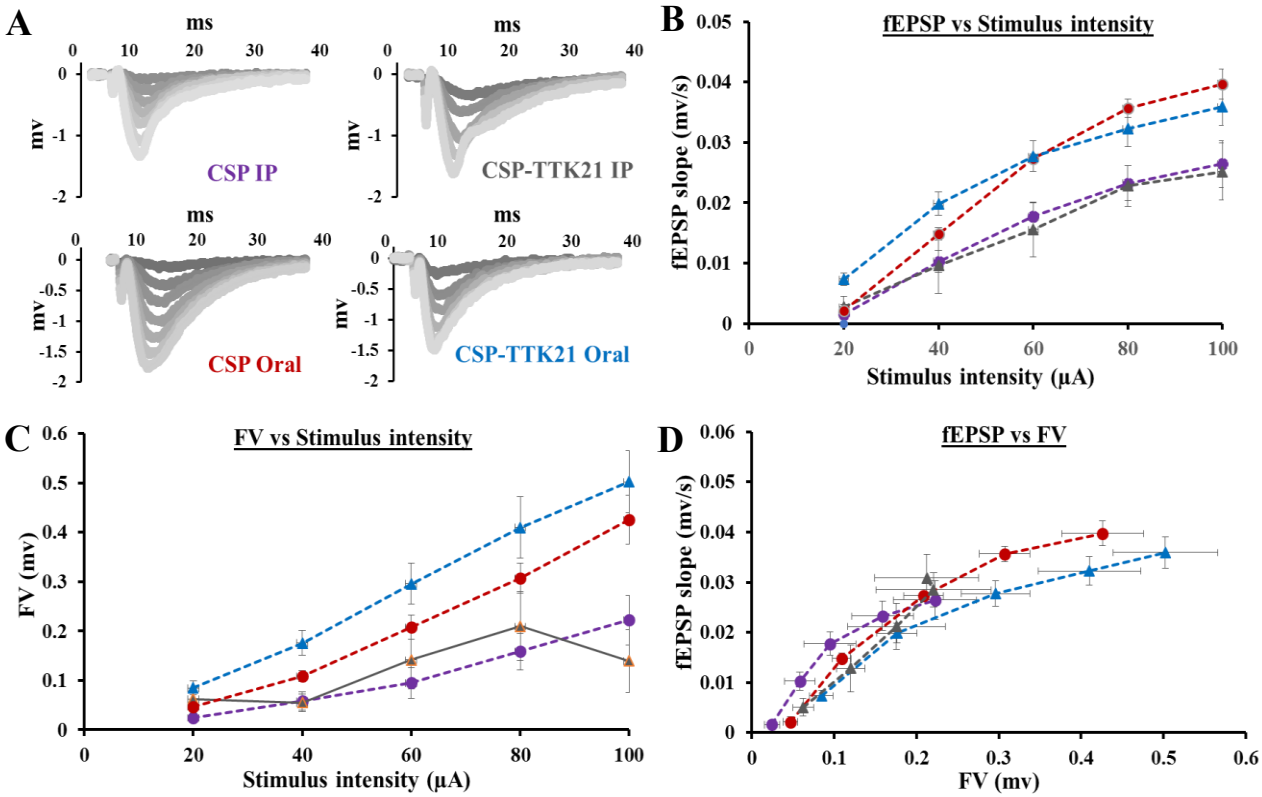

Figure 3.5. CSP-TTK21 does not alters basal synaptic transmission. (A) Sample traces for input-output curve analysis for each group. (B) FV vs Stimulus intensity (C) fEPSP vs Stimulus intensity showing increase in FV amplitude and fEPSP slope with increasing stimulus intensity. (D) FV vs fEPSP curve depicting there is no alteration in basal synaptic transmission upon CSP-TTK21 treatment. Error bar represents SEM.

3.5. CSP-TTK21 induces LTP in mice hippocampal slices:

Next, we wanted to check the effect of this molecule on LTP, we found that this molecule significantly induces long-term potentiation in mice treated with single dose of CSP-TTK21 as compared to mice treated with CSP only. Interestingly, when we compared the magnitude of LTP induction in both cases (oral and IP administration) the magnitude of LTP induction was remarkably similar in both cases (fig 3.7Aand B). This result further validate our previous observation that Oral delivery of CSP-TTK21 is as efficient as IP injections. These results show that CSP-TTK21 can cross the BBB upon oral delivery, and could induce histone acetylation that does not alter basal synaptic transmission but significantly induce long-term potentiation (LTP). Since the major motif of our study is to bring this molecule into the clinical trials followed by its

usage as a drug for human neurodegenerative and neuro-developmental disorders, the findings suggests that CSP-TTK21 could be delivered orally to the brain.

