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ROLE OF ACTIVATED TRANSCRIPTION FACTOR 4 (ATF4) IN LEARNING AND MEMORY

Coordinator: Professor Guido CAVALETTI

Tutor: Professor Giovanni TREDICI

Doctoral thesis of:

Silvia PASINI

061480

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to Marta

my constant source of inspiration

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ABSTRACT

The aim of this study is to understand the role of Activated Transcription Factor 4 (ATF4) in the processes of learning and memory. The topic of learning and memory has always aroused great interest from time immemorial and although a lot of researches have been focused on this subject for a long time, many mechanisms have not yet been fully understood.

Identifying the players and the mechanisms involved in learning and memory is of utmost importance because deficits in these cognitive functions are symptoms of common neurological diseases like stoke, depression, dementia and Alzheimer's disease, one of the most wide spread neurodegenerative disease.

It has already been established that new gene expression and protein synthesis are required for long term memory, providing the basis to think that transcription factors may play a key role in these processes. Several studies have demonstrated the involvement of different transcription factors in memory formation such as cAMP response element binding protein (CREB), CCAAT enhancer binding protein (C/EBP), activated protein 1 (AP1), early growth response factor (Egr) and Rel/nuclear factor kB (Rel/NFkB).

Very little is known about the involvement of another transcription factor, Activated Transcription Factor 4. ATF4 is a member of the activated transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family. It was originally described as a repressor of CRE-dependent gene transcription but recent studies have shown it to be a transcriptional activator. It is also a stress responsive gene, regulating the adaptation of cells to stress stimuli such as anoxia, endoplasmic reticulum stress and oxidative stress. ATF4 plays an essential role in development, and is particularly required for proper skeletal and eye development and is also involved in tumor progression and metastasis.

ATF4 has always been reported as a memory repressor that blocks new gene expression required for memory formation but no study has ever investigated it in a specific and direct way. The aim of this thesis is to study, in a specific and direct manner, the role of ATF4 in the processes of learning and memory. To reach this goal, ATF4 expression was modified in mouse hippocampi, the brain region

mainly involved in learning and memory, with the injection of lentivirus carrying ATF4 gene, for the gain-of-function analysis, and lentivirus carrying shATF4, for the loss-of-function studies.

Before starting the experiments of ATF4 overexpression and downregulation, preliminary experiments were conducted to set up the injection coordinates to target the mouse hippocampi, to verify the lentiviral tropism and most importantly to evaluate the lentiviral spread, within the hippocampus, after the injection.

The consequence of ATF4 gain- and loss-of-function was then studied in the performance of standard behavioral tests such as Water Maze tests and Fear Conditioning, widely used to assess spatial and associative memory respectively. The behavioral test results showed that ATF4 protein overexpression enhances spatial memory, under the weak training paradigm in the Morris Water Maze test, and associative memory while ATF4 downregulation impairs spatial memory under the standard training condition.

After completing the behavioral tests, ATF4 overexpressed and downregulated mice were subjected to electrophysiological and neuronal spine analysis to verify if the alteration in cognitive functions, as a result of ATF4 modification, is supported by changes in synaptic potentiation and spine density and morphology.

Long Term Potentiation (LTP) is a long lasting enhancement in neuronal transmission and is widely considered as a cellular model of learning and memory in the central nervous system. The long-term memory impairment of ATF4 downregulated mice is supported by electrophysiological analysis, in which ATF4 downregulated slices showed an impairment in LTP. Unexpectedly, LTP impairment was also found in ATF4 overexpressed slices, maybe due to the difference in the time between the injection and the behavioral tests or the electrophysiological recordings.

Most of the intracellular pathways responsible for LTP require new gene expression and protein synthesis. This, in turn, leads to morphological changes required to sustain the enhancement of signal transmission. One of these morphological changes is the modification of the density and the morphology of dendritic spines. ATF4 up- and downregulation in hippocampal neurons does not affect spine density but ATF4 overexpression causes a significant increase in the percentage of mushroom spines as compared to that found after ATF4 downregulation. Mushroom spines with a large head are the most stable neuronal spines and contribute to strong synaptic connections, hence it has been hypothesized that they represent the "memory spines".

Collectively, these results support the hypothesis that the transcription factor ATF4 plays a positive role in synaptic plasticity and memory formation. Further studies need to be done to understand the molecular mechanisms through which ATF4 acts.

This thesis represents only a step on the road towards understanding the complicate mechanisms of learning and memory, not forgetting that the most important discoveries were the result of small knowledge acquired step by step.

CHAPTER I: INTRODUCTION

1.1. LEARNING AND MEMORY

1.1.1. HISTORICAL OVERVIEW

The topic of learning and memory has always aroused great interest from time immemorial. The study of memory started 2000 years ago with Aristotle when he tried to understand memory in his treatise "On the Soul". He compared the human mind to a white slate saying that humans were born without any knowledge and they are the sum of their experiences. In antiquity, it was generally assumed that there were two kinds of memory: "natural memory" (innate to a person) and "acquired memory" (developed through learning). This idea was expanded in the "art of memory" by Cicero and then passed down to the medieval Scholastics and later scholars of the Renaissance era like Matteo Ricci and Giordano Bruno.

Prior to the 19th century the philosophical approaches to study this subject used methods of introspection and logic to determine what was innate or acquired. The late 19th century brought for the first time quantitative study of mental processes. In 1885, Hermann Ebbinghaus published the monograph "On Memory" in which he described systematic measurement of memory in terms of accuracy, retention and capacity. In 1887, Sergei Korsakoff published his work on a syndrome that established the use of memory disorders as a means of studying mnemonic processes. "Principles of Psychology" written by William James in 1890 for the first time brought out the notion of the existence of different forms of memory, primary and secondary, that we now call Short-Term (STM) and Long-Term Memory (LTM) (Andrew Hudmon, 2005).

The 20th century was the "behaviorist era" during which scientists demonstrated systematic ways of modifying behavior through experience. At the beginning of the 19th century Pavlov published his work on classical conditioning, a reflexive type of associative learning in which a stimulus (bell sound: conditional stimulus) acquires the capacity to evoke a response (dog salivation) that was originally evoked by another stimulus (food: unconditional stimulus). In 1911, Thorndike demonstrated the principles of trial-and-error learning or instrumental/operant conditioning. When something causes animal's satisfaction, the animal draws an association between the behavior and positive outcome. This association forms the basis for later behavior (reinforcement). When the animal makes an error,

no association is formed between the behavior that led to the error and a positive outcome, so the ineffective behavior is less likely to recur (punishment) (see paragraph 1.1.4, Long Term Memory).

In addition to all these studies examining the principles of learning at the behavioral level there was an effort to identify the mechanisms responsible for learning and memory at the cellular level. In the 1890's Ramon Cajal suggested that structural changes in the brain might underlie learning and memory. He suggested that plasticity in the number and strength of neuronal connections is the basis of learning and consolidation of memory. Beginning in the 1920's Karl Lashley began studying the effect of removal of different amounts of cerebral cortex on maze learning. From his work the idea of "mass action" was formulated in which brain function was non-localized. In 1949, Donald Hebb published his most important work the "Organization of Behavior" in which he analyzed the processes of learning. His theory about how learning works became famous as Hebbian Theory and it is best expressed by this quote from his book:

"When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased".

This is usually summarized as "Neurons that fire together wire together. In 1957, Scoville and Milner published their work examining the patient's H.M. in which removal of regions of the medial temporal cortex led to profound loss in memory capabilities while sparing intellectual or cognitive abilities demonstrating clear functional heterogeneity within the brain in contrast to the earlier notions of Lashley. Subsequent work in the 1960's by Brenda Milner demonstrated that procedural memory in H.M. was also unaffected, further demonstrating that different types of memory could be maintained by different brain regions. In 1967, Seymour Benzer working in the fruit fly Drosophila used chemical mutagenesis to alter the function of individual genes implicated in behavior introducing for the first time molecular genetic approaches in the study of memory(Andrew Hudmon, 2005). These are only few of the most important authors whose studies during the last two centuries contributed significantly to our understanding of learning and memory. In the following part of the background I will discuss in more detail about recent discoveries of molecular mechanisms underlying these processes and in particular my attention will focus on the role of Activated Transcription Factor 4 (ATF4).

1.1.2 DEFINITIONS

Before discussing in detail the mechanisms that regulate learning and memory, I want to give some definitions concerning these two processes and briefly describe the anatomy of the hippocampus, the brain area mainly involved in learning and memory and the target of the experiments described in this thesis.

What is learning? In literature there are a lot of definitions of learning and the most inclusive one says that learning is the strengthening of existing response or the formation of a new response to existing stimuli that occurs because of practice or repetition (Hull 1943).

What is memory? According to Kandel, memory is a faculty of the mind through which the "information" is encoded, retained and recalled when needed. It is the ability to remember past experiences, and the process of recalling to mind previously learned facts, experiences, impressions, skills and habits. It is the store of things learned and retained from our activity or experience (Kandel et al., 2000).

Etymologically, the English word "memory" comes from the Middle English *memorie*, which comes from the Anglo-French *memorie*, which derives from the Latin *memor*, meaning "mindful" or "remembering". In more neurological terms, memory is a set of encoded neural connections in the brain. It is a reconstruction of past experiences by the synchronous firing of neurons that were involved in the original experience.

1.1.3. ANATOMY OF THE HIPPOCAMPUS

Hippocampus is part of the limbic system with cingulate cortex, olfactory cortex, anterior thalamic nuclei and amygdala. It is located in the forebrain in the medial temporal lobe beneath the cortical surface (Fig 1.1).
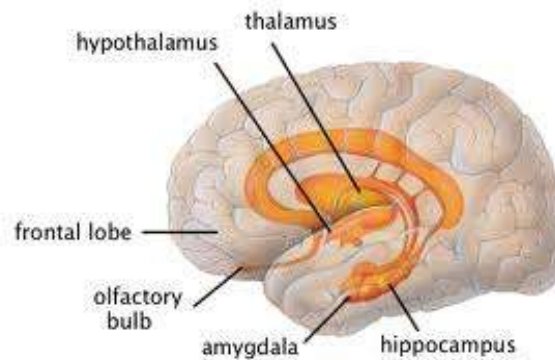
Fig 1.1. The limbic system.

Hippocampus is divided into two major parts: the fascia dentata with the area dentate and the dentate gyrus, and the hippocampus proper, called Cornu Ammonis (CA) for its shape, further subdivided into 4 different parts, CA1 through CA4. The Dentate Gyrus (DG) is mainly composed of small granule cells, while the CA areas are composed of pyramidal cells. The CA areas are connected with each other and also with the Subiculum, Presubiculum, Parasubiculum and the Entorhinal Cortex (EC) (Fig 1.2).
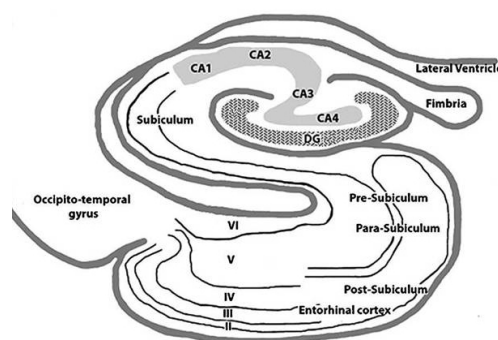
Fig 1.2. The hippocampus.

At cellular level the fascia dentata is divided into 3 layers:

- Granule layer, containing the cell body of the granule cells;

-        Molecular layer, with the apical dendrites of the granule cells and their afferents;

-        Polymorph layer, which contains the initial segment of the granule cell axons and the basket cells (interneurons).

Instead, the hippocampus proper is divided into 5 layers:

-        The alveus, which contains the axons of the pyramidal neurons that go towards the Subiculum;

-        The stratum oriens, that contains the basal dendrites of the pyramidal cells and some basket cells;

-        The stratum pyramidale, which contains the cell bodies of the pyramidal neurons;

- The stratum radiatum and moleculare, which contain the distal and the apical segment of the apical dendritic tree, respectively (O'Keefe and Nadel, 1978).

1.1.3.a. Afferents to the hippocampus

The hippocampal neurons receive afferents from other cells located in the same area. There are two types of interactions, a direct excitatory one and an indirect inhibitory one, mediated by an interneuron.

The hippocampal neurons also receive afferents from other areas of the brain. It has been demonstrated that the major interconnections between the different areas of the hippocampus are largely unidirectional with signals propagating from the 2nd layer of the EC to the DG (Fig 1.3). Granule cells of the DG send their axons to CA3 through Mossy Fibers. Pyramidal cells of CA3 send their axons to CA1 through the "Schaffer Collateral" fibers. The Schaffer collateral pathway is widely used for electrophysiological studies, in particular for the Long Term Potentiation (LTP, see paragraph 1.1.7). Pyramidal cells of CA1 send their axons to the Subiculum and deep layers of the EC. Subicular neurons send their axons out of the hippocampus to the EC (Fig 1.3).
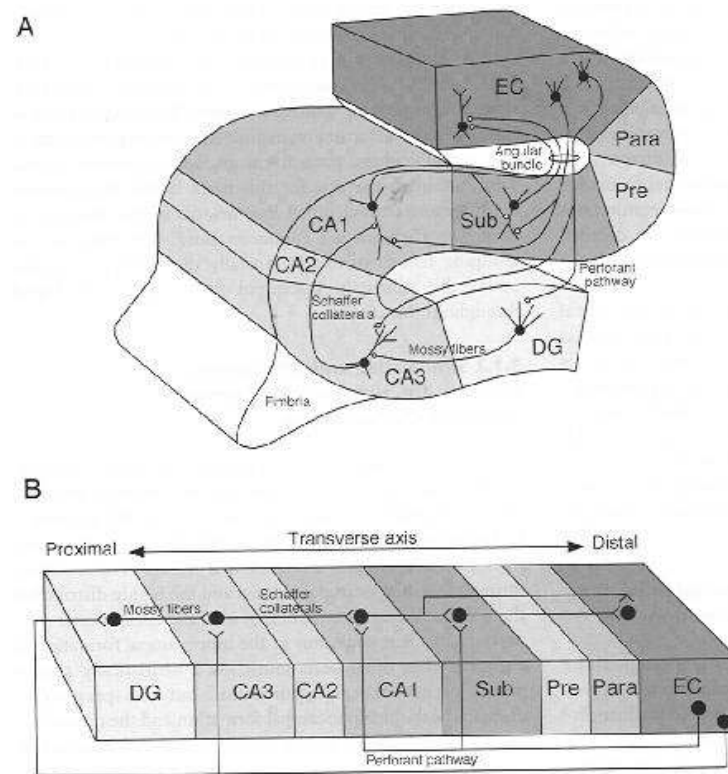
Fig 1.3. Hippocampal connections.

Moreover the hippocampus can receive extrinsic afferents not only from EC but also from other structures including the medial septal area and several brain-stem sites (O'Keefe and Nadel, 1978).

1.1.4. TYPES OF MEMORY

Human memory can be divided into Sensory Memory, Short Term Memory (STM) and Long Term Memory (LTM). Each of these types of memory has its own particular mode of operation but all of them cooperate in the process of memorization. In this modal-model of memory developed by Richard Shiffrin in 1968, memory can be seen as a sequence of different steps important for the formation of a lasting memory.
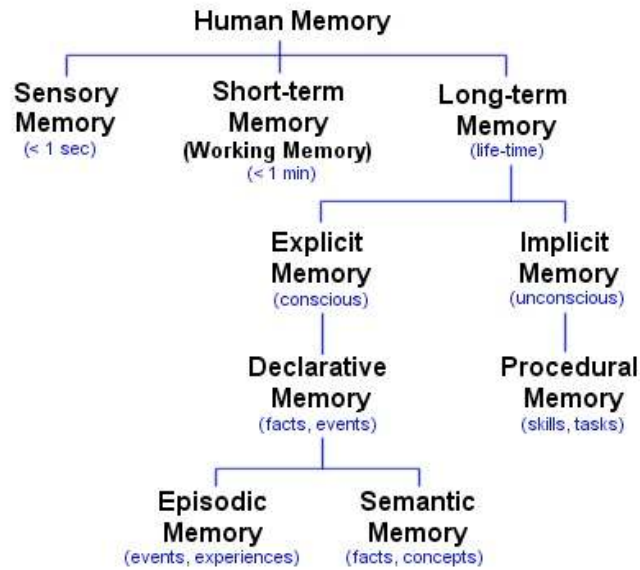
Fig 1.4. Classification of the different types of human memory (Diagram by Luke Mastin).

Sensory Memory is the shortest form of memory. It is the ability to store impressions of stimuli received through the five senses of sight, hearing, smell, taste and touch, which are retained accurately, but for very short time. It corresponds to the initial 200–500 milliseconds after an item is perceived. This type of memory is usually considered to be completely outside of conscious control. When an information is perceived, it is stored in sensory memory automatically and unbidden. Unlike other types of memory, the sensory memory cannot be prolonged via practice.

George Sperling (1960) was the first scientist who explored this form of sensory memory using the "partial report paradigm". He showed his subjects a grid of letters for 50 milliseconds. After this brief exposure, subjects were exposed to either a high, medium or low tone, to indicate them which rows to report. Based on these experiments, he suggested that the upper limit of sensory memory was approximately 12 items, but it degraded very quickly (within a few hundred milliseconds) (Alan D. Baddeley, 1997).

Short Term Memory (STM) is the kind of memory able to hold a small amount of information for a short period of time, typically from 10-15 seconds to 1 minute. One example of STM in action is remembering some information temporarily in order to complete a task. However, these information will disappear soon unless we make a conscious effort to store them. In this way STM is a necessary step towards the next stage of retention, the Long Term Memory.

Sometimes the term "working memory" is used instead of STM, although working memory represents more the structures and the processes used for the temporary storage and manipulation

of information. The limited capacity of STM was showed for the first time by George Miller in 1956. He described that a human being can hold in STM an average of 5-9 objects.

The part of the brain important for STM is located in the anterior part of the prefrontal cortex. It serves as temporary store where information are kept available for the current reasoning process but also as a place where to recall information from elsewhere in the brain. STM process requires neither new gene expression nor protein synthesis. It is based on the post-translational modification of existing proteins (Alan D. Baddeley, 1997).