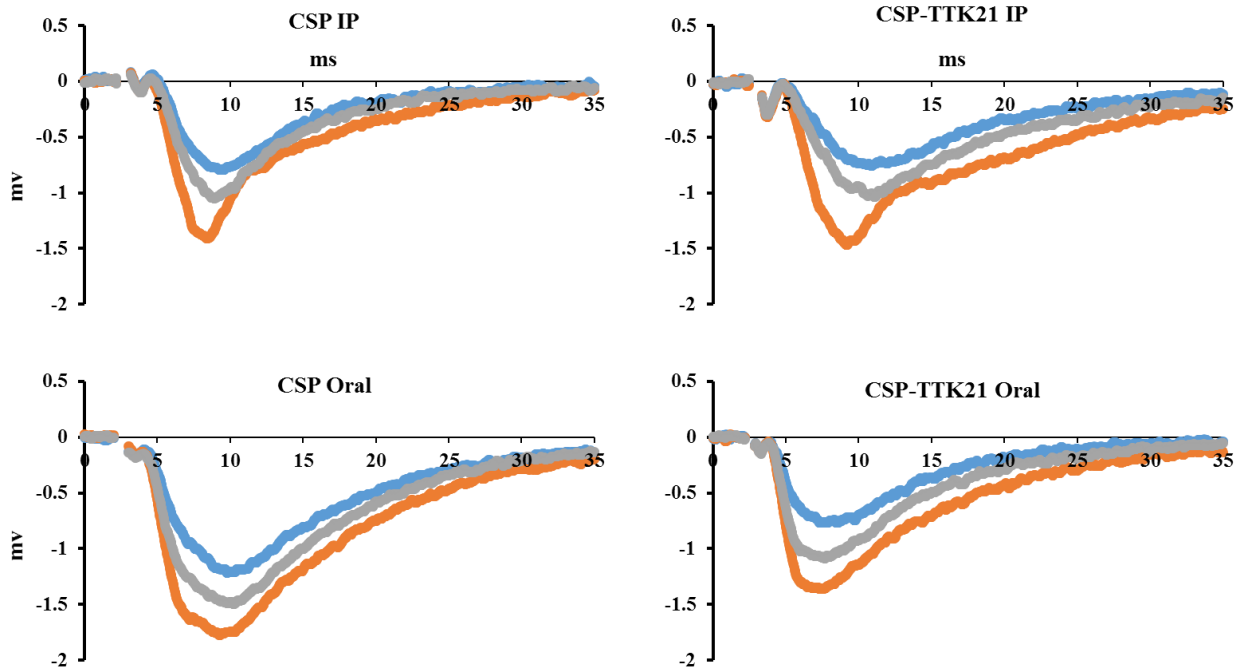

Figure 3.6. Representative sample traces for each group, taken 1 min before (blue), 1 min after (orange) and 35 min after (grey) LTP induction.

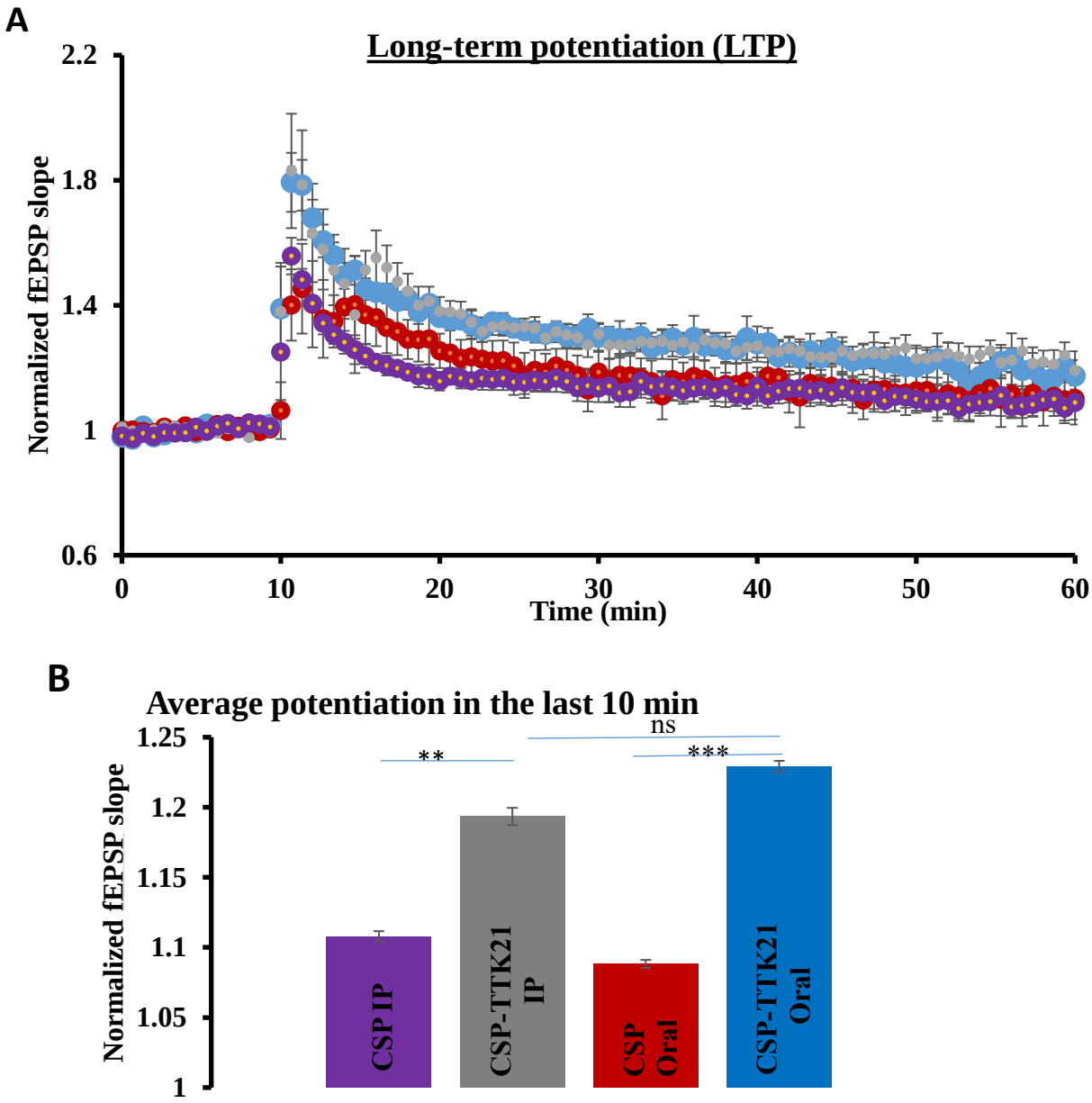

Figure 3.7. CSP-TTK21 induces long-term potentiation in mice hippocampal slices. Mice were treated with CSP or CSP-TTK21 either through Oral Gavaging or IP injections and extracellular recordings were done from hippocampal slices (n=6 for each group) after 84 hours of treatment from Schafer collateral pathway. LTP was induced by 1 train of 100Hz stimulation for 1 sec. (A) Induction of LTP upon CSP-TTK21 treatment both with oral and IP injection. (B) Quantitation showing significant increase in potentiation during the last 10 min of recordings in CSP-TTK21 treated group. Error bars represent SEM. Two way Anova (repeated measures) was done for significance test and the p-value obtained, **p<.05 (*), P<.01 (**) and p<.001 (***).**

CHAPTER 4

Therapeutic Potential of CSP-TTK21 in Syngap1 Heterozygous mouse model for Intellectual disability

This Chapter briefly describes about the role of SYNGAP1, a synaptic RAS-GAP in synaptic plasticity and cognitive processes. It also describes about the pathogenic mutations of the gene encoding this protein and its impact on dendritic spine maturation and its involvement in Intellectual disability. Here in this chapter, we have investigated the therapeutic potential of CSP-TTK21 in a Syngap1 heterozygous mouse model for intellectual disability (ID). Our initial results show that CSP-TTK21 rescues LTP deficits associated with these mice and could be a potential therapeutic option for treating ID.

Outline of the chapter:

4.1. *SYNGAP1*: A synaptic RAS-GAP

4.2. p300/CBP: Role in synaptic plasticity and cognitive processes

4.3. CSP-TTK21 induces histone acetylation in SynHet mice

4.4. CSP-TTK21 rescues LTP deficits associated with SynHet mice

4.5. CSP-TTK21 acts at post-synapse:

4.1. *SYNGAP1*- A synaptic RAS-GAP:

SYNGAP1 is a synaptic activator of RAS-GAP (GTPase activating protein) highly enriched at dendritic spines of excitatory neurons, which plays a crucial role in synaptic plasticity during learning and memory process [163, 171]. SYNGAP1 acts downstream of NMDAR (N-methyl D-Aspartate receptor) where it suppresses the signaling pathways associated with NMDAR mediated long-term potentiation (LTP) and synaptic plasticity [172]. It acts downstream of NMDAR and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). At basal condition, it suppresses NMDAR mediated signaling pathway by inhibiting the downstream protein kinases such as ERK, MAPK [160]. During and after LTP induction, it gets phosphorylated by CaMKII and dispersed from the dendritic spines of excitatory neurons, leading to activation of downstream protein kinases important for LTP and synaptic plasticity. Therefore, it seems SYNGAP1 imparts negative role on

synaptic plasticity and LTP. However, SYNGAP1 heterozygous knock out mice show deficits in LTP and synaptic plasticity, which is contrasting. This contrasting role can be explained by recent studies that show that impairment in LTP and cognitive behavior in these mice is due to imbalance of the excitatory and inhibitory neuronal activity which is required for proper synaptic function [164, 166, 173]. In these mice, excitation precedes over inhibition leading to accelerated maturation of spines which shifts the critical period of dendritic spine development by 1 week, i.e., dendritic spines mature 1 week before than their normal developmental time [164]. Mutations in *SYNGAP1* had been reported in several patients with intellectual disability (ID) [158, 161]. These patients have severe impairment in learning and cognitive processes as reflected by their lower IQ levels (below 70). Moreover, the number of ID cases resulting from *SYNGAP1* mutations had highly increased in the past one decade. In current time, there is no therapeutic option available for these patients. Recently, one group have shown that in adulthood even genetic rescue of *Syngap1* in *Syngap1* heterozygous mouse model did not rescue the deficits in behavioural and cognitive processes, even after showing rescue of the LTP deficits [166]. Hence identification of new therapeutic options for treating Syngap1-related intellectual disability is currently a new field.

4.2. p300/CBP: Role in synaptic plasticity and cognitive processes

It is well-established now that NMDAR mediated activation of CREB transcription factor plays a crucial role during synaptic plasticity and long-term memory (LTM) formation [115, 174]. This requires activation of several protein kinases downstream of NMDAR such as ERK, MAPK and CaM kinase IV (CaMKIV). Several studies suggest that CREB recruits CBP, a transcription co-activator at promoters of specific neuronal genes (such as c-Fos and BDNF) reviewed in [175]. And there are reports that show CBP play important role in NMDAR-dependent transcription of CREB regulated immediate early genes [176]. Molecular studies show that CaMKIV activate transcriptional activity of CBP by phosphorylating CBP at S301 [177]. In addition to this, p300/CBP are essential KAT for neural development and reported to induce differentiation and maturation of neurons [47]. Moreover, p300 and CBP also play critical roles in memory and cognitive processes. Transgenic mice with inhibitory truncated form of p300 or sub-region specific conditional knock-out of p300 show deficits in cognition and long-term memory retention [117, 118]. Reduced expression and heterozygous mutations in p300/CBP have been reported in patients suffering from Rubinstein-Tyabi syndrome (RTS) [106, 111, 178], a type of intellectual disability

characterized by growth defects, severe deficits in cognition and synaptic plasticity. Using mice model for RTS, several studies show that treatment with KDAC inhibitors such as TSA and SAHA significantly recuses deficits in cognition and synaptic plasticity [120, 178]. These data suggest that p300/CBP play an important role in synaptic and cognitive processes. Hence, modulating the activity of this transcriptional coactivator could be a potential therapeutic option for *SYNGAP1*-related ID. However, human trials with these inhibitors are lacking due to their pleiotropic effects and limited specificity. Thus, there is need for alternative strategies to target such disorders that occurs due to dysregulation of KATs. One such alternative approach is focused on identifying and utilizing KAT activators. Recently some KAT activators have been identified (discussed previously in chapter 1) but due to their cell-impermeable nature there is lack of in-vivo studies, utilizing these activators for treatment of this type of disorders. Previously, our laboratory have synthesized and characterized a small molecule KAT activator (CSP-TTK21) that specifically activates p300/CBP KAT activity. In-vivo studies show that this molecule is cell-permeable and efficiently induces histone acetylation. Upon intra-peritoneal injection in mice, this molecule could be delivered to the brain without apparent toxicity and induces adult neurogenesis via increased histone acetylation. In addition to this, CSP-TTK21 also extends duration of long-term spatial memory. The work discussed in the previous chapter, show that this molecule could be delivered to the brain through oral gavaging and is almost as effective as IP injection in inducing histone acetylation and LTP. In this chapter we are focusing on the effect of the CSP-TTK21 in a mouse model of ID (SynHet mouse) through oral delivery.