Long Term Memory (LTM) is a storage of information for a long period of time. STM can become LTM after consolidation, through rehearsal and meaningful association. Physiologically, the establishment of LTM requires changes in the structure of neurons in the brain, this process is known as Long Term Potentiation (LTP), which will be discussed in detail later. When something is learned, circuits of neurons in specific areas of the brain are created, altered or strengthened.

Long Term Memory can be further divided into two types, explicit (or declarative) and implicit (or procedural). These different types of LTM are stored in different regions of the brain and undergo quite different processes. Declarative memories are encoded within the medial temporal lobe: hippocampus, entorhinal cortex and perirhinal cortex but they are consolidated and stored mainly in the temporal cortex. Procedural memories, on the other hand, do not appear to involve the hippocampus, and are encoded and stored by the cerebellum, putamen, caudate nucleus and the motor cortex, all of which are involved in motor control. Without the medial temporal lobe a person is able to form new procedural memories (for example how to ride a bicycle), but cannot remember the events during which it happened.

Explicit memory is the memory of facts (semantic) and events (episodic) that can be consciously recalled. Semantic memory, in particular, is a structured record of facts, meanings and knowledge about the world that we have acquired while episodic memory is our memory of experiences. It is the memory of autobiographical events, times, places, emotions and other contextual knowledge related to a particular event.

Semantic and episodic memories are stored in different brain areas. Semantic memory tends to involve frontal and temporal cortex while episodic memory is mainly correlated to the hippocampus (Alan D. Baddeley, 1997).

The hippocampus is the area where humans and animals form an internal cognitive map of their spatial environment. The idea of this map was proposed for the first time by Tolman in 1948 and two

decades later O'Keefe and Dostrovsky (1971) demonstrated that this cognitive map of space is created thanks to specific cells known as "place cells".

These "place cells" are pyramidal neurons that exhibit a high rate of firing when the animal is in a specific position in the environment and the population of these cells generates a spatial map of the place. More recent studies showed the involvement of other types of cells, located outside the hippocampus, important for spatial mapping: the grid cell (Fyhn et al., 2004), the head-direction (Muller et al., 1996) and the boundary vector cells (Lever et al., 2009). These cells are located in the medial entorhinal cortex and the subiculum and form other maps of the environment. A lot of studies, mainly performed in rats, have been conducted to understand the spatial representation system in the brain. All of these studies showed that these cells generate different dynamic maps representing different places, at different times, in different brain regions that interact to create a dynamic representation of self-location (Brown et al., 1998, Fuhs et al., 2005).

Implicit memory, on the other hand, is the unconscious memory of skills and how to perform tasks. Implicit memory can be further divided into associative and non-associative. Regarding associative memory there are two kinds of associative learning that have been well studied: the classical conditioning and the operant one.

In the classical conditioning there is an association between two stimuli. This kind of learning was demonstrated by Ivan Pavlov (1927) in an experiment in which he started presenting meat to a dog, and the dog started salivating (unconditioned response) whenever he saw the meat (unconditioned stimulus). Then before presenting the meat he started ringing a bell (conditioned stimulus). The dog started to associate the sound of the bell with meat and started salivating (conditioned response) whenever he listened to the bell even without seeing the meat.

Operant conditioning, term coined by Skinner B.F., forms an association between a behavior and the consequence for that behavior. Depending on the consequence of a certain behavior it is possible to modify the frequency with which that behavior occurs. Reinforcement and punishment represent the tools for operant conditioning. Both of them can be positive or negative giving a total of 4 basic consequences:

1. Positive reinforcement: occurs when a behavior is followed by a stimulus that is rewarding, thus increasing the frequency of that behavior.

2. Negative reinforcement (Escape): occurs when a behavior is followed by the removal of an aversive stimulus, thereby increasing that behavior's frequency.

3. Positive punishment: occurs when a behavior is followed by an aversive stimulus, such as introducing a shock or loud noise, resulting in a decrease in that behavior.

4.Negative punishment: occurs when a behavior is followed by the removal of a stimulus, resulting in a decrease in that behavior.

On the other hand, non-associative learning is one of the most basic forms of learning. It is also called single event learning. There are two well-known types of non-associative learning: habituation and sensitization.

1.1.5. HABITUATION

Habituation is a process in which there is a decrease in the response to a stimulus after repeated exposure to that stimulus over a duration of time. The learning underlying habituation is a basic process of biological systems and does not require awareness to occur. Habituation can result in a decrease in behavior, subjective experience, or synaptic transmission. The changes in synaptic transmission that occur during habituation have been well-characterized in the marine mollusk Aplysia californica (Pinsker et al., 1970). If the siphon of Aplysia is mechanically stimulated, the animal withdraws his gill. The response of gill withdrawing occurs because the stimulus activates receptors in the siphon which activate the motor neurons that withdraw the gill. When the stimulus is repeated over time it causes a depression at these sensory neuron to motor neuron synapses (Fig 1.5). There was a widespread belief that the major mechanism responsible for synaptic depression was the depletion of the readily releasable pool of vesicles. Recent studies have shown that this synaptic depression is mediated by an activity-dependent, but release-independent, switching off of individual release sites to a silent state rather than a decrease of releasable vesicles. This switching off of release sites is initiated by Ca2+ influx during individual action potentials. It seems that there is no modification in postsynaptic responses (Gover and Abrams, 2009).
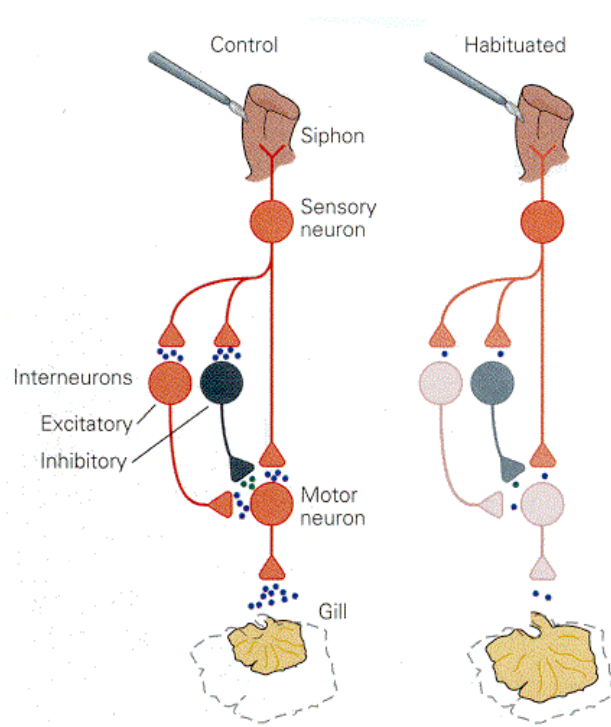
Fig 1.5. Habituation. Simplified neural circuits involved in the habituation process in Aplysia. The control condition is shown on the left, the habituated condition on the right (Kandel et al., 2000).

1.1.6. SENSITIZATION

Aplysia exhibits another form of synaptic plasticity called sensitization. In general, this term refers to the process through which a stimulus to one pathway enhances reflex strength in another one. In Aplysia that has been habituated to siphon touching, a strong electrical stimulus to the tail elicits a strong gill withdrawal, as if the animal has not been habituated. Only one stimulus applied to the tail is sufficient to enhance the gill withdrawal reflex for an hour. With repeated pairing of tail and siphon stimuli, this behavior can be altered for days or weeks, demonstrating a simple form of long term memory.

This mechanism involved serotoninergic axon-axonic synapses. As shown in Fig 1.6 the shock that evokes sensitization activates the sensory neuron 2 that innervates the tail. These sensory neurons in turn activate facilitating interneurons that release serotonin to the presynaptic terminals of the sensory neurons of the siphon. This neurotransmitter enhances transmitter release from the siphon's sensory neuron terminals leading to an increase of motor neuron synaptic excitation. The consequence of this process is to increase the size of Excitatory Post Synaptic Potential (EPSP) in the motor neurons without modifying the response of sensory neuron 1.
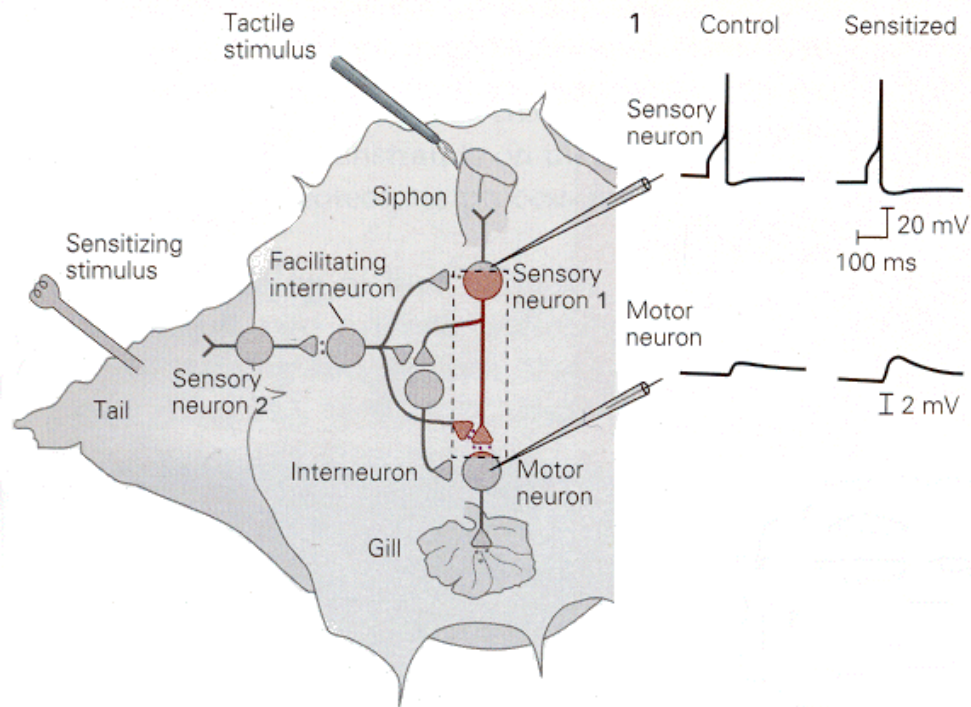
Fig 1.6. Sensitization. Schematic representation of the neuronal circuits underlying sensitization in the marine mollusk Aplysia californica (Kandel et al., 2000).

The biochemical mechanism responsible for this short-term sensitization, depicted in Fig 1.7 is that facilitating interneurons release serotonin which binds to G–protein coupled serotoninergic receptors on the presynaptic terminal of the siphon sensory neurons, leading to the activation of G-protein. The G-protein activates adenylyl cyclase that produces cAMP which, in turn, activates Protein Kinase A (PKA). Once active, PKA phosphorylates different proteins including K^+ channels leading to their closure. Moreover G-protein also activates Protein Kinase C (PKC) which, together with PKA, leads to opening of L-type Ca^{++} channels which mobilizes vesicles for exocytosis. When a sensory neuron synapse is depolarized, N-type Ca^{++} channels are opened and more Ca^{++} comes into the terminal. The closing of the K^+ channel prolongs the duration of the depolarization leading to an increase of Ca^{++} influx. This causes the release of synaptic vesicles. This cascade ultimately enhances synaptic transmission between the sensory and the motor neurons within the gill withdrawal circuit.
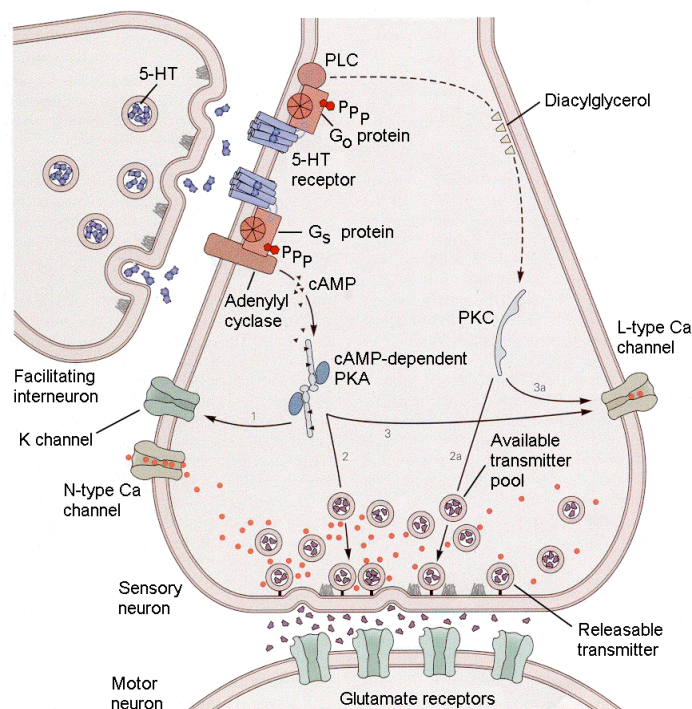
Fig 1.7. Sensitization. Schematic portrayal of the biochemical presynaptic mechanisms involved in the sensitization process (Kandel et al., 2000).

The sensitization generated by a short-term stimulation of the tail will quickly disappear once the stimulus is removed. However it is possible to prolong the duration of this form of plasticity by repeated tail stimulation. This long-term sensitization occors because of structural changes in the presynaptic terminals. It has been demonstrated that with sensitization there is an increase in the number of presynaptic vescicles in both sensory and motor-neurons. This change requires new gene expression and new protein synthesis. In mammals and vertebrates there is a similar mechanism of synaptic plasticity called Long Term Potentiation that has been correlated with learning and memory.

1.1.7. LONG TERM POTENTIATION (LTP)

Neuroscientists have always been interested in understanding the molecular mechanisms underlying learning and memory. At the end of the 19th century, scientists generally believed that the number of neurons in the adult brain doesn't increase with age, thus concluding that learning and memory are linked to changes in patterns of communication between existing neurons. After the discovery of hippocampal neurogenesis in adult brain (Altman and Das, 1965) scientists now have to figure out how these new neurons participate in learning and memory processes (for detail read Deng et al., 2010).

In the middle of the 20th century different clinical studies showed the connection between human memory dysfunction and the hippocampus (Scoville and Milner 1957) and investigators started to develop hypotheses that could explain the mechanism of learning. The neuroanatomist Cajal in 1894 suggested that memory is formed by strengthening the connection between existing neurons. Later on Hebbian Theory, described by Donald Hebb in 1949, proposed that the concomitant activation of neurons leads to an increase in synaptic strength between them. All of these discoveries stimulated the research in the field of synaptic plasticity in mammalian brain with the aim to figure out the mechanisms through which strengthening of synaptic connections can be achieved.

In 1973, Bliss and Lomo showed that in rabbit hippocampus after stimulation of perforant path fibres with a single high frequency tetanization they could record the postsynaptic response in the granule cells of the dentate gyrus for several hours. They proposed this long lasting enhancement in neuronal transmission, now known as Long Term Potentiation (LTP), as the mechanism that underlies learning and memory (Bliss and Lomo, 1973).

After this discovery a number of papers on LTP have been published. on PubMed there are around 11 thousands papers on this topic and still not all the mechanisms by which LTP occurs have been understood in detail.

LTP is restricted not only to the hippocampus but it is also observed in other areas of the brain such as cerebral cortex (Jung et al., 1990; Sakamoto et al., 1987), cerebellum (Racine etal., 1986), amygdala (Racine et al., 1983; Chapman et al., 1990), and others. Different parts of the brain exhibit different kinds of LTP that can be distinguished by the signaling pathway involved in the generation of the process. For example, some forms of LTP depend on the activation of NMDA receptors while others require only the metabotropic glutamate receptors (mGluR) (Malenka and Bear, 2004).

This thesis will focus on LTP that occurs in the hippocampus, particularly in the Schaffer Collateral pathway that connects neurons from CA3 region to CA1 pyramidal neurons and which is NMDA receptor dependant (O'Dell and Kandel, 1994). The neurons in this area exhibit high synaptic plasticity and hippocampal CA1 synapses are considered to be representative of many types of central synapses so the mechanisms described are applicable to other synapses in the brain (De Sevilla et al., 2010, Lauri et al., 1999, Stanton et al., 1984, Collingridge G. L. et al., 2004).

In general the LTP process can be divided into two phases: Early phase (E-LTP) that lasts for 30 to 60 min and is independent of new protein synthesis followed by the late phase (L-LTP) that can last for hours to days and requires new gene expression and new protein synthesis.

<u>1.1.7.a. E-LTP</u>

High frequency stimulation of presynaptic neurons causes the release of neurotransmitters, typically glutamate, from the axon terminal into the intra-synaptic space. Here glutamate binds to both AMPA and NMDA receptors located on the post-synaptic membrane. Both AMPA and NMDA receptors are permeable to Na^+ and K^+ but the conductance of NMDA receptors is limited by Mg^{2+} ions that block the NMDA channels. With a sufficiently strong stimulus, AMPA receptors alone can depolarize the membrane thus reaching the voltage required for the expulsion of Mg^{2+} ions from the NMDA channels making them permeable to Ca^{2+}. Intracellular Ca^{2+} acts as a second messenger and activates different kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII), Protein Kinase C (PKC), Protein Kinase A (PKA) and Mitogen Activated Protein Kinase (MAPK). These activated kinases (CaMKII and PKC), in turn, phosphorylate the existing AMPA receptors increasing their conductance for cations. Moreover, they modulate the insertion of new AMPA receptors into the postsynaptic membrane. Ca^{2+} ions are also able to facilitate the synthesis and release of a retrograde messenger such as carbon monoxide, platelet-activating factor and nitric oxide. These retrograde messengers travel from the postsynaptic neuron to the presynaptic neuron where they can activate pathways that increase the release of the neurotransmitter vesicles contributing to the maintenance of LTP (Voronin et al., 1995).

<u>1.1.7.b. L-LTP</u>

The late phase of LTP is not separate entirely but represents a natural extension of the early phase. As mentioned above, this phase of LTP requires new gene expression and protein synthesis that seem to occur both postsynaptically and presynaptically (Lisman and Harris, 1993). Postsynaptically, the increase in intracellular concentration of Ca^{2+} and cAMP leads to the activation of different intracellular pathways. It has been demonstrated that cAMP activates PKA which is important for the formation of L-LTP. PKA inhibitors block the formation of L-LTP induced by three high frequency trains of stimuli. Activated PKA phosphorylates the transcriptional activator cAMP-response element binding protein (CREB) at serine 133. The activation of CREB dependent gene expression also requires the formation of the complex between pCREB and Coactivator Binding Protein (CBP). Recent studies have also shown the involvement of *Transducer Of Regulated CREB activity 1* protein (TORC1) as CREB coactivator. TORC1 in the presence of high levels of intracellular Ca^{2+} and cAMP translocates in the nucleus where it binds the ZIP domain of CREB and regulates activity-dependent CREB target gene expression, important or the maintenance of L-LTP (Shuai Li et al., 2009). More recent studies have shown the involvement of an atypical PKC isoform, protein kinase Mzeta (PKMz) in the maintenance of L-LTP. PKMz is a constitutively active kinase, independent of second

messengers. It has been demonstrated that this protein sustains LTP by increasing the number of postsynaptic AMPA receptors. PKMz regulates the AMPA receptor trafficking that involves the N-ethylmaleimide-Sensitive Factor (NSF) which directly binds to the AMPA receptor subunit GluR2 to maintain the receptor at the postsynaptic site (Nishimune et al., 1998, Yao et al., 2008). Higher level of cAMP also leads to the activation of Extracellular signal Regulated Kinase (ERK) which seems to be involved in the regulation of potassium channel Kv4.2. By phosphorylating the potassium channels, ERK decreases their the voltage activation leading to an increase in cell excitability that enhances LTP. ERK is also able to translocate into the nucleus to regulate gene transcription. The downstream genes regulated by ERK include MAP2 and Tau, two cytoskeletal proteins, c-Myc, c-fos, c-jun, Elk1, CREB, C/EBPβ and ATF proteins.

Other players important in the maintenance of L-LTP are neurotrophins, in particular Brain Derived Neurotrophic Factor (BDNF). A number of studies have been conducted to prove the involvement of BDNF in synaptic potentiation. Intrahippocampal infusion of BDNF in anesthetized rats causes enhancement in the synaptic response in dentate gyrus neurons (Messaoudi et al., 1998) The BDNF-induced potentiation seems to be correlated with NMDA receptor activation and dependent on ERK and CREB activation. Moreover, BDNF up-regulates activity-regulated cytoskeleton-associated protein (Arc), a member of the immediate-early gene (IEG) family. Arc protein interacts with actin so they can contribute to cytoskeletal remodeling during synaptic plasticity (Mesaoudi_et al., 2007). Most of the intracellular pathways responsible for LTP require new gene expression and protein synthesis. This, in turn, leads to morphological changes required to sustain the enhancement of signal transmission. One of these morphological changes is the modification of the number and the morphology of dendritic spines.

1.1.8.DENDRITIC SPINES

Dendritic spines were discovered by Cajal in 1888 using the Golgi staining method (García-López P et al., 2007). Spines are small membranous protrusions from dendrites and represent the postsynaptic component of most excitatory, mainly glutamatergic, synapses in the brain. Spine density is usually between 1 to 10 spines per μm of dendritic lenght, so one dendrite can contain thousands of spines (Bourne and Harris, 2008). Dendritic spines are usually composed of a head connected to the dendritic shaft by a narrow neck. Based on their shape and length, spines can be classified into different categories (Fig 1.8):

- **Mushroom spines** with a large head and narrow neck;
- **Thin spines** with a small head and narrow neck;

- **Stubby spines** without a distinction between head and neck;

- **Filopodia** that are long thin spines without a head;

- **Branched spines** with two heads connected to the dendrite by the same neck.
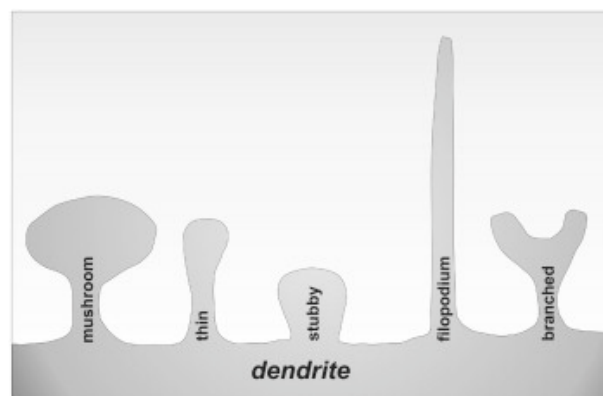
Fig 1.8. Classification of dendritic spines with respect to their shape (Oliver von Bohlen Und Halbach,2009).

Spine size is correlated with the size of its excitatory synapse. Spine head is a local compartment where all the molecules and proteins of the postsynaptic machinery are concentrated. Inside the spine head there is an area called Post Synaptic Density (PSD) which is attached to the postsynaptic membrane and is opposite to the presynaptic active zone. This area contains glutamate receptors (AMPA and NMDA), cytoskeletal proteins (actin, ProSAPs/Shanks), cell adhesion molecules (PSD95), proteins that activate different intracellular pathways (CaMKII), and many other proteins involved in the synaptic functioning. PSD95 protein is located next to the postsynaptic membrane where it interacts with the NMDA receptors regulating their distribuition (Fujita and Kurachi, 2000). Moreover, PSD95 interacts with other membrane proteins and signaling proteins facilitating the transduction of intracellular signals (Kim and Sheng, 2004). Dendritic spines usually contain few mitochondria because these are mainly localized in the dendritic shafts; instead spines contain polyribosomes for local protein translation, endosomal vescicles important for endocytosis and membrane recycling, and smooth endoplasmic reticulum (SER) for Ca^{2+} storage (Chicurel and Harris, 1992).

Dendritic spines develop from filopodia. During synaptogenesis, dendrites rapidly sprout and retract filopodia, small membrane organelle-lacking membranous protrusions, that represent immature form of spines, without a complete PSD region and synaptic activities. To become mature spine, filopodia have to find appropriate presynaptic partners that release glutamate which drives the PSD expression thanks to the help of two proteins, Telenchephalin, an adhesion molecule, and SynGAP, a RAS GTPase protein, which maintains filopodia in an active state during synaptogenesis.

Telenchephalin will be substituted by N-cadherin and α–catenin once filopodia become mature spines, while SynGAP remains in the spines and binds PDS95 (Bourne and Harris, 2008). The development and stabilization of the spines also requires astroglia. Astrocytes secrete soluble factors such as thrombospondin and cholesterol which influence spine formation and synapsis maturation (Allen and Barres, 2005).

Once spines become mature they won't stay in the same "state" (shape, volume, activity) for long time. Yasumatsu demonstrated the "intrinsic fluctuation" of spines, term that underlines their native instability (Nobuaki Yasumatsu et al., 2008). In fact spines change in number and morphology in relation to the neuronal activity. Experimental evidence has suggested that the growth and morphological changes of spines are governed by modification of the dynamic cytoskeleton composed mainly of actin filaments and requires a complex network of proteins. CaMKs and other molecules, N-cadherin and BDNF, located at PSD phosphorylate guanine nucleotide exchange factor (GEF), Kalirin7, which is expressed at high levels in the dendritic spines of the hippocampus and cortex of adult brain. When Kalirin7 is phosphorylated it activates Rac1. Rac1 is a small GTPase member of the Rho family of GTPases, with Rho a and Cdc42. Once active, Rac1 can control spine morphogenesis and plasticity through other kinases like PAK 1 and 3, which activate LIMK that in turn phosphorylates cofilin leading to a modulation of actin cytoskeleton (Fig 1.9) (Morgado-Bernal, 2011).
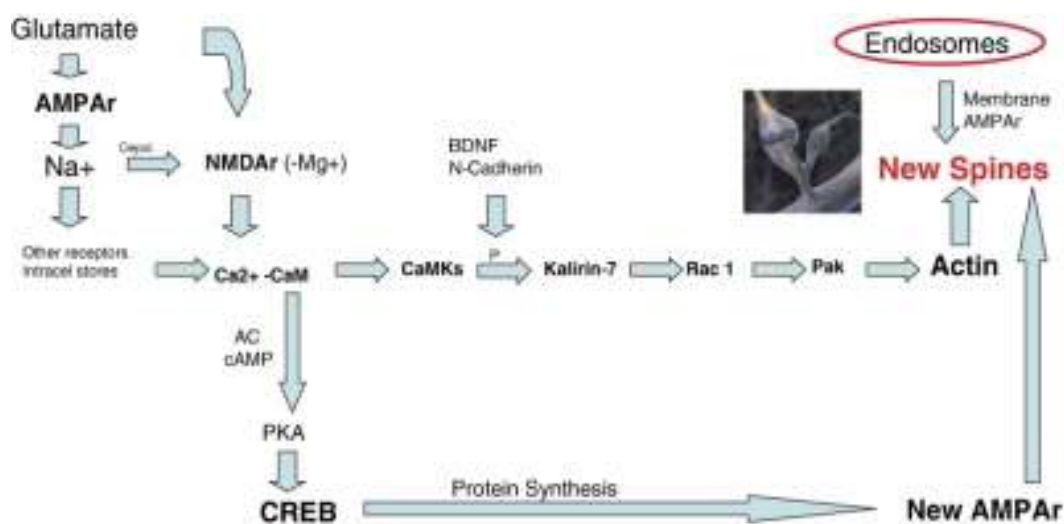
Fig 1.9. Link between LTP and spine formation (Morgado-Bernal, 2011).

Spines contain both the monomeric form, G actin, and the polymers, F actin, and the ratio between the two forms determines the size, the number and the motility of the spines (Okamoto et al., 2004). It has been demonstrated that LTP induction shifts the ratio towards F actin leading to an increase in spine volume, while during Long Term Depression (LTD), another kind of synaptic plasticity

characterized by a reduction in the efficiency of synaptic transmission, the balance is in favor of the G form of actin that causes spine shrinkage (Fukazawa et al., 2003, Zhouet al., 2004).

Memory consolidation is the process by which information that have been acquired (STM) are stored in different brain regions such as prefrontal cortex, anterior cingulate cortex and hippocampus and they can be recalled days, weeks or even years after their stabilization (LTM). Kandel in 2001 linked this process to "a dialog between genes and synapses" (Kandel, 2001) where new gene expression and protein synthesis are required for the formation and the morphological changes in spines. Spines under this point of view represent the substrate upon which LTM can be established (Engert and Bonhoeffer, 1999). It has been shown that the number of dendritic spines increases with learning and LTP causes an enlargement of the spine head. This enlargement of the spine head is due to the activation of CaMKI and subsequent synaptic recruitment of AMPAR leading to the activation of NMDAR and the regulation of a signaling pathway that drives spine enlargement via actin polymerization (Fortin et al., 2010).

It has also been hypothesized that spines with a large head such as mushroom spines are more stable and contribute to strong synaptic connection so they represent the "memory spines" while small spines are motile and unstable and can better adapt to changes in synaptic activity and are thus called "learning spines" (Bourne and Harris, 2007). In literature there are a lot of reports that show a positive correlation between dendritic spines and learning. In 2010, Chen and coworkers published a paper in which they demonstrated that stress impairs learning and memory via mechanisms that disrupts the integrity of hippocampal dendritic spines. They showed the degree of memory deficit in individual mice, tested with novel object recognition test, correlated significantly with the reduced density of area CA3 apical dendritic spines in the same mice (Chen et al., 2010). Another study conducted in zebra finches birds showed that when the birds learn to sing by imitating a tutor song during a juvenile sensitive period, there is a stabilization, accumulation and enlargement of dendritic spines in the forebrain nucleus HVC assessed by two-photon microscopy (Roberts et al., 2010). Moreover, it has been demonstrated that the passive avoidance task increases the density of dendritic spines in the dentate gyrus of rodents (O'Malley A. et al., 1998) and a similar result was also found after training in a spatial water maze test (O'Malley A. et al., 2000). In 2003, Leuner and coworker showed that the formation of associative memory during trace eye blink conditioning task was correlated with an increase in the number of dendritic spines in the neurons located in CA1 region. In this study the authors demonstrated that spine density increase is due to the process of learning and not the training experience (Leuner B., 2003).

1.1.9. LTP AS A MECHANISTIC MODEL OF LEARNING AND MEMORY

A lot of studies support the hypothesis that LTP can be considered the cellular model of learning and memory. Both processes are Ca^{2+} and protein synthesis dependent and they share the same biochemical mechanisms. Both processes are associated with glutamate release (McGahon et al., 1996), activation of protein kinases such as PKC and ERK, activation of transcription factors that lead to new gene expression and protein synthesis and up regulation of BDNF levels (Gooney et a., 2002). Moreover, LTP can be evoked in the main afferent pathways of the hippocampus which is also extremely important for learning and memory. Thus both processes are associated with the same part of the brain and both are impaired in stress and aged animals. (Rapp et al., 1987; Bach et al., 1999; Thiels et al., 2000). In addition, different studies have demonstrated that LTP and memory can be inhibited by the same agents, for example by the treatment with AP5, an NMDAR antagonists (Morris et al., 1986). There are different forms of memory and each has multiple facets so we should keep in mind that ascribing such a complex modality only to the LTP process would be too simplistic. Anyway, based on the evidence from the studies mentioned above, LTP can be considered as one of the mechanisms underlying learning and memory.

1.1.10. TRANSCRIPTION FACTORS

As quoted above, new gene expression and protein synthesis are required for the late phase of LTP and for memory consolidation. In the last 50 years a lot of studies have been conducted in different species using transcriptional inhibitors like actinomycin D, adenosine analog DRB and RNA polymerase II inhibitor α-amanitin, and translational inhibitors, like anysomicin to demonstrate that transcription and translation are essential steps for memory formation. In these studies when RNA or protein synthesis were blocked, before or after training, memory consolidation was disrupted (Gold PE., 2008; Harnandez et al., 2008; Sutton et al., 2006; Pedreira et al.; 1996, Squire et al., 1970; Thut et al., 1974). Since transcription is an essential step for memory storage, transcription factors should play a key role in LTM and synaptic plasticity (Alberini, 2009).

Transcription Factors (TFs) are proteins that bind specific DNA sequences and regulate the transcription of the genetic information from DNA to RNA, performed by the RNA polymerase II enzyme. TFs act either by directly interacting with the RNA polymerase or by regulating its catalytic function by binding to *cis*-acting DNA sequences or another TF. TFs are extremely important for the regulation of gene expression and are present in all living organisms. Their number increases with genome size, and larger genomes tend to have more transcription factors per gene. In eukaryotes, an important class of TFs is the General Transcription Factors (GTFs) that cooperate for the

transcription of each gene and have been remarkably conserved throughout evolution. Many of these GTFs don't bind DNA but are part of the large transcription pre-initiation complex that interacts directly with RNA polymerase. They include the TATA box binding protein (TBP) and the TBP associated factors (TAFs) which together form the transcription factor IID. Other GTFs are TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH. The pre-initiation complex binds to the promoter region of DNA located upstream of the gene that it regulates (Hampsey et al., 1998).

Other TFs interact directly with the DNA by binding specific DNA sequences. Once the TFs bind this site, their regulatory domain can regulate transcription leading to an up or a down regulation of gene transcription. The TF families that are known to be critically involved in synaptic plasticity and memory formation include cAMP response element binding protein (CREB), CCAAT enhancer binding protein (C/EBP), activated protein 1 (AP1), early growth response factor (Egr) and Rel/nuclear factor kB (Rel/NFkB) (Thiel, 2006).

Very little is known about the involvement of another class of TFs, the Activated Transcription Factors (ATFs) particularly one member of this family, ATF4, in cognitive processes.

1.2 ACTIVATED TRANSCRIPTION FACTOR 4 (ATF4)

1.2.1. GENERAL PROPERTIES AND REGULATION

Activated Transcription Factor 4 (ATF4) has been classified as a member of the activated transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family. (Hai & Curran, 1991). Other members of ATF/CREB family are ATF1, CREB/CREM, CREB314, CREB-H, ATF2, ATF3, ATF5, ATF6, ATF7 and B-ATF (Brindle & Montminy, 1992; Ziff, 1990).

Partial human ATF4 cDNA was isolated for the first time in 1989 (Hai et al., 1989) on the basis of its ability to bind to the consensus ATF/CRE site GTGACGT (C/A) (G/A) (Lin and Green, 1988). Later on, full-length cDNA clones encoding homologous proteins were isolated and named differently. These include the human clone TAXCREB67 (Tsujimoto et al., 1991), CREB-2 (Karpinski et al., 1992), the mouse clone mATF4 (Mielnicki & Pruitt, 1991), mTR67 (Chevray & Nathans, 1992) and C/ATF (Vallejo et al., 1993). The human clones are 85% homologous to the mouse ones at the amino acid level. In this thesis I will refer to these proteins as ATF4. The ATF4 gene is located on chromosome 22 in humans and chromosome 15 in mice.

ATF4 mRNA includes two short open reading frames (uORFs) (Fig1.10. green boxes) in the 5' UTR before the authentic one (white rectangle) important for the translation of ATF4 under stress condition (Harding et al., 2002, Vattem & Wek, 2004). The first uORF works as a positive-acting element that leads to ribosome scanning and translation initiation at downstream coding regions. Under normal condition (left panel) where the level of eIF2-GTP is high, the ribosome scans downstream of the first uORF, reading the second one which works as an inhibitor element, blocking the ATF4 mRNA translation. Under stress condition, for example during amino acid deprivation (right panel) the phosphorylation of eIF2 causes a decrease in the level of eIF2-GTP and a decrease in the eIF2-GTP Met-tRNA^{Met} complex formation, leading to a delay in translation initiation, allowing ribosomes to bypass the inhibitory uORF2 and start the translation at the ATF4 coding region, after the ribosome acquires eIF2-GTP Met-tRNA^{Met}. (Vattem & Wek, 2004).
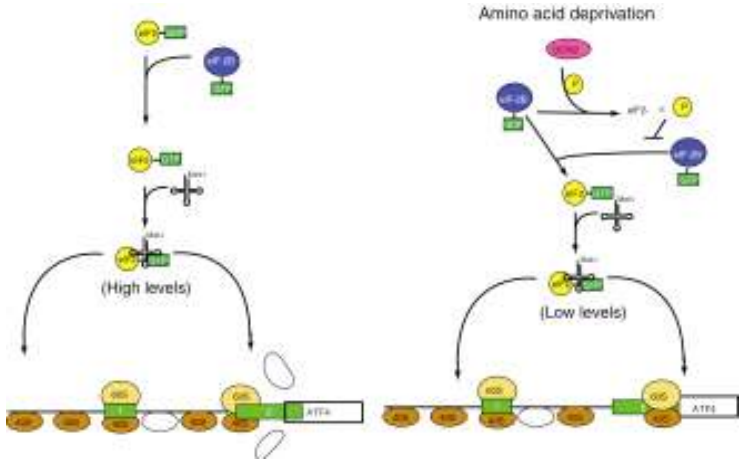
Fig.1.10. Schematic diagram of ATF4 mRNA translation (Costa-Mattioli & Sonenberg, 2008).