4.3. CSP-TTK21 induces histone acetylation in SynHet mice:

Previously we have reported that CSP-TTK21 induces histone acetylation majorly in dorsal hippocampus and frontal cortex region of the brain. Because hippocampus is one of the site where adult neurogenesis occurs and it also plays critical role in most of the learning and memory processes. Therefore, here we have focused specifically on different histone acetylation marks in dorsal hippocampus. Mice were treated with single dose (20 mg/Kg) of CSP or CSP-TTK21 and sacrificed after 84 hours of treatment followed by lysate preparation from hippocampus and cortex region of the brain. Upon western blotting, we found significant increase in histone acetylation level (H3K14Ac) in hippocampus of mice treated with CSP-TTK21 (either WT or SynHet) as compared to CSP-treated mice, we observed almost 2 and 4 fold increase in histone acetylation

level in SynHet and WT mice respectively (fig 4.1).However, there was no significant fold change in histone acetylation marks in the cortex region of mice treated with CSP-TTK21.

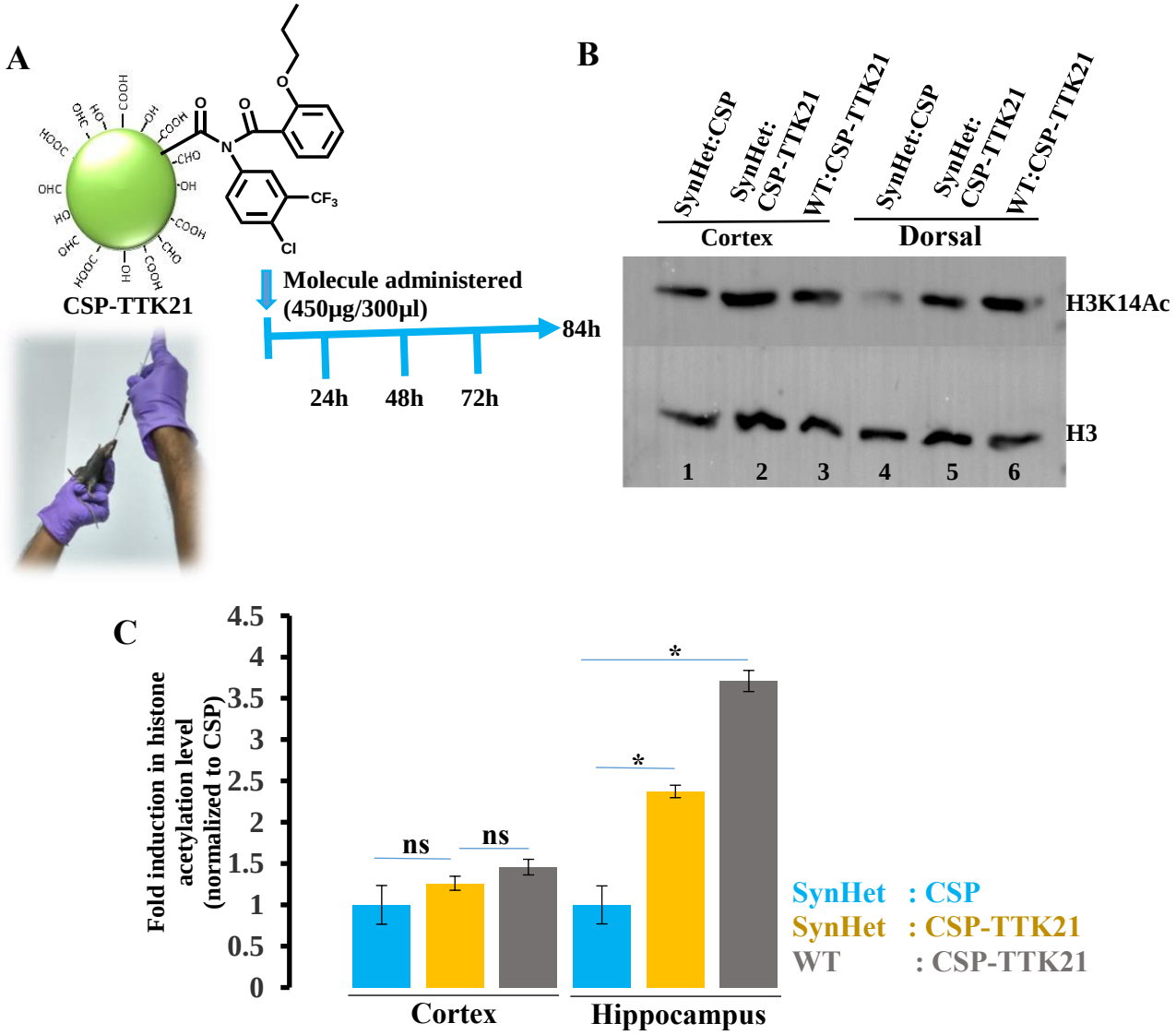

Figure 4.1. Western blotting from mice lysate. (A) Schematic showing the timeline for treatment (3 mice for each group). (B) CSP-TTK21 treatment increases histone acetylation in hippocampal region and not in cortex. Lanes1 and 4- SynHet: CSP; lanes2 and 5- SynHet: CSP-TTK21; and lanes3 and 6- WT: CSP-TTK21. (C) Quantitation showing significant increase in H3K14Ac level in hippocampal region**.** Error bars represent SEM. Student's t-test was done for significance test and the p-value obtained, ***p<0.05, **p<0.01 and ***p<0.001.**

This observation was further validated by performing immunohistochemistry from another batch of mice treated with CSP and CSP-TTK21. Mice treated with CSP-TTK21 showed increased histone acetylation marks such as H3K14, H4K12 (figure 4.2A) but no change in H3K9 mark as

compared to CSP control (fig 4.2B). Thus, these results demonstrate that CSP-TTK21 can induce site-specific histone acetylation in SynHet mice.