ATF4 protein is made by 351 amino acids and it contains different motifs (Fig 1.11): transcriptional activation domain located at N-Terminal, bTrCP recognition motif between amino acids 218 and 224, a basic domain leucine zipper motif (bZIP) at C-Terminal and an Acetylation site at K311 (Ameri & Harris, 2008).
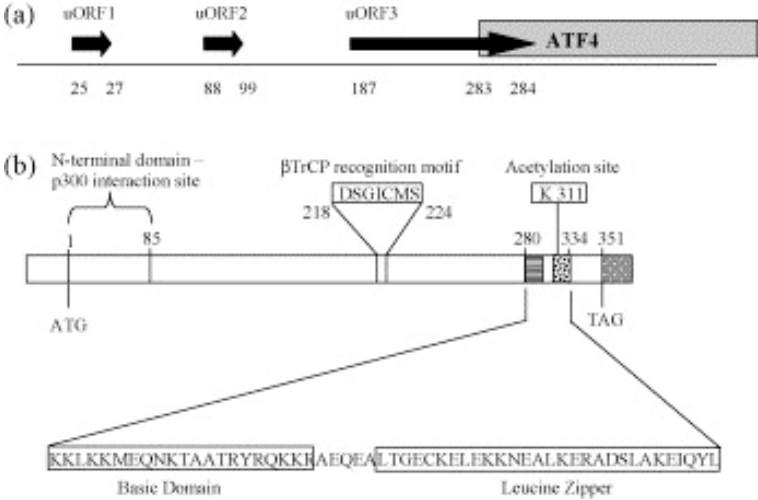
Fig 1.11. (a) ATF4 mRNA: the uORFs in the ATF4 5 ′ UTR human. (b) Schematic representation of the ATF4 structure (Ameri & Harris, 2008).

Through its basic domain ATF4 interacts with DNA and through its leucine zipper domain it can dimerize, generating homodimers and heterodimers, with a member of the AP-1 and C/EBP family proteins including Fos (Hai & Curran, 1991) and Jun (Chevray & Nathans, 1992). ATF4 can also interact

with p300, RSK2, a growth factor regulated kinase (Yang et al., 2004), Satb2, a nuclear matrix protein (Dobreva et al., 2006), NIPK, a neuronal cell-death-inducible putative kinase (Ord D. & Ord T., 2003), CHOP, the C/EBP-homologous protein (Gachon et al., 2001) and others.

ATF4 mRNA is ubiquitously expressed, and the protein is present at very low level (Vallejo et al., 1993); ATF4 protein has a short half-life, between 30 and 60 minutes. ATF4 protein amount can be upregulated by different extracellular signals /stressors such as oxygen deprivation (Ameri et al., 2004), amino acid deprivation, endoplasmic reticulum stress and oxidative stress (Harding et al., 2003). ATF4 regulation can occur at transcriptional, translational and posttranslational level. The growth factor heregulin $\beta 1$ (HRG) is able to induce ATF4 mRNA in human cancer cells (Talukder et al., 2000). Moreover, amino acid and glucose deprivation increase the level of ATF4 messenger (Siu et al., 2002). EIF2α is the central mediator in the translational regulation of ATF4. EIF2α phosphorylation leads to a general translational reduction but a specific induction of ATF4 mRNA translation (Harding et al., 2000). The stability of ATF4 protein can be modulated mainly by SCFbTrCP class of ubiquitin ligase, that interacts with ATF4 protein leading to its proteasomal degradation (Lassot et al., 2001), and by histone acetyltransferase p300, that induces ATF4 stabilization preventing SCFbTrCP-ATF4 interaction and subsequent ATF4 degradation (Lassot et al., 2005). Moreover, CBP as well as p300 acetylate ATF4 in its bZIP domain enhancing its transcriptional activity (Liang & Hai, 1997, Lassot et al., 2005).

1.2.2. BIOLOGICAL FUNCTIONS

1.2.2.a. Transcriptional activity of ATF4: activator or repressor?

ATF4 was originally described as a repressor of CRE-dependent gene transcription. Deletional studies showed that the transcriptional repressor activity of ATF4 is located between amino acids 249 and 351, a region that contains the leucine zipper and the basic domain which are involved in its dimerization and DNA binding (Karpinski at al., 1992). Further studies demonstrated the transcriptional activity of ATF4 as a repressor of long term memory storage (Bartsch et al., 1995; Chen et al., 2003).

Recent studies have shown that ATF4 is also a transcriptional activator. It can induce different kinds of genes such as receptor activator of nuclear factor-kappa B ligand (RANKL) (Elefteriou et al., 2005), osteocalcin (Yang & Karsenty, 2004), E-selectin (Talukder et al., 2000), VEGF (Roybal et al., 2005), Gadd153 (Fawcett et al., 1999) and gadd34 (Ma & Hendershot, 2003). Moreover, ATF4 can up-regulate genes involved in mitochondrial function and amino acid metabolism (Harding et al., 2003).

ATF4 can also activate gene expression through its interaction with different binding partners. It has been shown that the interaction between ATF4 and Nrf2 induces heme oxygenase-1 (HO-1) expression (Cullian & Diehl, 2006).

<u>1.2.2.b. ATF4 as a developmental gene</u>

Transgenic studies have demonstrated the involvement of ATF4 in eye development. ATF4 deficient mice exhibit severe microphthalmia which is caused by degeneration of the lens (Tanaka et al., 1998). ATF4 knock-out mice have a structurally normal lens until embryonic day 14.5. Later on, the lens fiber cells undergo apoptotic cell death, which is p53 mediated (Hettmann et al., 2000). These cells are not replaced by new ones suggesting that ATF4 plays an important role in lens fiber differentiation. In these mutant mice retinal development is normal (Tanaka et al., 1998).

ATF4 is also involved in bone development; it has been shown that ATF4 is a crucial factor promoting osteoblast maturation. In osteoblasts, the interaction between ATF4 and Runx2, mediated by the nuclear matrix protein SATB2, activates the osteocalcin promoter (Dobreva et al., 2006). Moreover, a recent study demonstrated that general transcription factor IIA (TFIIA) interacts with both Runx2 and ATF4, preventing the degradation of ATF4, which in turn enhances osteocalcin expression (Yu *et al.,* 2008). ATF4 is also a critical substrate for RSK2 which is required for the terminal differentiation of osteoblasts and osteoblast-specific gene expression. Additionally, RSK2 and ATF4 post-transcriptionally regulate the synthesis of type I collagen, the main constituent of bone matrix (Yang et al., 2004).

<u>1.2.2.c. ATF4 and cancer</u>

ATF4 is involved in tumor progression and metastasis. ATF4 protein level was found to be higher in primary human tumors as compared to normal tissues (Ameri et al., 2004). Hypoxia is one of the most important factor that contributes to cancer progression because chemotherapeutic drugs are less affective at killing hypoxic cells.

Cells can respond to hypoxic stress by inducing Hypoxia-Inducible Factors 1 and 2 (HIF1-2) or by activating HIF-independent mechanisms that lead to a general inhibition of protein synthesis. Hypoxia attenuates general translation through the activation of PERK and the subsequent phosphorylation of the translation initiation factor eIF2α. When eIF2α is phosphorylated, there is a general inhibition of translation but select stress-response genes, such as ATF4 and CHOP, are up regulated (Koumenis, 2006). The role of ATF4 and CHOP in the cellular response to hypoxia is not completely understood. Different studies have demonstrated that xenograft tumors grown from

cells with a compromised PERK-eIF2α-ATF4 pathway grow slower and smaller than tumors with wild-type PERK- eIF2α-ATF4 function, suggesting that inhibiting PERK-eIF2α- ATF4 activity negatively affects tumor growth (Bi et al., 2005).ATF4 is able to induce VEGF and E-selectin which may be associated with an increase in metastasis (Talukder et al., 2000; Roybal et al., 2005).

1.2.2.d. ATF4 as a stress responsive gene

ATF4 gene can be up-regulated by several stressors, for example metabolic deficit such as glucose and amino acid deprivation, oxidative stress and ER stress. In mammals, different stress signals activate 4 different eIF2α kinases. The nutritional stress activates General Control Nonderepressible-2 (GCN2) (Harding et al., 2000), heme deficiency and heavy metal ions trigger Heme Regulated Inhibitor (HRI) (Liu et al., 2001), viral infections and interferon treatment activate dsRNA induced protein kinase R (PKR) (Patel et al., 2000) and Endoplasmic Reticulum (ER) stress activates eIF2α kinase (PEK, also known as PERK) (Yan et al., 2002). All four kinases are able to phosphorylate the α subunit of eIF2 on serine residue 51. eIF2 forms a ternary complex, eIF2.GTP.methionyl-tRNAi, which is an obligate intermediate in the binding of the initiator methionyl-tRNAi to the 40S ribosomal subunit. The eIF2 factor is subsequently released from the ribosome as an eIF2.GDP complex. Under steady-state condition of translation, eIF2 must be recycled in order to participate in another cycle of translation initiation. Conversion of the inactive eIF2.GDP to the active eIF2.GTP form is catalyzed by a guanine nucleotide exchange factor, eIF2B. Phosphorylation of the *a* subunit of eIF2 inhibits translation initiation by impairing the eIF2B-catalyzed guanine nucleotide exchange reaction (Moldave, 1985) leading to a global protein synthesis inhibition and a concurrent expression of genes that function to alleviate stress damage in cells. One of these stress response genes is ATF4 which is strongly induced after eIF2α phosphorylation.

Once ATF4 is expressed, it can act as a prodeath transcription factor as well as a prosurvival one. In 2003, Harding and coworkers showed that wild type mouse fibroblasts treated with thapsigargin, that causes ER stress leading to the activation of PERK pathway, were resistant to cell death as compared to ATF4 -/- fibroblasts. In this model, ATF4 acts as an activator driving the expression of genes involved in amino acid import like asparagine synthase, glycine transporter 1 which provides glycine and a precursor of glutathione biosynthesis. Glutathione is a major antioxidant in the adult brain. ATF4 also up-regulates genes involved in intracellular metabolism of sulfur-containing amino acids like cystathionine γ-lyase and methylenetetrahydrofolate dehydrogenase, suggesting that the defective import and metabolism of certain amino acids predisposes ATF4 -/- cells to oxidative stress eventually causing their death (Harding et al., 2003). Moreover, ATF4 protein can dimerize with the transcription factor NRF2 inducing Hmox-1 gene, which in turn promotes resistance to oxidative

stress. (He et al., 2001).

ER stress, usually originating from the accumulation of unfolded secretory proteins in the lumen of the ER, activates a response signaling pathway called Unfolded Protein Response (UPR). The UPR pathway initially has the aim to restore normal functions of the cell by halting protein translation and by increasing the production of molecular chaperones involved in protein folding. If the ER stress is severe or prolonged, the UPR leads to apoptosis. The UPR starts with the activation of 3 key proteins: two kinases, PERK and IRE1, and the transcription factor ATF6. These 3 proteins activate three different pathways which coordinate the response against the misfolded protein by downregulating protein translation, upregulating ER chaperone that promotes protein refolding such as GRP78 and by the activation of proteases involved in the degradation of the misfolded proteins (Ron & Walter, 2007).

Another recent paper (Bouman et al., 2010) showed that ER stress, through the pathway that involves activation of PERK, upregulates ATF4 protein which binds to the CREB/ATF site within the parkin promoter inducing the upregulation of parkin gene which helps to preserve the cellular function and survival in the adaptive phase of UPR. The protective role of parkin in response to ER stress is independent of proteasome. Indeed, the inhibition of proteasome does not interfere with the ability of parkin to prevent ER stress-induced apoptosis. If the stress is severe, there is a preferential binding of c-jun to the parkin promoter leading to a repression of parkin expression. C-jun binds to the same ATF4-binding site within the parkin promoter, acting as a dominant negative protein that suppresses the parkin cytoprotective role and favors the proapoptotic pathway (Bouman et al., 2010).

On the contrary, in 2008 Lange and colleagues demonstrated that primary cortical neurons derived from ATF4-/- brain are resistant to oxidative stress-induced cell death. Oxidative stress, which is pathogenic in different neurological diseases like stroke, induced by treating primary cortical neurons with a glutamate analogue like homocysteate (HCA) causes an up-regulation of ATF4 gene with a subsequent neuronal death. Conversely, ATF4 -/- neurons are resistant to oxidative death. ATF4 is also a prodeath factor in vivo. They showed that in a transient middle cerebral artery occlusion a model of ischemia–reperfusion injury, ATF4 null mice showed a smaller infarct area in comparison to WT (Lange et al., 2008).

In literature there are a number of papers that support the ATF4 prodeath activity in different models. Some of them show that ATF4 leads to cell death through the up-regulation of another transcription factor, CHOP/GADD153 (Harding et al., 2000; Kim et al., 2010). It has been demonstrated

that in an animal model of Parkinson's disease, generated by intrastriatal infusion of 6OHDA in adult rats, the loss of dopaminergic neurons is mediated by the expression of CHOP protein. They showed a positive correlation between CHOP positive profile and apoptotic profile: the cells that undergo apoptosis express CHOP protein. They confirmed that CHOP is a mediator of neuronal death in the 6OHDA animal model of Parkinsonism, showing that CHOP null animals treated with 6OHDA have a 65% reduction in the number of apoptotic cells.

Regarding the mechanism by which ATF4 up-regulates the transcription of CHOP, Cherasse and colleagues (2007) showed that in the context of amino acid starvation, the N-terminal region of ATF4 directly interacts with p300/CBP-associated factor (PCAF) recruiting it to the CHOP AARE DNA sequence. PCAF acts as a co-activator of ATF4 and it seems that its HAT activity is required for the activation of AARE-dependent transcription of CHOP (Cherasse et al., 2007). CHOP is a small nuclear protein that dimerizes with members of the C/EBP family of transcription factors. Normally undetectable under basal conditions, its expression is strongly up-regulated under stress conditions. CHOP expression in stressed cells is mainly linked to the development of programmed cell death. CHOP can cause apoptosis through the activation of PUMA, a Bcl-2 homology 3 (BH3) –only member of Bcl-2 family, as showed by Galehdar in 2010. In this study, PUMA is transcriptionally activated in primary cortical neurons by ER stress. PUMA activation depends on ATF4-CHOP pathway and it is p53 independent. ER stress, caused by treatment with tunicamicin (N-glycosylation inhibitor) or thapsigargin (Ca^{2+}-ATPase inhibitor), up-regulates ATF4 which induces the expression of the transcription factor CHOP, that in turn induces PUMA expression by binding to its promoter. PUMA causes neuronal apoptosis (Galehdar et al., 2010).

Since an uncontrolled response to stress can be deleterious, cells have developed a negative feedback loop to control the response to stressful conditions. One of the proteins involved in this loop is TRB3. Under the stress condition of amino acid deprivation, the up-regulation of ATF4 results in the induction of a large number of target genes including TRB3 which inhibits ATF4 transcriptional activity in a negative feedback loop (Jousse et al., 2007).

The studies described above underline that the stress response gene ATF4 plays a double role. Indeed, it can act as a prodeath factor as well as a prosurvival one. This dichotomy can depend on the experimental conditions: for example, the cellular or animal model used to conduct the evaluation and the level of cellular damage. The cell first attempts to recover the damage. However, if the injury is too severe and the cell is not able to repair it, the cell will activate pathways involved in cell death.

1.2.3. ATF4 AND MEMORY: IMPLICATION IN NEURODEGENERATIVE DISEASES

Since transcription plays an important role in memory and synaptic plasticity, in the last several years a lot of effort has been made to understand the involvement of transcription factors in these processes. Different families of transcription factors have been extensively studied including CREB, c/EBP, Erg, AP-1 and others. All of them seem to have essential functions in both processes. Little is known about the role of another class of transcription factors, the Activated Transcription Factor (ATF) particularly the implication of one of its member, the Activated Transcription Factor 4 (ATF4).

The first study involving the role of ATF4 in the process of memory was conducted in the marine mollusk Aplysia Californica, a valuable laboratory animal. It was used to study neurobiology of learning and memory because of its seemingly simple nervous system, capable of a variety of non-associative and associative learning tasks, described before. ApCREB2, the Aplysia homologous form of human CREB2 and mouse ATF4, is constitutively expressed in sensory neurons of Aplysia. Injection of anti- ApCREB2 antibody in these neurons elicits long term facilitation which lasts up to 24h, after only a single brief application of 5-hydroxytryptamine (5-HT or serotonin). This stimulation under normal conditions produces only short-term facilitation that usually decays after 10 minutes. The authors attributed this enhancement to the mechanism by which the antibody, anti ApCREB2, blocks the direct or indirect repression activity of ApCREB2 on the ApCREB1 mediated transcription, which is required for long term synaptic plasticity and memory formation. ApCREB is the Aplysia homologue to the rodent and human CREB (Bartsch et al., 1995).

Similar results were found in transgenic mice that express in the hippocampus a broad dominant negative inhibitor of C/EBP proteins, called AZIP, after doxycycline treatment. This inhibitor dimerizes with endogenous C/EBP proteins blocking DNA binding and gene transcription. The transgenic mice showed a downregulation of ATF4 and C/EBPβ protein levels and an altered transcript profile in comparison with the control mice without doxycycline treatment. The protocol that produces only E-LTP in WT mice is sufficient for establishing transcriptional dependent L-LTP in transgenic mice. Similarly, a weak training protocol that is insufficient, in the WT mice, to form LTM on the Morris Water Maze Task, allows consolidation of LTM in transgenic mice. The mice that overexpress AZIP inhibitor show an enhancement in synaptic plasticity and an improvement in LTM (Chen et al., 2003).

As mentioned before, L-LTP and LTM are closely dependent on new protein expression. The phosphorylation of the α subunit of eIF2 inhibits general translation but selectively causes translation of ATF4 mRNA. Modification of proteins involved in this pathway, located upstream of

ATF4, alters synaptic plasticity and memory formation. GCN2 null mice, and mice homozygous for eIF2α $^{+/S51A}$ mutation, in which the phosphorylation of the α subunit of eIF2 is reduced, have a reduction in the level of ATF4 and an increase in CREB activity. These mice showed a decreased threshold for eliciting L-LTP and an enhancement in Morris Water Maze performance after a weak training protocol, while strong stimulation and a standard training protocol caused an impairment in LTP and memory formation in GCN2 null mice (Costa Mattioli et al., 2005) and an enhancement in eIF2α $^{+/S51A}$ mutant mice (Costa Mattioli et al., 2007). Strong stimulation might activate an inhibitory pathway, which seems to be potentiated in GCN2 null mice, to counteract excessive neuronal activities that can be dangerous for the cell, like during seizure. It has been hypothesized that neurons have two different thresholds: one for the activation of gene expression and the second where excessive stimulation decreases gene expression thus blocking synaptic plasticity (Costa Mattioli et al., 2007).

Understanding the mechanisms that underlie the processes of synaptic plasticity and memory formation is important, since loss of memory is the cardinal symptom of one of the most diffuse neurodegenerative diseases, Alzheimer's Disease (AD). Alzheimer's disease is an incurable chronic progressive neurodegenerative disorder characterized by loss of synapses and decrease in cell density in distinct regions of the brain (Burns & Iliffe, 2009). AD exhibits two pathological features: neurofibrillary tangles (NFT) and senile-plaques. NFTs are intraneuronal accumulation of paired helical filaments composed of abnormally hyperphosphorylated tau protein. Senile plaques are mainly composed of β-amyloid peptide (Aβ) derived from the amyloid precursor protein (APP) after proteolytic processing by β–secretase (BACE-1) and γ–secretase.It has been shown that ATF4 regulates γ-secretase activity during amino acid imbalance by inducing PS1 expression. ATF4 binds to the regulatory region of PS1 gene (Mitsuda et al., 2007). PS1 is a key component of the γ–secretase enzyme. Soon after synthesis, its hydrophobic loop domain is cleaved to generate two different fragments: N-terminal fragment (NTF) and C-terminal fragment (CTF), which are stabilized by other cofactors including Aph1, Pen2 and nicastrin. Together all these factors form the γ–secretase complex (Takasugi et al., 2003). The increase of γ-secretase activity leads to an increment of Aβ production that is toxic for the cells. (Ohta et al., 2010).

Uncover the role of ATF4 in learning and memory is important to identify new players and new mechanisms involved in these processes and to develop new therapeutic approaches for the treatment of the pathologies whose symptoms are characterized by learning and memory deficit.

CHAPTER II: EXPERIMENTAL PROCEDURES

2.1 LENTIVIRAL VECTOR PRODUCTION

2.1.1. VECTOR PLASMID PRODUCTION

Vector for the ATF4 protein overexpression was made by amplifying the full-length of rat ATF4 cDNA by PCR using the following primers:

Forward: 5' AAC ATG ACC GAG ATG AGC TTC 3'

Reverse: 5' TTA TTT GTC GTC GTC GTC TTT GTA GTC CAT CGG AAC TCT CTT CTT CCC-3'

The primers were designed to add a Flag-tag at the C terminal of the ATF4 sequence. The ATF4 cDNA sequence was then inserted into the pWPI plasmid (Add gene, 12254) using PmeI as the restriction enzyme. We used the linearized pWPI empty vector, without ATF4 gene, as control.

Vector for the downregulation of ATF4 protein was made using a shRNA that binds to a specific region inside the ATF4 cDNA. The sequence we chose for binding extends from bp 576 to bp 596 of the rat ATF4 cDNA. The shRNA sequences were the following:

Forward: 5' CGCGT GCCTGACTCTGCTGCTTAT ttcaagaga ATAAGCAGCAGAGTCAGGCtttttt A 3'

Reverse: 5'CGCGT aaaaaaGCCTGACTCTGCTGCTTAT tctcttgaa ATAAGCAGCAGAGTCAGGC A 3'

As control for ATF4 downregulation, we introduced 6 mutations in the shRNA sequence used to downregulate ATF4, so this mutant shRNA is not able to bind ATF4 mRNA.

shRNA: 5' AGG AAG CCT GAC TCT GCT GCT TAT ATT ACT 3'

Mutant shRNA: 5' G CC<u>A</u> GA<u>T</u> TC<u>A</u> GC<u>G</u> GC<u>C</u> TA<u>C</u> AT 3'

The sequences used to generate the mutant shRNA were the following:

Forward:

5'.CGCGTGCCAGATTCAGCGGCCTACATttcaagagaATGTAGGCCGCTGAATCTGGCtttttttA 3'

Reverse:

5' CGCGTaaaaaaGCCAGATTCAGCGGCCTACATtctcttgaaATGTAGGCCGCTGAATCTGGCA 3'

Sticky ends were added to both sides of the sequences to insert the shRNA inside the pLVTHM vector (Addgene, 12247). Both shRNA sequences were inserted into the pLVTHM vector using Mlu I restriction enzyme. All the vectors were amplified in Stbl3 chemically competent E. coli bacteria and purified using maxi prep protocol (HiSpeed Plasmid Maxi kit, Quiagen) according to the vendor's instructions.

2.1.2. LENTIVIRAL VECTOR PRODUCTION

All of the following procedures were performed under sterile conditions.

1- Preparation of HEK293T cells

150 mm dishes were coated with 0.02 mg/ml of Poly-D-lysine (Sigma) dissolved in distilled water (15 ml of solution/dish) and left Over Night (ON) at 37°C. The day after, Poly-D-lysine solution was removed and the dishes were washed with Double Distilled Water (DDW). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) plus 10% FBS medium on the pre-coated dishes. 3 dishes for each type of lentivirus were prepared.

2-Lentivirus preparation

Lentivirus was prepared using 2nd generation packaging system. For each lentivirus, three different vectors were used. The lentivirus for ATF4 overexpression was made by transfecting HEK293T cells with the following vectors:

a) pWPI (with or without ATF4 Flag-Tag gene),

b) Packaging vector, psPAX2 (Addgene,12260),

c) Envelope vector, pMD2.G (Addgene, 12259).

The lentivirus for ATF4 downregulation was made by transfecting HEK293T cells with the following vectors:

a) pLVTHM (with shRNA against ATF4 or the mutant shRNA),

b) Packaging vector, psPAX2,

c) Envelope vector, pMD2.G.

The solution for transfection was prepared as follows:

For each 150 mm dish:

2M CaCl2 150ul, PMD2G 10μg, psPAX2 15μg, WPI or pLVTHM 22μg and DDW to a final volume of 1.2ml.

This solution was added, drop by drop, to 1200 ☐l of Hank's Balanced Salt Solution (HBSS, Invitrogen) 2X and mixed thoroughly. The solution was added into each 150 mm dish with HEK293T cells. The dishes were incubated at 37°C and the medium was replaced with fresh one after 6 to 8 hrs. Two days after transfection, the medium was collected in a 50 ml tube and stored at 4°C. After collecting the medium, the dishes were refilled with fresh medium. 24 hrs later, the medium from the dishes was collected again. The media from the first and second harvests were pooled together. The media were centrifuged 3000 rpm for 5 minutes at Room Temperaturte (RT) to eliminate the cells that remained on the pellet. The supernatant was collected and again cleared from cell debris by filtering it through 0.45μm filter under vacuum. The filtered medium was added to a centrifugal filter tube (cutoff 100K, Millipore) and centrifuged at 3000 rpm at 4°C for 20 minutes. The medium left above the filter inside the centrifugal filter tube was collected and the viral particles were concentrated by ultracentrifugation (50000 rpm for 1h at 4°C). The supernatant was removed and the pellet was resuspended in 100☐l of PBS. The tube was shaken at 4°C for 1h after which it was centrifuged at high speed for two minutes. The supernatant containing the lentivirus particles was aliquoted and stored at -80°C.

3-Virus titer quantification

To quantify the lentivirus titer, primary hippocampal neurons extracted from mouse pups (P1) were dissociated and plated on a poly-D-lysine precoated 24 multiwell and cultured in NeuroBasal plus B27 medium (Invitrogen). Tenfold serial dilutions of the lentiviral preparation were made in the neuronal medium (from undiluted to a dilution of 10^{-7}) by adding 5μl of each precedent sample in 45 μl of

medium to generate the subsequent one. 45μl of each viral dilution was used to infect dissociated hippocampal neurons.

3 days after infection, neurons were fixed with 4% Paraformaldehyde (PFA) in PBS and stained for Green Fluorescence Protein (GFP) (see below) and the percentage of GFP+ cells was calculated as follows:

There were 50 GFP$^+$ cells in the 10^5 dilution well, meaning that 5μl of virus preparation diluted 100,000 times was able to infect 50 cells. 5μl of undiluted virus from the same preparation would have infected 5X 10^6 cells. Assuming that each cell has been infected by only one lentivirus particle, 1ul of this lentivirus preparation contains 1x 10^6 viral infectious particles.

4-Immunostaining to determine lentiviral titer

Infected neurons were fixed with 4% PFA for 10 minutes at RT. After washing with PBS, the cells were permeabilized with 0.5% Triton X-100/PBS for 10 minutes. Blocking solution was made with 3% Bovine Serum Albumin (BSA) in PBS and added to the cells for 1h at RT. The cells were then incubated with primary antibody, anti Green Fluorescence Protein (GFP), (1:1000, Invitrogen) diluted in Blocking Buffer for 1h at RT. After washing with PBS, the secondary antibody diluted in Blocking Buffer (1:1000, Alexa Fluo 488) was added to the cells for 1h at RT. The cells were washed again with PBS and analyzed under epifluorescent microscope (Nikon Eclipse TE300). 3 pictures of each well of 2 chosen lentivirus dilution were taken (usually 10^5 and 10^6) and the number of GFP+ cells was counted.

2.2. DUAL LUCIFERASE REPORTER ASSAY (Promega)

PC12 cells were cultured in a 48 multi well plate in Roswell Park Memorial Institute (RPMI, Invitrogen) medium plus 1% horse serum and Nerve Growth Factor (NGF, Sigma) 50 ng/ml.

Each well of PC12 cells was transfected with the following plasmids, using Lipofectamine 2000 (Sigma) (2.5μl of Lipofectamine/ 1μg DNA) as reagent:

1- 0.1 μg/well pGL3/basic (Promega) with Firefly Luciferase gene (experimental reporter enzyme) under the control of the 25Kb human RTP801 promoter which contains the binding site for ATF4 protein. The expression of Firefly Luciferase is activated by ATF4 binding to the RTP801 promoter;

2- 0.05 μg/well pRL-TK plasmid (Promega) with Renilla Luciferase gene under TK promoter (control reporter enzyme);

and 0.25 μg/well of one of the following plasmids as described previously:

a- pWPI plasmid, without ATF4 gene;

b- pWPI ATF4 plasmid, with ATF4 gene without Flag-tag;

c- pWPI ATF4 tag plasmid, with ATF4 gene and the Flag-tag.

Each combination of 3 plasmids was prepared in RPMI medium (solution 1) and Lipofectamine 2000 was also diluted in RPMI medium (solution 2). Solution 1 and 2 were mixed together and incubated for 20 min at RT and then added to the cells. 2 wells of PC12 cells for each plasmid (a, b or c) solution were transfected. 5 hours after transfection, the medium was replaced with RPMI plus 1% horse serum and NGF (50ng/ml). Two days after transfection the growth medium was removed and cells were rinsed with PBS. The rinse solution was removed and 65ul of 1X Passive Lysis Buffer (PLB) was added into each culture well. The wells were then gently shaken for 15 minutes at RT and the reagent solutions were prepared as follows:

1. 1X PLB: 1 volume of 5X Passive Lysis Buffer (PLB) was added to 4 volumes of DDW.

2. LAR II: lyophilized Luciferase Assay Substrate was resuspended in Luciferase Assay BufferII

3. Stop & Glo® Reagent: 0.2ml of 50X Stop & Glo® Substrate was added to 10ml of Stop & Glo® Buffer to make a 1X solution of Stop & Glo® Reagent.

100μl of LAR II solution and 20μl of PLB lysate were predispensed into luminometer tube and the Firefly Luciferase activity was measured. 100μl of Stop & Glo® Reagent was added to quench the Firefly Luciferase activity and activate Renilla Luciferase whose activity was measured, again by the luminometer.

2.3. PRIMARY MOUSE HIPPOCAMPAL NEURON CULTURE

P0-P1 Wild Type C57BL/6J mice were washed with 75% ethanol and then decapitated. Their brains were placed in a 6cm dish filled with 5ml ice cold MEM (Invitrogen). Through microdissection, their hippocampi were removed and placed in a 3.5cm dish containing ice cold MEM. 200ul of sterile trypsin was added to each dish containing around 8 hippocampi and the dish was placed in incubator at 37°C for 15 minutes (chemical dissociation). By using a pasteur pipet, the hippocampi were transferred into a 15 ml tube containing 2ml plating medium composed by DMEM Glutamax (Invitrogen) plus 10% Calf Fetal Serum (CFS) (GIBCO). A flame-polished glass pasteur pipet was used

to disrupt tissue by pipetting up and down (mechanical dissociation). Cells were then spun down for 8 minutes at 8000 rpm at 4°C. The supernatant was removed and the cells were suspended with plating medium. 200ul of cell suspension plus 800ul of plating medium was plated in each well (24 well plate). The wells were pre-coated with Poly-D- lysine (20ug/ml in Hanks Buffer) (Sigma). After 24h, the plating medium was replaced with 1 ml of formulated A/B27 media /well (Neurobasal A (Invitrogen), 2% B27 (Invitrogen), 1% CFS, 0.4 L- glutamine, 0.8 mM HCl and 8 mM FluorodeoxyUridine (FdU) (16.7mg uridine (Sigma) + 6.7mg FDU (Sigma)). FDU was added to remove glial cells from the cultures.

2.4. WESTERN BLOT

Cells were rinsed with cold 1x PBS before being lysed with 100 µl of lysis buffer (Cell Signaling) containing 1 mM PMSF. Samples were sonicated for 30 seconds, centrifuged for 15 minutes at 4°C and the supernatant was collected. The Bradford Assay system (Pierce) was used to measure protein concentration. The desired volume of sample was added to NuPAGE® LDS Sample Buffer (NuPage, Invitrogen) with 5% beta-mercaptoethanol and boiled for 15 minutes. Samples were run on 10% or 12% bis-tris SDS-PAGE gradient gels (NuPage, Invitrogen). Separated proteins were transferred to nitrocellulose membrane, and blocked for 1 hour in 5% bovine serum albumin in 1x PBS + 0.2% Tween-20 (5% BSA/PBST). The membrane was incubated with primary antibody overnight at 4°C in a blocking buffer. The next day, the membrane was washed with PBST and then incubated with secondary antibody in blocking buffer for 1 hour at RT. Following washes in TBST, membranes were developed with ECL (Thermo Scientific).

Following primary antibodies were used: mouse anti Flag-tag (1:1000, Sigma), rabbit anti ATF4 (1:1000), mouse anti GAPDH (1:2000, Millipore), rabbit anti 4EBPI (1:500, abCAM) and mouse anti actin (1:2000, Millipore). Following secondary antibodies were used: goat anti mouse HRP conjugated (1:5000, Invitrogen) and goat anti rabbit HRP conjugated (1:5000, Invitrogen).

2.5. MICE

All animal studies were conducted according to protocols examined and approved by the Animal Use and Care Committee of Columbia University. All the experiments were performed on 3 month old male C57BL/6 WT mice (Jackson Laboratory). Mice were housed in cage of five until the day before behavioral tests, when they were caged individually. Mice were maintained on a 12h light/dark cycle in temperature and humidity controlled rooms of the animal facility, and allowed ad libitum access to food and water in their home cage. All the experiments were conducted blind during the light cycle.

2.6. SURGICAL PROCEDURES

3 month old male C57BL/6 WT mice were anesthetized with 500 mg/kg avertin (stock solution: 10g of tribromoethanol in 10ml tert-amyl alcohol. Working solution: 0.5 ml of the stock solution in 19.5 ml of warm 1x PBS, injection volume 0.5 ml/25g body weight) and placed in a stereotaxic apparatus. 2 ul of viral preparation (viral titer varied between 10^7 and 10^9 IU/µl) was injected bilaterally through a 31G needle attached to a 50µl Hamilton syringe with a speed of 0.5 µl/min over a period of 4 minutes. After infusion, the needle was left in place for another minute to allow diffusion. The injection coordinates with respect to bregma were; 2.45 mm posterior, 1.8mm lateral and 2mm depth, according to the mouse brain atlas (Fig 2.1).
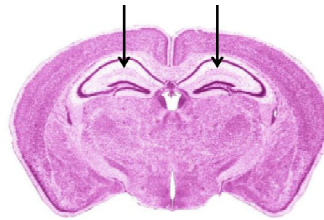
Fig 2.1. Injection site in the dorsal hippocampus

The mice were kept in a warm place until they fully recovered from anesthesia. Mice were divided into 4 groups, 2 for ATF4 overexpression experiment: pWPI (ctrl) and ATF4 and 2 for ATF4 downregulation experiment: shMUT (ctrl) and shATF4. Within each group mice received the respective lentiviral preparation.

2.7. BEHAVIORAL TESTS

The effect of ATF4 overexpression and downregulation on spatial and associative learning and memory was assessed by Morris Water Maze (MWM), Two-day Radial arm Water Maze (RAWM) and fear conditioning (FC) performed one month after lentiviral injection.