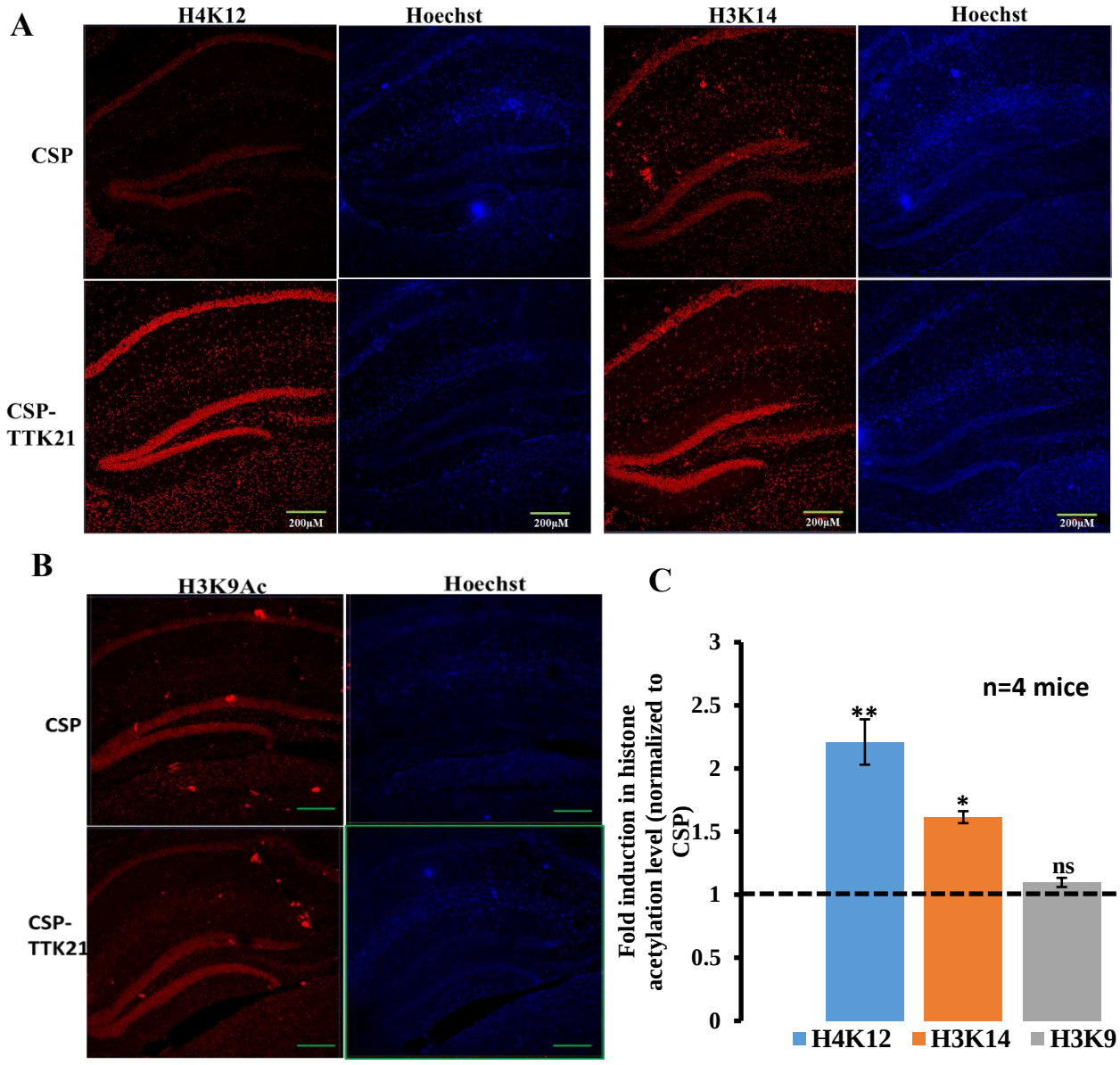

Figure 4.2. Increased histone acetylation in dorsal hippocampus of CSP-TTK21 treated mice. Immunohistochemistry was performed on 20µm thick sections with anti-acetylated antibodies (H3K14, H4K12, and H3K9). Increase in acetylation was observed for H4K12 and H3K14 marks (A) but not for H3K9 (B). Quantitation for fold induction in histone acetylation level (C). Error bars represent SEM. Student's t-test was done for significance (n=4 mice) and the p-value obtained, ***p<0.05, **p<.01.**

4.4. CSP-TTK21 rescues LTP deficits associated with SynHet mice:

Next we went on to check the effect of CSP-TTK21 on memory processes through electrophysiological analysis. One such analysis is LTP induction a phenomenon that leads to persistent strengthening of synapses in response to high frequency stimulation. It is thought to be a direct correlate of molecular mechanism underlying long-term memory. *Syngap1* het mice show deficits in LTP induction. Therefore, we started a pilot experiment where we treated these mice with a single dose of either CSP or CSP-TTK21 and sacrificed after 84 hours followed by extracellular field recordings from the hippocampal region especially from the Schaffer-collateral pathway. Significantly, we observed that CSP-TTK21 treatment could restore the LTP deficits in these mice. Thus, for further studies we have used the same set of dose and treatment time. We found that a single dose of CSP-TTK21 significantly restored the LTP deficits associated with these mice as compared to CSP and saline control which show complete LTP deficits as reported previously (fig 4.4). Previously, it has been reported that adult restoration of Syngap1 rescues the LTP deficits in these mice but didn't improve behavioural, social and cognitive abnormalities associated with these mice. Therefore, further experiments have to be done to check the rescue of behavioural, social and cognitive deficits associated with these mice model to verify whether CSP-TTK21 could acts a potential drug for treatment of Syngap1-related IDs.