2.7.1. MWM

Mice were trained to find a hidden platform in a 1.10m diameter circular pool located in a room with extra maze cues. The location of the platform (10cm diameter) was kept constant for all mice during training and was 1cm beneath the surface of the water which was maintained at 24°C +/- 2°C throughout the duration of the test. Mice were trained with two different training protocols. In the standard protocol, mice were trained in two daily sessions (3h apart), each consisting of three trials

(1 minute each), for three consecutive days. In the weak training protocol, mice were trained in one daily session consisting of three trials (1 minute each), for three consecutive days. Mice were started in different quadrants on a random basis during each trial of training. If a mouse failed to reach the platform within 60s, it was guided to the platform and kept there for 15s to observe visual cues. The training was followed by 4 probe trials (1 minute each) in which the platform was removed from the pool to test the retention of the spatial memory. The maze was divided into 4 quadrants. The percent of time spent in each quadrant, the escape latency and the crossing frequency of the platform area, were recorded and analyzed using Etho Vision XT Tracking System (Noldus information Technology). After the probe trials, visual, motor, and motivation skills were also tested using a visible platform to measure the time and the speed to reach a visible platform placed within the same pool by means of the same video tracking system. During the task mice were tested for two consecutive days, 2 sessions/day with 3 trials/session and the platform position was changed randomly from trial to trial.

2.7.2. RAWM

Mice were tested to find a platform in a 1.10m diameter circular pool containing six swim paths (arms) extending out of an open central area (Fig 2.2). The pool was located in a room with extra maze cues. The water was maintained at 24°C +/- 2°C throughout the duration of testing and it was made opaque by the addition of non-toxic white paint to hide the platform. The location of the platform (10cm diameter) was kept constant for each mouse for the whole duration of the test. Each mouse was tested for 15 trials /day for two consecutive days. On the first day mice were trained for 15 trials, with the first 12 trials alternating between visible (platform 1 cm above the water surface) and hidden (platform 1 cm beneath the water surface) platform. The last 3 trials of the first day and all the 15 trials of the second day were done with hidden platform. Each mouse in each trial started from a different starting position. In each trial, the mouse was allowed to swim freely for 60 seconds in the maze to find the platform. Once on the platform, the mouse was allowed 15 seconds to observe visual cues before starting the next trial. If a mouse was unable to find the platform within 60 seconds, the experimenter guided him towards the platform for the 15 seconds stay. During the 1minute period, each time the mouse entered an arm other than the goal arm (in which the platform was located) or if the mouse did not take any decision regarding which arm to explore within 15 seconds, an error was registered. Entry was defined as the entry of all the four paws of the mouse into the particular arm. The result was shown by dividing the 30 trials into 10 blocks. Each block represents the error average of 3 consecutive trials.
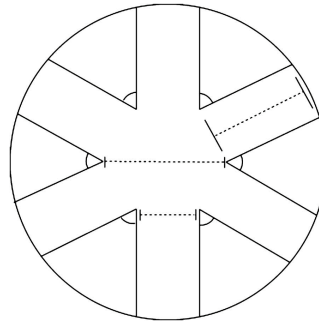
Fig 2.2. Schematic representation of RAWM apparatus.

2.7.3. FEAR CONDITIONING

Our conditioning chamber was located inside a sound-attenuating box (72cm × 51cm × 48cm). A clear plexiglas window (2cm × 12cm × 20cm) allowed the experimenter to film the mouse performance with a camera placed on a tripod and connected to the Freezeframe software (MED Associates Inc.). The conditioning chamber (33cm × 20cm × 22cm) was made of transparent plexiglas on 2 sides and metal on the other 2. One of the metal sides had a speaker and the other had a 24-V light. To provide background white noise (72 dB), a single computer fan was installed in one of the sides of the sound-attenuating chamber. The chamber had a 36-bar insulated shock grid floor. The floor was removable and after each use it was cleaned with 70% ethanol and then with water. Only 1 animal at a time was present in the experimentation room. For the fear conditioning experiments, mice were placed in the conditioning chamber for 2 minutes before the onset of a discrete tone (CS) (a sound that lasted 30 seconds at 2,800 Hz and 85 dB). In the last 2 seconds of the CS, mice were given a foot shock (US) of 0.8 mA for 2 seconds through the bars of the floor. After the CS/US pairing, the mice were left in the conditioning chamber for another 30 seconds and were then placed back in their home cages. "Freezing" behavior, defined as the absence of all movement except for that necessitated by breathing, was scored using the Freezeview software (MED Associates Inc.). To evaluate contextual fear learning, freezing was measured for 5 consecutive minutes in the chamber in which the mice were trained 24 hours after training. To evaluate cued fear learning, 24h after contextual testing, mice were placed in a novel context (triangular cage with smooth flat floor and with vanilla odorant) for 2 minutes (pre-CS test), after which they were exposed to the CS for 3 minutes (CS test), and freezing was measured.

2.8. ELECTROPHYSIOLOGY

Field Excitatory Post-Synaptic Potential (fEPSP) recording. For electrophysiology experiments, mice were killed by cervical dislocation followed by decapitation. Hippocampi were quickly removed.

Transverse hippocampal slices (400 μm) were cut and transferred to a recording chamber where they were maintained at 29℃ and perfused with artificial cerebro-spinal fluid (ACSF) continuously bubbled with 95% O_2 and 5% CO_2. The ACSF was composed of: 124mM NaCl , 4.4mM KCl, 1mM Na_2HPO_4, 25mM $NaHCO_3$, 2mM $CaCl_2$, 2mM $MgSO_4$, and 10mM glucose. The slices were allowed to recover for 90 minutes in the recording chamber before starting recording. Field extracellular recordings were performed by stimulating the Schaffer Collateral fibers through a bipolar tungsten electrode and recording in CA1 stratum radiatum with a glass electrode filled with ACSF. A 15 minute baseline was recorded at an intensity that evoked a response 35% of the maximum evoked response. LTP was induced using a theta-burst stimulation (four pulses at 100 Hz, with the bursts repeated at 5 Hz and each tetanus including three 10-burst trains separated by 15 s). Responses were recorded for 2 h after tetanization and measured as fEPSP slope expressed as percentage of baseline.

2.9. STATISTICAL ANALYSIS

Experiments were performed in blind. All data were presented as mean ± SEM. Results were analyzed with Student t-test and the electrophysiology data with two-way ANOVA with Bonferroni as post-test. The level of significance was set at $p < 0.05$.

2.10. HISTOCHEMISTRY

Once behavioral tests were completed, mice were deeply anesthetized with avertin and transcardially perfused with cold 1x PBS and their brains were removed and fixed in 4% PFA in PBS for two hours. After fixation, brains were rinsed with 1x PBS and transferred to 30% sucrose in PBS until they sunk. Following dehydration in the sucrose, the brains were embedded in OCT (Tissue – Tek, Sakura) cryosectioning matrix and frozen in dry ice. Specimens were sectioned on a cryostat (Micom HM505E) at 12 to 14 μm thickness and collected on Superfrost Plus microscope slides (Fisherbrand) and stored at -80℃.

The slides were thawed at RT for 30 minutes. Tissues were permeabilized with 1x PBS + 0.4% Triton X-100 (PBST) for 20 minutes at RT and then blocked with 10% Fetal Bovine Serum in PBS for 1h at RT. The tissue sections were incubated with primary antibody at 4℃ ON in a blocking buffer. The following day, slides were washed with PBST and then incubated with secondary antibody in blocking buffer for 1 h at RT. Following washes in PBST, the sections were stained with TO-PRO3 or DAPI, a nuclear marker (1:2000 in 1x PBS), for 10 minutes at RT and were then mounted with ProLong Gold antifade reagent (Invitrogen) and observed with Confocal Microscopy (Nikon Eclipse TE200).

Regarding immunostaining of primary hippocampal neurons, cells were fixed with cold 4% PFA in PBS for 15 minutes following the same protocol used for the permeabilization step.

Following primary antibodies were used: mouse anti-Flag tag (1:1000, Sigma), rabbit anti-GFP (1:1000, Invitrogen) and mouse anti NeuN (1:2000, Millipore). Following secondary antibodies were used: Alexa Fluor® 568 goat anti-mouse IgG (1:1000, Invitrogen) and Alexa Fluor® 488 donkey anti-rabbit IgG (1:1000, Invitrogen).

2.11. DIOLISTIC LABELING AND SPINE ANALYSIS

2.11.1. COATING PARTICLES WITH LIPOPHILIC DYE

100mg of tungsten particles (1.1µm, Bio-Rad) were measured and spreaded on a glass slide. A few drops of methylene chloride (Sigma) were added to the tungsten particles and left for drying. 5-8 mg of DiI, a lipophilic tracer, (Invitrogen) was dissolved in 100µl of methylene chloride; this dye solution was added to the particles on the same glass slide. As methylene chloride evaporated quickly, the dye precipitated onto the tungsten particles. The dye coated particles were then dissolved in 3 ml of DDW, vortexed for 1 minute and sonicated in a sinicator bath for 10 minutes to prevent the formation of large clusters.

2.11.2. BULLET PREPARATION

To prepare the "bullets", a tefzel plastic tube (Bio-Rad) was cut according to the prep station size. The tube was treated with 10mg/ml solution of PolyVinylPyrrolidon (Sigma) by sucking it up using a 10 ml syringe connected on one side of the tube and then gently pushing it out. Next, the tube was filled with dye particles solution and placed in the prep station. The excess of liquid was removed by using a syringe and the particles's solution was spreaded by rotation of the prep station for 5 minutes with the dryer on. Once all the liquid was removed, the tubes were cut in 13mm pieces using specialized chopper and stored in a jar at RT.

2.11.3. IMMUNOHISTOCHEMISTRY AND DIOLISTIC LABELING

Mice were anesthetized with avertin and fixed with 4% PFA by transcardial perfusion. Their brains were removed and fixed for another 2 h in 4% PFA at 4°C. The PFA was substituted with 30% sucrose in PBS until the brains sunk to the bottom of the tube. After settling, the brains were embedded in 4% agarose and sectioned coronally (200µm) using vibratome (Leica). Tissue sections were collected

in 24well plate in PBS and stored at 4°C. Tissue sections were stained for GFP protein according to the following protocol:

The sections were first blocked with 10% Fetal Bovine Serum in 1x PBS for 1h at RT. Then they were incubated with primary antibody, anti GFP (1:1000, Invitrogen), 2 ON at 4°C in the blocking buffer solution. The day after, the sections were washed with PBS for 1h at RT and then incubated with secondary antibody (1:1000 A488 goat anti-rabbit fitch) in blocking buffer for 6 h at RT. The sections were then washed with PBS ON.

The following day, the tissue sections were shot with Dil-labeled bullets using Helios gene gun system (Bio-Rad), which label the neuronal architecture in red. During the shooting, PBS was removed from the well and a membrane filter (Millipore 3μm pore size) was placed between the tissue and the gene gun with a makeshift filter holder to prevent large cluster of particles from landing on the tissue together. After shooting, PBS was replaced and the sections were mounted using ProLong Gold antifade reagent (Invitrogen). The sections were analyzed using Confocal microscopy (Nikon). 20 pictures for each sample were taken. Spine density and morphology were analyzed in neurons labeled with both GFP and Dil using the NeuronStudio (Beta) software (Rodriguez et al., 2008).

2.12. qRT-PCR

1 month after lentiviral injection, animals were sacrificed by cervical dislocation followed by decapitation and their brains were removed and the hippocampi were isolated and snap frozen in liquid nitrogen.

2.12.1. TOTAL RNA EXTRACTION

Total RNA was extracted from the mouse hippocampus according to the RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissue (Quiagen kit).During the RNA extraction, the optional DNase digestion step was performed following the protocol, Optional on–column DNase Digestion with RNase-Free DNase set provided by the same QUIAGEN kit. Once eluted, the RNA was quantified using BioPhotometer machine (eppendorf) by diluting 5ul of RNA sample in 45 ul of Nuclease free DDW.RNA integrity was assessed by running the RNA samples on a 1% Agarose gel made by dissolving agarose powder in TAE buffer (1X). 3ul of Ethidium Bromide was added to the solution to visualize the RNA bands under UV light. The samples were prepared by adding 1x loading buffer and

about 0.5ug of RNA. The gel was run at 140V for 30 minutes.RNA integrity was checked under UV lamp.

2.12.2. CDNA RETROTRANSCRIPTION

cDNA strand was generated from RNA using the 1st Strand cDNA Synthesis System for quantitative RT-PCR (Origene) following the protocol instructions provided by the kit. Briefly, all the components were thawed and mixed thoroughly. 0.2ml thin-walled PCR tubes were prepared as follows:

RNA (10pg to 1ug total RNA): x µl, Nuclease free DDW: 15-x µl, 5x cDNA Synthesis Mix: 4 µl,Reverse Transcriptase: 1µl, Total volume: 20µl.

The tubes were placed in a thermocycler programmed as follows:

1 cycle 22 C, 5 min, 1 cycle 42 °C, 30 min, 1 cycle 85 °C, 5 min, 4 °C hold.

The real-time PCR thermocycling parameters were: 22°C for 5 min (1 cycle), 42°C for 30 min (1 cycle) and 85°C for 5 min (1 cycle).

2.12.3. qRT-PCR

Primers specific for mouse ATF4 were designed using the Primer 3 design program:

Forward: 5' ATG CCA GAT GAG CTC TTG ACC AC 3'

Reverse: 5' GTC ATT GTC AGA GGG AGT GTC TTC 3'

Parameters for qRT-PCR were as follows: preheat at 95°C for 10 min; followed by 40 cycles of 95°C for 15s, 58°C for 15s and 72°C for 20s. qRT-PCR (Eppendorf) was performed in triplicate and Alpha Tubulin was used as housekeeping gene to normalize input cDNA using the following primers:

Forward: 5' ATG AGG CCA TCT ATG ACA TC 3'

Reverse: 5' TCC ACA AAC TGG ATG GTA C 3'.

CHAPTER III: RESULTS

To study the role of ATF4 in learning and memory, lentivirus carrying ATF4 gene, for the gain-of-function analysis, and lentivirus carrying shATF4, for the loss-of-function analysis were inoculated into the hippocampi of adult mice. For the ATF4 overexpression studies, the lentiviral constructs were prepared by cloning the ATF4 Flag-tag gene in the pWPI plasmid and used the linearized pWPI, without ATF4 gene, as control. Regarding the downregulation experiments, a shATF4 sequence was cloned in the pLVTHM vector and the same shATF4 sequence with 6 mutations, to prevent it from binding ATF4 mRNA, was used as control. Each of these constructs also carried the Green Fluorescence Protein (GFP) gene that allowed us to visualize the infected cells.
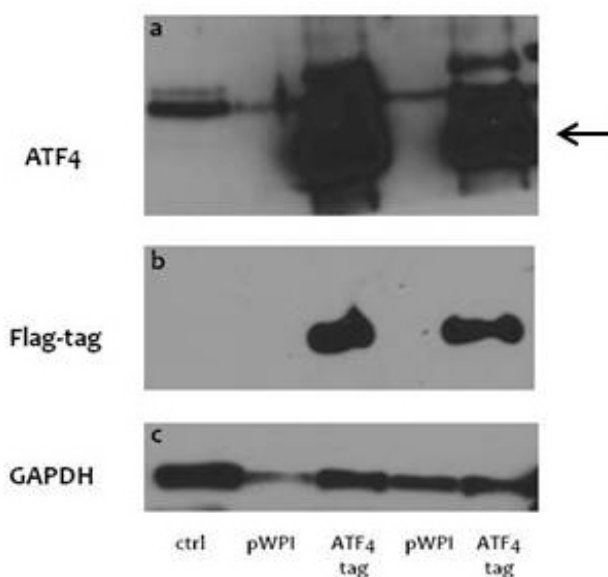
3.1. *IN VITRO* TESTING OF LENTIVIRAL CONSTRUCTS

Before injecting the lentivirus into the mouse brain, the lentiviral constructs were tested *in vitro,* to check their ability to overexpress and silence ATF4 in primary hippocampal neurons. The lentiviral constructs were tested by western blot 1 week after the infection.
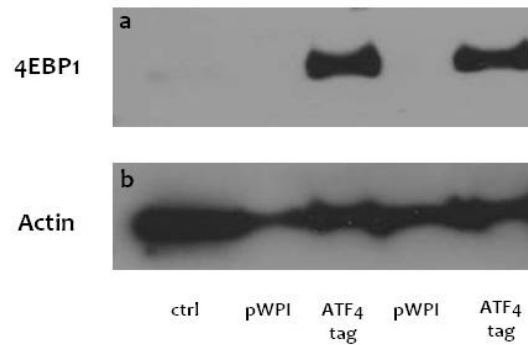
3.1.1. ATF4 OVEREXPRESSING LENTIVIRAL CONSTRUCTS

Fig 3.1a shows that hippocampal neurons not infected with lentivirus (ctrl) and those infected with control lentivirus without ATF4 gene (pWPI) do not express ATF4 protein, while neurons that have been infected with lentivirus carrying ATF4 gene show a strong expression of this protein (ATF4 tag). In the ctrl and pWPI samples, ATF4 protein was not detected because under normal condition ATF4 protein is expressed at a very low level which is not detectable with a short-time film exposition. This result showed that the lentiviral construct carrying the ATF4 gene is able to infect neurons and use their "machinery" to strongly overexpress ATF4 protein. Since there are no commercially available antibodies to detect ATF4 protein in tissue, a Flag-tag sequence was cloned at the C-terminal of ATF4 gene to detect the overexpression of ATF4 protein after injection. The Flag-tag antibody did not detect ATF4 protein in ctrl neurons without infection (Fig 3.1b, ctrl) and in neurons infected with control lentivirus (pWPI), while two strong bands were detected by the Flag-tag antibody in neurons infected with lentivirus carrying the ATF4 gene (ATF4 tag).

The Flag-tag antibody was able to detect the ATF4 protein whose expression was due to the lentiviral infection. Moreover the expression of an ATF4 downstream target gene, 4EBP1, was tested to see if the ATF4 protein overexpression causes the modification of 4EBP1 expression (Fig 3.2). 4EBP1 protein, under normal conditions, is expressed at very low level but its expression is strongly upregulated after ATF4 overexexpression. Cultured neurons in which ATF4 was upregulated (ATF4 tag) also expressed 4EBP1 protein, while in non-infected neurons (ctrl) and neurons infected by control lentivirus (pWPI), 4EBP1 protein was not detected.