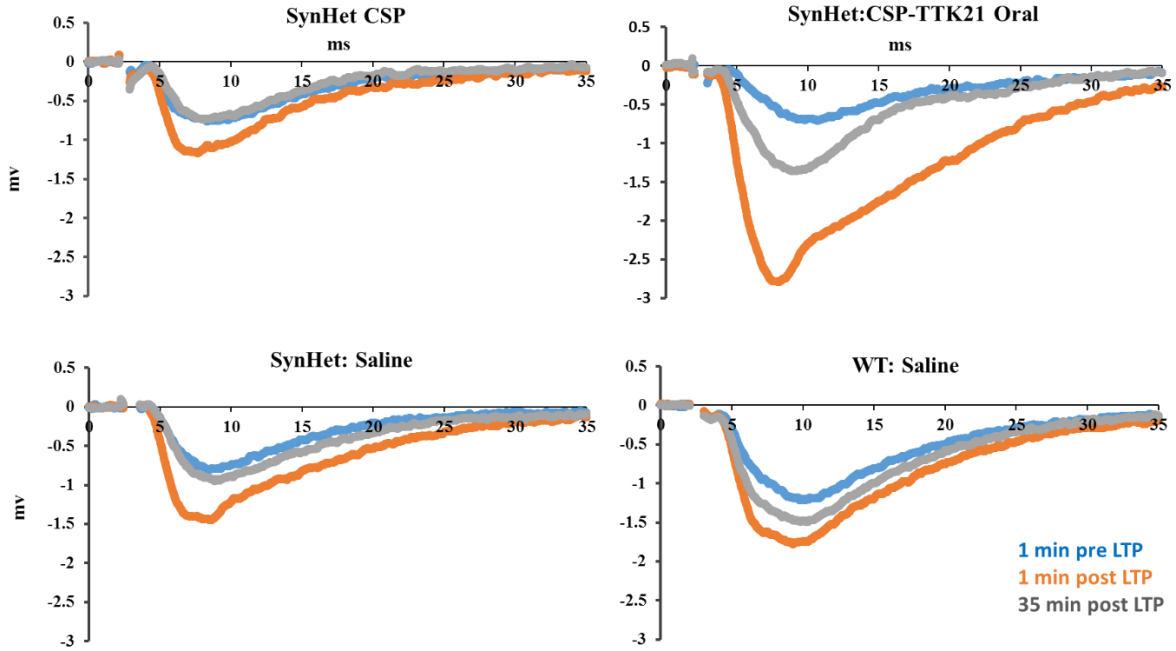

Figure 4. 3. Representative sample traces for each group, taken 1 min before (blue), 1 min after (orange) and 35 min after (grey) LTP induction.

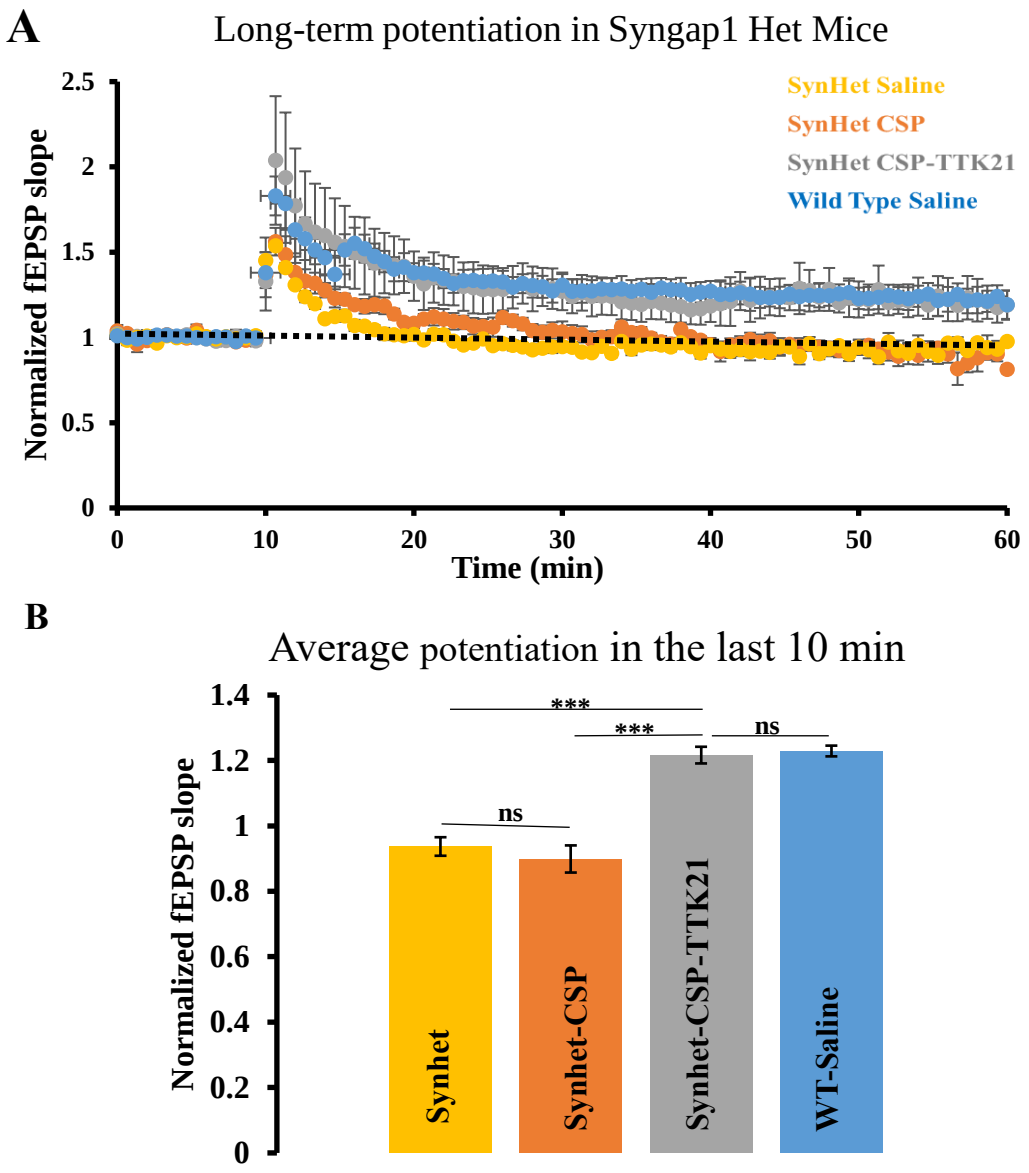

Figure 4.4. CSP-TTK21 rescue LTP deficits in SynHet mice. Mice were treated with CSP or CSP-TTK21 through Oral Gavaging. After 84 hours of treatment extracellular recordings were done in Schafer collateral pathway from hippocampal slices (n=6 for each group except n=4 for SynHet saline control). LTP was induced by 1 train of 100Hz stimulation for 1 sec. (A) Induction of LTP upon CSP-TTK21 treatment in SynHet as well as WT saline. (B) Quantitation showing significant increase in potentiation during the last 10 min of recordings in CSP-TTK21 treated and WT group. Error bars represent SEM. Two way Anova (repeated measures) was done for significance test and the p-value obtained, ***p<0.05, **p<0.01 and ***p<0.001.**

4.5. CSP-TTK21 acts at post-synapse:

There are billions of neurons in the nerves system that forms trillions of connections called synapses to pass electrical/chemical signal from one neuron to another. Hence these synapses are essential for proper signal propagation and neuronal function. These synapses are not continuous/direct connections, but are formed when the plasma membrane of the signal passing neuron (pre-synapse) comes into close proximity of the target neuron (post-synapse). These synapses play important role during memory processing. There is extensive modulation of these synapses during information processing, and strengthening of these synapses between neurons are linked with storage of information, resulting in memory. This phenomenon is termed as long-term potentiation (LTP). Since CSP-TTK21significantly induces LTP in SynHet mice, we next wanted to see whether this molecule is acting at pre-synaptic level or post-synaptic level. Paired-pulse facilitation (PPF) is a phenomenon in which postsynaptic potentials (EPSPs) evoked by an impulse are increased when that impulse closely follows a prior impulse. It measures the pattern of neurotransmitter release from presynaptic neurons when two stimuli are given simultaneously at an increasing interval. Therefore, the phenomenon of PPF is directly linked to pre-synaptic effect. Previously it has been shown that CBP/p300 act downstream of MEK-ERK pathway in the post-synaptic neurons and could get activated by kinase mediated phosphorylation leading to increased transcriptional activity, eventually resulting in expression of genes involved in long term memory formation. As expected, we did not find any significant variation in PPF in CSP-TTK21 mice as compared to CSP and saline controls (fig 4.5). Thus, CSP-TTK21 mediates its action at post-synaptic level.

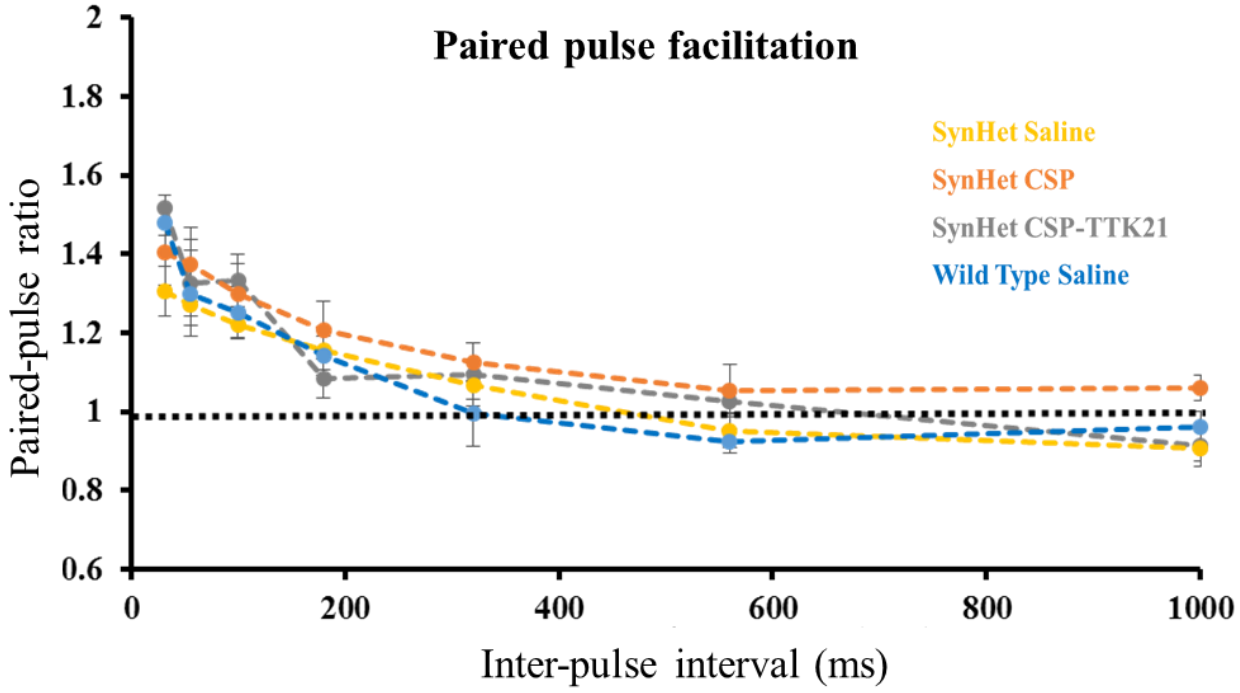

Figure 4.5. No alteration in PPR upon CSP-TTK21 treatment. Two presynaptic spikes were evoked simultaneously at different time interval and the ratio of the two post-synaptic response (fEPSP2/fEPSP1) were measured and plotted against time interval. No significant alteration was seen in paired pulse facilitation, $p>0.06$. Significance was tested by Two-way Anova (repeated measures). Error bars represent SEM. n= 4 slices.

CHAPTER 5

Summary, Discussion and Future perspectives

This chapter briefly discusses and summarizes all the results obtained during this thesis work. It also sheds light on the future work that have to be done in this field to further validate our results.

Summary of the entire work:

1. CSP-TTK21 could be delivered orally to the mice brain.
2. Oral delivery of CSP-TTK21 shows similar activity as IP administration.
3. CSP-TTK21 induces Long term potentiation (LTP) in hippocampal slices without altering the basal synaptic transmission.
4. CSP-TTK21 could rescue LTP deficits in Syngap1 heterozygous mouse model for Intellectual Disability (ID).