Fig 3.1. *In vitro* **testing of lentivirus for ATF4 overexpression.** Total protein extraction was performed on mouse primary hippocampal neurons not infected with the lentivirus (ctrl), infected with control lentivirus without ATF4 Flag-tag gene (pWPI) and infected with lentivirus carrying the ATF4 Flag-tag gene (ATF4 tag). The blot probed with anti ATF4 antibody (a) showed an increase in ATF4 expression in neurons infected with the lentivirus carrying ATF4 Flag-tag gene, as compared to the other two experimental conditions. The blot probed with anti Flag-tag antibody (b) showed the presence of the Flag-tag in neurons infected with the lentivirus carrying the ATF4 Flag-tag gene. Blot c shows GAPDH loading control.
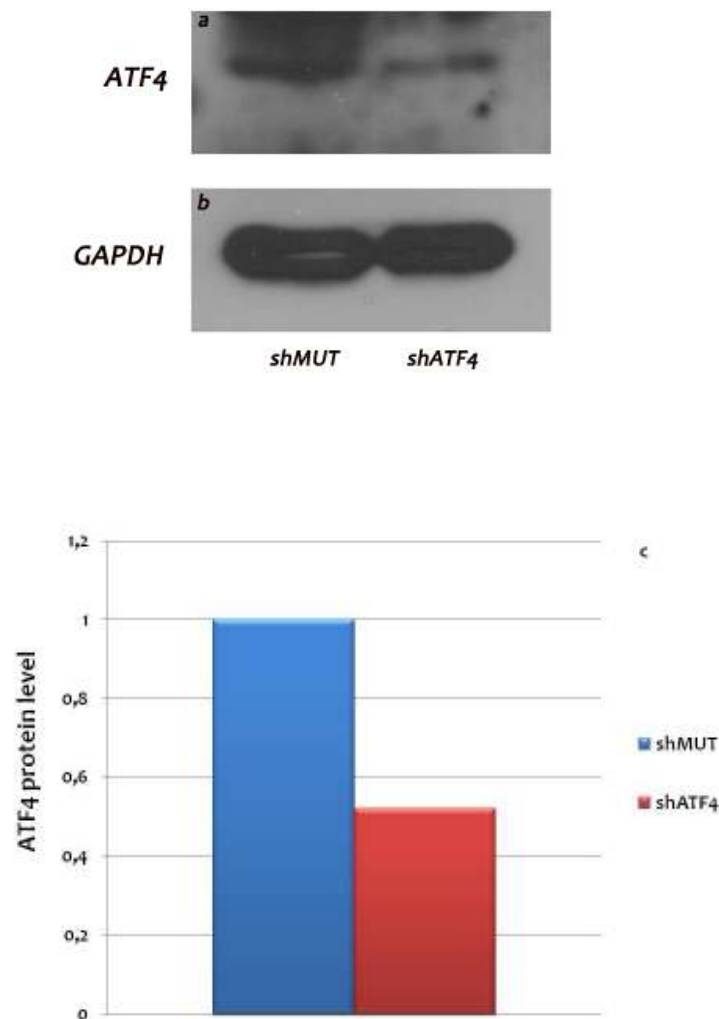
Fig 3.2. *In vitro* testing of lentivirus for ATF4 overexpression. Total protein extraction was performed on mouse primary hippocampal neurons not infected with the lentivirus (ctrl), infected with control lentivirus without ATF4 Flag-tag gene (pWPI) and infected with lentivirus carrying the ATF4 Flag-tag gene (ATF4 tag). The blot probed with anti 4EBP1 antibody (a) showed that neurons infected with lentivirus carrying the ATF4 Flag-tag gene (ATF4 tag) have an increase in 4EBP1 protein expression as compared to both non-infected neurons (ctrl) and neurons infected with the control lentivirus (pWPI). Blot b shows Actin loading control.

## 3.1.2. ATF4 DOWNREGULATING LENTIVIRAL CONSTRUCTS

shATF4 binds to ATF4 mRNA and blocks its translation, thus downregulating ATF4 expression. ATF4 downregulation control lentivirus (shMUT) was prepared using the same shATF4 sequence by inserting 6 mutations into it to prevent it from binding ATF4 mRNA. A shMUT construct was used as control to make sure that any effects seen after ATF4 silencing were not an artifact due to the presence of a shRNA.

Primary hippocampal neurons infected with shATF4 lentivirus (Fig 3.3a) showed a downregulation of ATF4 protein as compared to those infected with shMUT lentivirus. After normalization for the housekeeping gene GAPDH, a 50% reduction in ATF4 protein level was found in neurons infected with shATF4 lentivirus as compared to ctrl (Fig 3.3c). The 4EBP1 protein level analysis revealed that shATF4 lentivirus caused approximately 40% downregulation of 4EBP1 as compared to the control shMUT lentivirus (Fig 3.4). These results confirmed that shATF4 lentivirus is able to downregulate both ATF4 and 4EBP1 proteins in primary hippocampal neurons 1 week after infection.

Fig 3.3. *In vitro* **testing of lentivirus for ATF4 downregulation.** Total protein extraction was performed on mouse primary hippocampal neurons infected with control lentivirus carrying a mutant shRNA (shMUT) and with lentivirus carrying a shRNA against the ATF4 mRNA (shATF4). The blot probed with anti ATF4 antibody (a) showed a reduction in ATF4 protein level in neurons treated with shATF4 lentivirus (shATF4) as compared to those infected with control lentivirus (shMUT). Blot b shows GAPDH loading control. The graph in panel c represents the quantification of ATF4 protein level after normalization with the housekeeping GAPDH. The ATF4 expression level in neurons treated with shATF4 lentivirus decreased by 50% as compared to neurons treated with shMUT lentivirus, used as control.
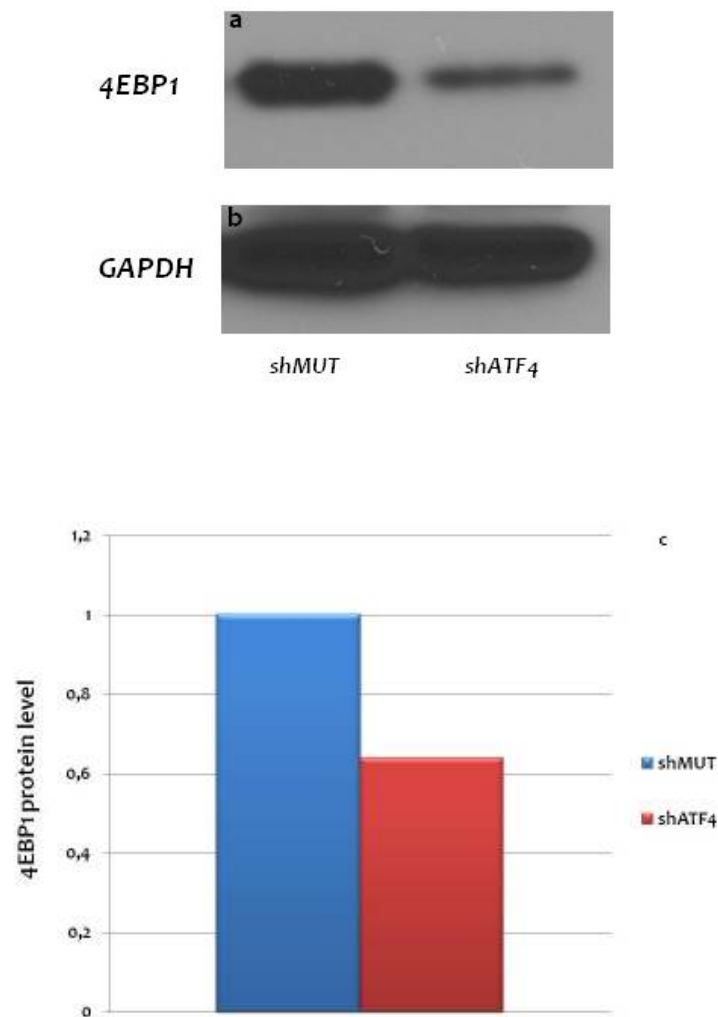
Fig 3.4. *In vitro* **testing of lentivirus for ATF4 downregulation.** Total protein extraction was performed on mouse primary hippocampal neurons infected with shMUT lentivirus and shATF4 lentivirus. The blot probed with an anti 4EBP1 antibody (a) showed that neurons infected with shATF4 lentivirus have a reduction in 4EBP1 protein level (shATF4) as compared to those infected with shMUT lentivirus. Blot b shows GAPDH loading control. The graph in panel c represents the quantification of ATF4 protein level after normalization with the housekeeping GAPDH. The 4EBP1 expression level in neurons treated with shATF4 lentivirus decreased by 40% as compared to neurons treated with shMUT lentivirus, used as control.

3.2. ANALYSIS OF ATF4 FLAG-TAG PROTEIN'S TRANSCRIPTIONAL ACTIVITY

Since ATF4 is a transcription factor, its main cellular function is to activate or repress the transcription of target genes. To detect ATF4 protein overexpression, a Flag-tag sequence was cloned at the C-terminal of ATF4 gene. To check if the Flag-tag sequence could interfere with the ATF4 protein's transcriptional activity, the ability of ATF4 Flag-tag to activate the transcription of a reporter enzyme, Firefly Luciferase, was tested using a Dual Luciferase Reporter Assay. Firefly Luciferase gene was cloned under the RTP801 promoter which has an ATF4 consensus binding site, so its transcription is activated by ATF4 protein. In this assay, we also measured the expression of another reporter enzyme, Renilla Luciferase, to check for transfection efficiency and cell viability. The vector carrying the ATF4 gene, without Flag-tag, was used as positive control and the empty vector (pWPI), without ATF4 gene, was used as negative control.

Fig 3.5 shows that the ability of ATF4 Flag-tag protein to activate transcription of Firefly enzyme is comparable to that of ATF4 protein without Flag-tag. This indicates that the Flag-tag cloned at the end of the ATF4 gene does not interfere with ATF4's transcriptional activity. Moreover, PC12 cells transfected with the vector carrying the ATF4 gene, with and without Flag-tag, exhibit higher Firefly enzyme transcription compared to those transfected with the empty vector (pWPI), in which the activation of Firefly enzyme is due to the endogenous ATF4. The increase in Firefly transcription in PC12 cells transfected with the vector carrying the ATF4 gene, with and without Flag-tag, is due to the overexpression of ATF4 protein after transfection.
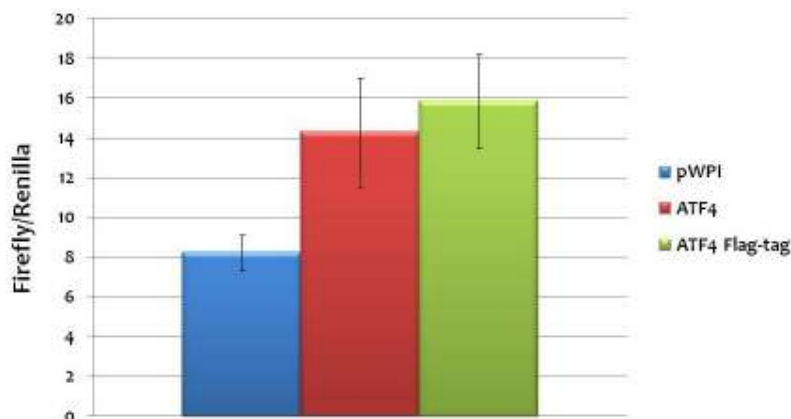
Fig 3.5. Dual Luciferase Reporter Assay for ATF4 Flag-tag's transcriptional activity analysis. After normalization with Renilla enzyme, the Firefly level of differentiated PC12 cells transfected with the vector carrying the ATF4 gene, with and without Flag-tag (ATF4, ATF4 tag), was almost double compared to that of PC12 cells transfected with the vector without ATF4 gene (pWPI). The increase in Firefly transcription is due to ATF4 overexpression after transfection. ATF4 Flag-tag protein is able to activate the transcription of Firefly enzyme at the same level as the ATF4 protein without Flag-tag, indicating that the Flag-tag does not interfere with ATF4's transcriptional activity.

3.3. ANALYSIS OF THE LENTIVIRAL SPREAD AFTER INTRAHIPPOCAMPAL INJECTION

3.3.1. ANALYSIS OF THE INJECTION COORDINATES

After verifying the lentiviral activity *in vitro*, mouse hippocampi were injected with the lentivirus. Injection coordinates were chosen according to the mouse brain atlas to target the hippocampus and were checked using our stereotaxic apparatus. 2ul of pWPI control lentivirus was injected into both hemispheres of adult mice at the chosen coordinates. 1 week after injection, immunostaining was performed using anti GFP antibody to detect the infected cells. Fig 3.6 shows that GFP staining (green) mainly localized in the Dentate Gyrus (DG) area (a,c) and at the beginning of the CA3 area (b,d), proving that our chosen coordinates are correct to target the hippocampus.
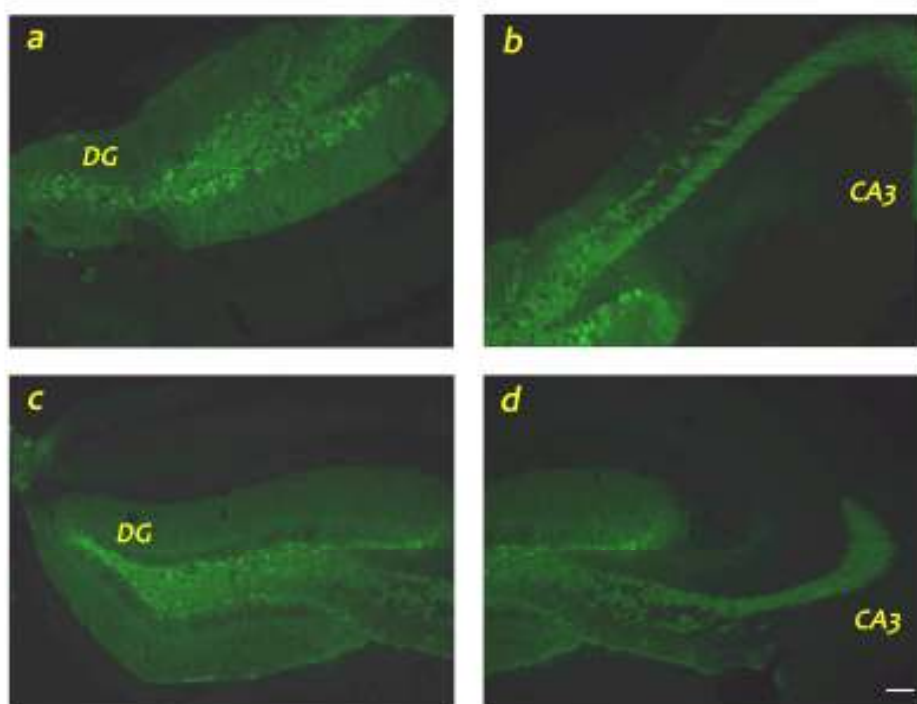
Fig 3.6. Evaluation of the injection coordinates. 2ul of pWPI control lentivirus was injected into the hippocampus following the coordinates reported in experimental procedures section. 1 week after injection, immunostaining for GFP protein was performed to visualize the cells that had been infected by the lentivirus. The GFP positive cells were found mainly in the dentate gyrus area (DG) of the hippocampus (a,b) and in the CA3 region that originates from the DG (CA3)(bar 50μm).

3.3.2. ANALYSIS OF LENTIVIRAL TROPISM

The next step was to verify *in vivo* the tropism of the lentiviral particles. 2ul of the pWPI control lentivirus was injected into both hippocampi of adult mice following the pre-selected coordinates. 1 week after injection, immonostaining was performed using anti GFP antibody, to detect the cells that had been infected by the lentivirus, and anti NeuN antibody as neuronal marker and the brain sections were counterstained with TO-PRO3, a blue nuclear marker. Cell counting was performed in 3 mouse brains choosing 3 different hippocampal sections per mouse. For each section, GFP$^+$ cells, NeuN$^+$ cells and all the nuclei were counted, and since only the cells in the DG were infected by lentivirus (GFP$^+$ cells), cell counting was performed only in this area. Table 1 shows that 1 week after injection, almost 30% of the cells in the DG expressed GFP protein so they had been infected by the lentivirus and that almost 90% of the GFP$^+$ cells were NeuN$^+$.This result shows that the lentiviral particles exhibit a major tropism for neurons, because almost 90% of the infected cells were positive for NeuN staining, a neuronal nuclear marker.

| TABLE3	TOPRO3$^+$	GFP$^+$	%GFP/TOPRO3	NeuN$^+$,GFP$^+$	%NeuN/GFP
Mouse 1	323	75	23	67	89
Mouse 1	205	45	20	35	83
Mouse 1	338	81	24	64	79
Mouse 2	226	37	16	27	73
Mouse 2	313	143	46	120	89
Mouse 2	267	95	32	75	79
Mouse 3	432	151	35	137	91
Mouse 3	378	83	22	76	92
Mouse 3	293	85	29	68	80

Table 1. Cell count to assess lentiviral tropism.