Discussion:

Previously, we have shown that CSP-TTK21 is a p300/CBP specific KAT activator and upon intra-peritoneal (IP) injection in mice stimulates adult hippocampal neurogenesis and extends long-term memory retention (spatial memory) without any apparent toxicity. Therefore, we speculate that this molecule could acts as a therapeutic drug for several neurological disorders characterized by cognitive and memory deficits. Thus, this thesis work is an attempt to understand the therapeutic potential of this KAT activator (CSP-TTK21) in neurodegenerative and neurodevelopmental disorders. For this purpose here we have addressed two important questions; (i) what happens to this molecule upon oral delivery will it cross the BBB and mediate its action? And (ii) its therapeutic potential in an intellectual disability (ID) mouse model (SynHet). BY utilizing different techniques, we found that CSP-TTK21 could be delivered to the brain orally, and could induce histone acetylation and long-term potentiation (LTP) in hippocampal region of WT mouse. Further, when we compared the effect of CSP-TTK21 in terms of its activity and function upon Oral Gavaging and IP administration; significantly, we found that in both cases the magnitude of its action was almost similar. The 3rd chapter shows that upon Oral Gavaging CSP-TTK21 could be delivered to the brain and is as active as upon IP administration. This is the first study where

we are reporting the direct role of CSP-TTK21 in synaptic plasticity through LTP induction. These all studies show that CSP-TTK21 has immense therapeutic potential for various neurological disorders and could be delivered orally.

The 4th chapter deals with the work related to therapeutic potential of CSP-TTK21 in SynHet mouse model for ID. As described previously in 4th chapter, SynHet mouse have imbalance in their excitation/inhibition neuronal activity where excitation precedes inhibition resulting in accelerated neuronal maturation/ development that leads to premature closure of critical period of development which results in failure of cognitive and behavioural maturation. In addition to this, these mice lack LTP induction upon high frequency stimulation. In this work, we found that mice treated with a single dose (450µg) of CSP-TTK21 have significant increase in histone acetylation especially in the dorsal hippocampus. Next, we found that CSP-TTK21 could rescue the LTP deficits associated with these mice at WT level but does not alters basal synaptic transmission/activity. PPF analysis gave us a hint that CSP-TTK21 is acting at the post synaptic level. Recent work shows that adult restoration of SYNGAP1 protein could completely rescue this deficits in LTP, but does not rescue the behavioural, social and cognitive abnormalities associated with these mice. Since CSP-TTK21 rescues LTP deficit in these mice, it will be interesting to see its effect on behavioural, social and cognitive deficit, as well as dendritic maturation. However, further experiments to be performed to elicit the molecular mechanism underlying this rescue of LTP deficits. Recently, one studies had shown that Syngap1 plays an important role in development of GABAergic neural connection and haploinsufficiency of Syngap1 results in impairment of inhibitory GABAergic synapse connection leading to cognitive defect. Here, we proposed that CSPTTK21 could be inducing expression of some neuronal genes crucial for development of GABAergic inhibitory synapses which restores the balance between excitatory/inhibitory neuronal activities eventually resulting in restoration of LTP in SynHet mice model.

Future perspectives:

Dysregulation of Lysine acetyltransferases (KATs) in several neuro-developmental and neurodegenerative disorders is a well-established field. But understanding the therapeutic potential of small molecule KAT modulators in neuropathologies is an emerging field. This is the first study

that show CSP-TTK21 a p300/CBP specific KAT activator could be a potential therapeutic option for patients suffering from Intellectual disability, a neurodevelopmental disorder. But several studies such as rescue of behavioural abnormalities, gene expression changes will be done to further validate and decipher the molecular mechanism underlying the positive effect of this molecule in ID. For that we planned to do some behavioural studies which are as follow: (a) Water Maze: for spatial memory, (b) T or Y maze: for reference memory, (c) Fear conditioning: for associative learning, (d) Open field: for locomotor activity and anxiety, (e) Three chamber assay: for social interaction.

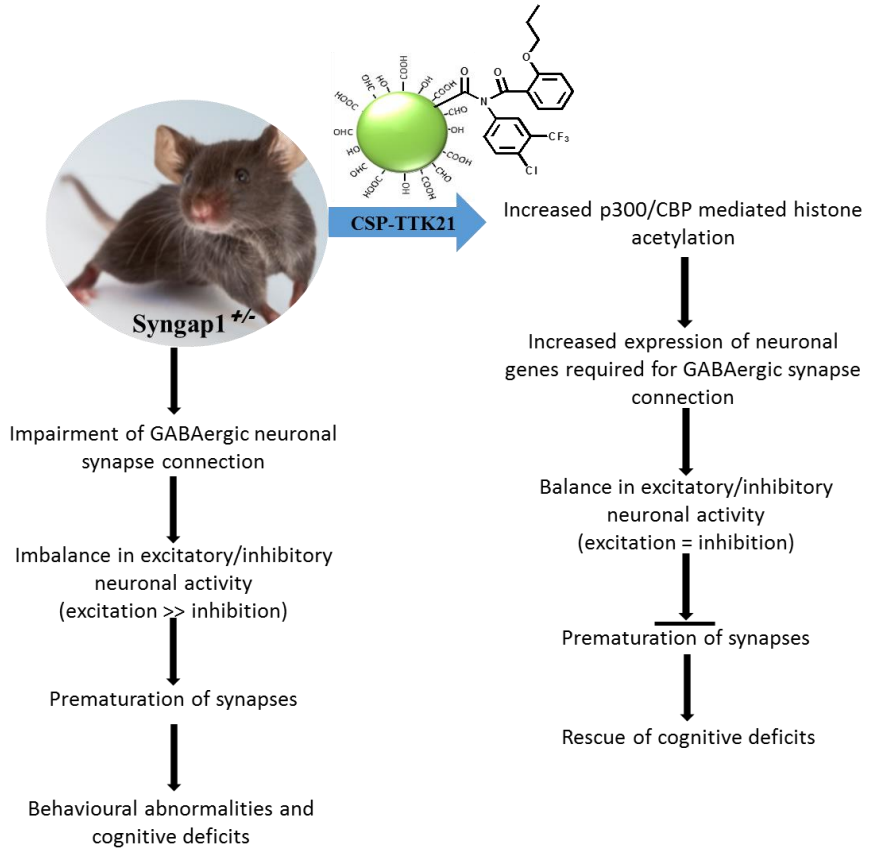

Figure 5.1. A proposed model for restoration of cognitive deficits through CSP-TTK21 in SynHet mice model.

We have planned to do whole genome RNA-seq to check the changes in overall gene expression which will help us to decipher the molecular network through which this molecule is acting. In addition to this, we will also be doing some biochemical and cytotoxicity assay to check the kinetics of CSP-TTK21.

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