3.3.3. ANALYSIS OF LENTIVIRAL SPREAD

Before starting the experiments of ATF4 overexpression and downregulation the lentiviral spread in mouse hippocampus was evaluated. Our aim was to allow the lentivirus to spread throughout the hippocampus before starting the behavioral tests, performed to assess the animal's cognitive functions. After the lentiviral injection, the animals were sacrificed at different time points to select the correct time window between the injection and the behavioral tests. 2ul of pWPI control lentivirus was injected into both hippocampi of adult mice following the coordinates mentioned before. Lentiviral spreading was analyzed at 1 week, 2 weeks and 4 weeks after injection by counting the GPF$^+$ cells in the DG. Cells were counted only in the DG because 1 and 2 weeks after the injection GPF$^+$ cells were found only in this area. For each time point, cells were counted in 3 mouse brains choosing 3 different hippocampal sections per mouse. The cell count analysis revealed that 1 week after injection around 30% of the cells in the DG had been infected by the lentivirus, as was found in the experiment described in the section 3.3.2. 2 weeks after injection, the GFP$^+$ cells in the same area increased to 40% while 1 month after injection, more than 50% of the cells in the DG were infected (Fig 3.7.1). Moreover, 2 weeks after injection the GFP$^+$ cells, visualized in green, were mainly localized in the DG even though some GFP$^+$ neurons were also seen in the CA1 region (Fig 3.7.2a). Fig 3.7.2 b, c and d show high magnification of DG region, 2 weeks after injection, where the infected cells expressed the GFP protein both in the cell body and along the processes. Fig 3.7.3 shows the lentiviral spreading 4 weeks after the injection. At this time point, the lentivirus also spread to the CA1 region (Fig 3.7.3a) meaning that, after the injection, the lentivirus took around 1 month to spread and infect neurons from DG to CA1 region. Fig 3.7.3 b, c and d are high magnifications of DG (Fig 3.7.3 b) and CA1 (Fig 3.7.3 c,d) region. Infected neurons expressed GFP protein (green) both in the cell body and along the processes. On the basis of these results, we decided to start the behavioral tests 4 weeks after lentiviral injection. At this time point, the lentiviral particles were spread throughout different regions of the hippocampus and at least 50% of the cells in the DG were infected.
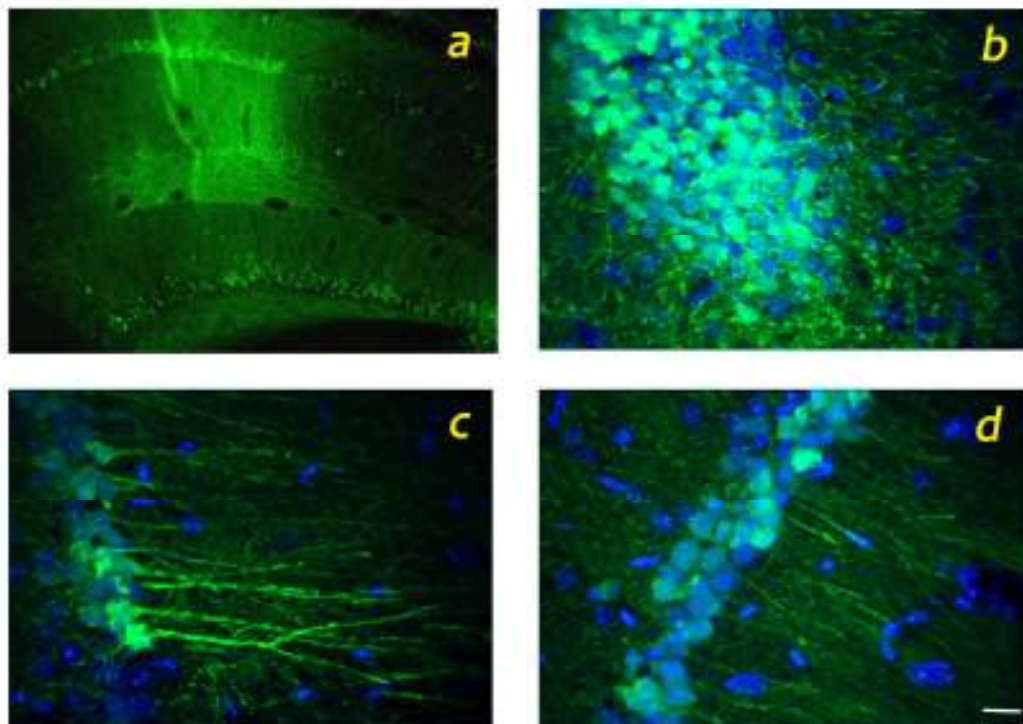
3.7.1
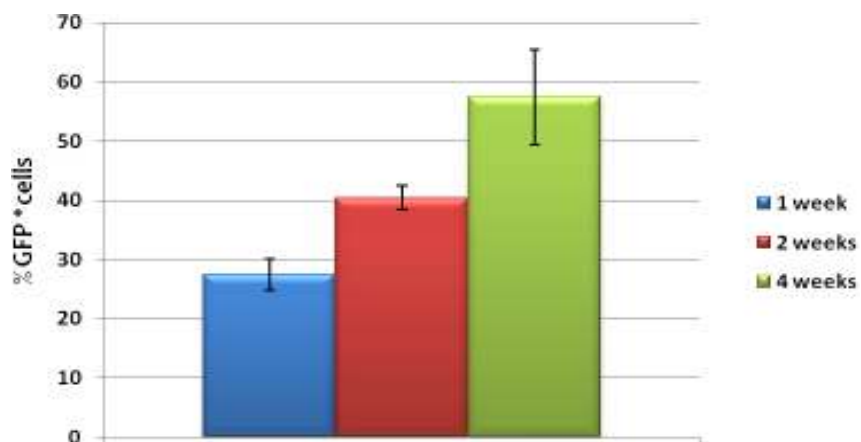
3.7.2
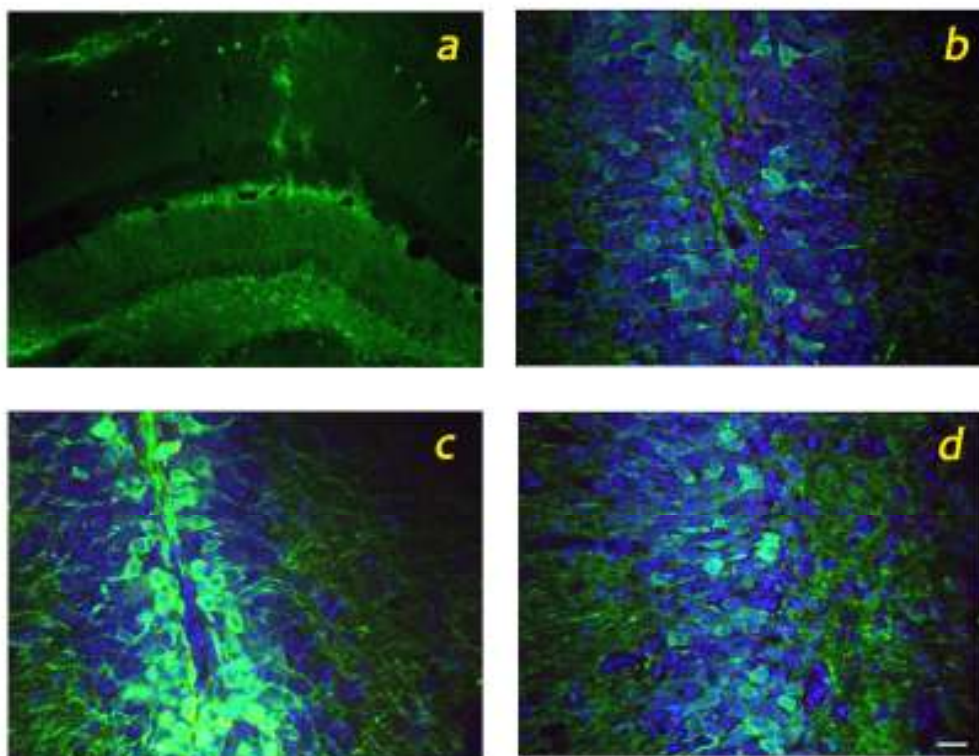
3.7.3

Fig 3.7. Analysis of lentiviral spread in the hippocampus. 2ul of pWPI control lentivirus was injected bilaterally into mouse hippocampus. The animals were sacrificed at different time points: 1 week, 2 weeks and 4 weeks after injection and immunostaining on brain slices was performed to count the GFP$^+$ cells. 3.7.1: number of GFP$^+$ cells in the DG: 1 week after injection around 27% of the cells were GFP$^+$; 2 weeks after injection around 40% of the cells were infected by the lentivirus and 4 weeks after injection 57% of the cells were positive for GFP protein. 3.7.2a: low magnification of CA1 and DG area 2 weeks after lentiviral injection, showing that at this time point there were a lot of GFP$^+$ cells (green) in the DG region while only a few cells had been infected by the lentivirus in the CA1 region. 3.7.2b, c and d: high magnification of DG area 2 weeks after injection. 3.7.3a: low magnification of CA1 and DG area 4 weeks after injection, showing that 1 month after injection the lentivirus also spread to the CA1 region and infected neurons in this area. 3.7.3b: high magnification of the DG area. 3.7.3c and d: high magnification of CA1. Blue represents the cell nuclei stained with TO-PRO3. (bar 10μm).

3.4. ATF4 OVEREXPRESSION

After analyzing lentiviral spread, the role of ATF4 protein in learning and memory was studied by using both gain-and loss-of-function approaches. First I will describe the results obtained after ATF4 overexpression followed by those obtained after ATF4 downregulation.
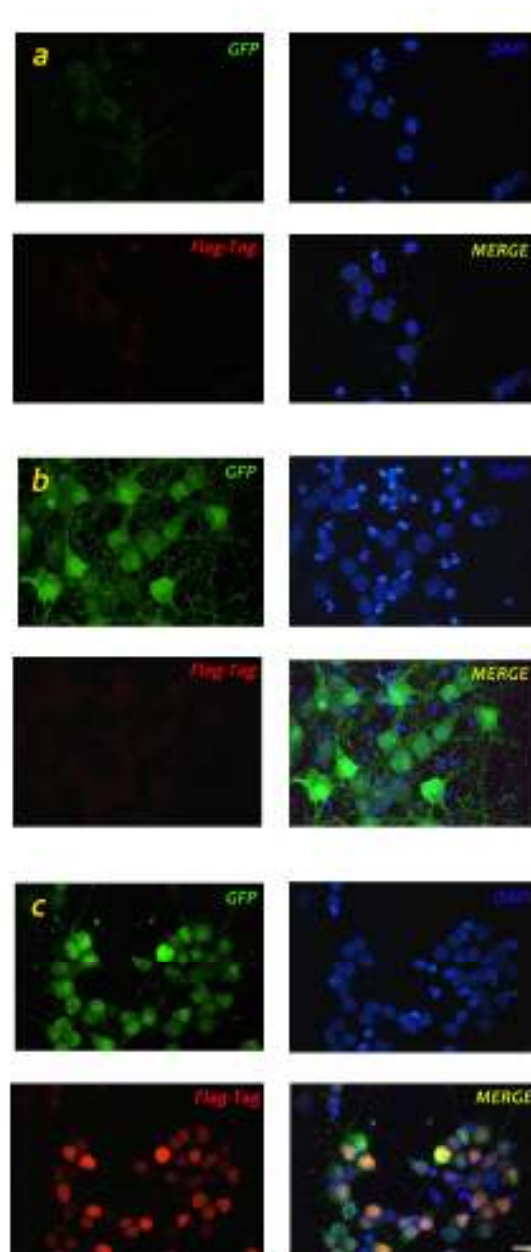
3.4.1. DETECTION OF OVEREXPRESSED ATF4 PROTEIN

Mouse cognitive function was assessed, under conditions of ATF4 overexpression and downregulation, 4 weeks after injection because at this time point a good number of hippocampal cells was found infected, hence it can be the best time to see a phenotype in behavioral test. Since specific antibodies, able to detect ATF4 protein in vivo, are not commercially available, a Flag-tag sequence was cloned at the C-terminal of ATF4 gene. Western blot was performed, using the Flag-tag antibody, to detect the overexpressed ATF4 protein in the infected mouse hippocampal protein extract. A very high background was obtained (data not shown) and the Flag-tag band was not visualized; hence the ATF4 protein overexpression was analyzed by immunostaining.

First the Flag-tag antibody was tested by immunostaining *in vitro*. Dissociated primary hippocampal neurons were infected with 2µl of control lentiviral preparation (Fig 3.8b) or 2µl of lentiviral particles carrying the ATF4 gene with the Flag-tag (Fig 3.8c). Dissociated primary hippocampal neurons without infection were used as control (Fig 3.8a). 1 week after infection, cells were fixed and immunofluorescence staining was performed to visualize the ATF4 overexpression mediated by the lentivirus. Non infected hippocampal neurons, visualized in blue by the DAPI nuclear staining, were negative for both GFP (green) and Flag-tag (red) staining (Fig 3.8a). Neurons infected with the control lentivirus without ATF4 gene were positive for GFP staining (green), index of infection, and negative for Flag-tag staining (red) (Fig 3.8b). Hippocampal neurons infected with the lentivirus carrying the ATF4 gene were positive for both GFP (green) and Flag-tag (red) staining (Fig 3.8c) meaning that 1 week after infection, the neurons overexpressed ATF4 protein that could be visualized by immunostaining using an antibody against the Flag-tag cloned at the C-terminal of ATF4 gene. GFP protein was localized mainly in the cell body and along the processes, while ATF4 protein with the Flag-tag was present only in the nucleus.

After verifying by immunostaining that the Flag-tag antibody works *in vitro,* overexpressed ATF4 protein was detected in the mouse hippocampus 1 month after lentivirus injection, the time point chosen for the following analysis. 3 months old C57BL/6J male mice were inoculated bilaterally with 2 µl of control lentivirus without ATF4 gene (pWPI) or with 2 µl of lentivirus carrying ATF4 gene with

the Flag-tag (ATF4). 4 weeks after injection, animals were transcardially perfused with 4%PFA. Their brains were removed and frozen in OCT and then sectioned. Immunostaining was performed to detect the ATF4 protein overexpression. pWPI mouse brain sections were positive for GFP staining, shown in green (Fig 3.9a), and negative for Flag-tag staining, shown in blue (Fig 3.9b), in the hippocampus. ATF4 mouse brain sections were positive for both GFP (Fig 3.9d) and Flag-tag staining (Fig 3.9e) in the hippocampus. The overlapping between figures 3.9d and e (Fig 3.9f merge) shows that the Flag-tag was not localized in the cell nucleus but mainly in the cytoplasm. Figures 3.9g, h and i show the cortical area of the same brain section obtained from mice inoculated with lentivirus carrying the ATF4 gene. Cells in this area were not infected as proved by the negativity for GFP (Fig 3.9g) and Flag-tag (Fig 3.9h) staining.

These results show that 1 month after injection, the hippocampal cells that were infected with the lentivirus carrying the ATF4 gene and the Flag-tag, expressed not only GFP but also ATF4 protein that could be detected thanks to the Flag-tag cloned at the C-terminal of ATF4 gene.

Fig 3.8. *In vitro* detection of overexpressed ATF4 protein. Dissociated primary hippocampal neurons were infected with control lentivirus without ATF4 gene (b) or with lentivirus carrying the ATF4 gene and the Flag-tag (c). Non infected neurons were used as control (a). Non infected neurons, visualized by the DAPI staining (blue), were negative for both GFP (green) and Flag-tag (red) staining (a). Neurons infected with the control lentivirus without ATF4 gene were positive for GFP (green) and negative for Flag-tag (red) staining (b). Neurons infected with the lentivirus carrying the ATF4 gene were positive for both GFP (green) and Flag-tag (red) staining (c). GFP protein was localized mainly in the cell body and along the processes, while ATF4 protein with the Flag-tag was present only in the nucleus (bar 15 μm).
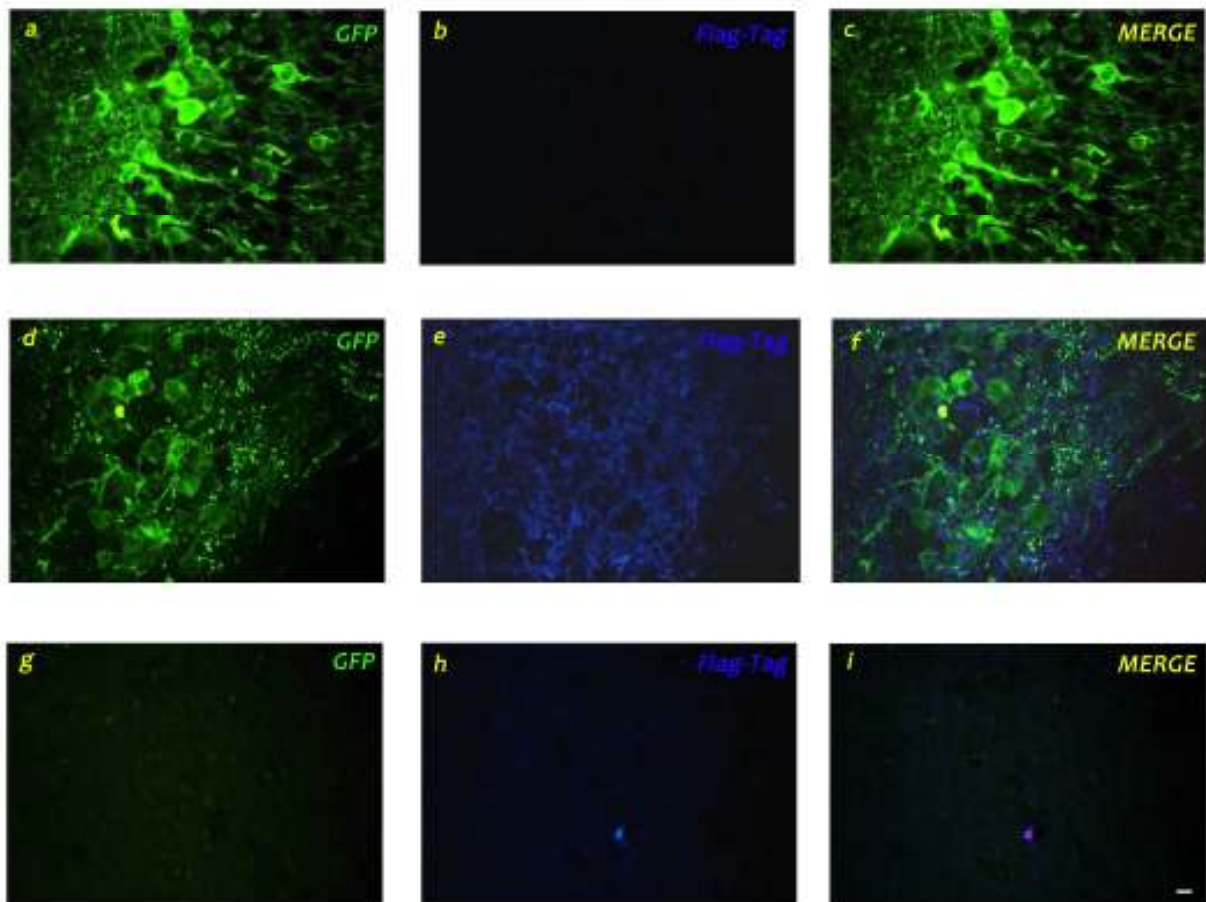
Fig 3.9. *In vivo* detection of overexpressed ATF4 protein. 3 months old C57BL/6J male mice were inoculated bilaterally with 2 μl of control lentivirus without ATF4 gene (a,b,c) or with 2 μl of lentivirus carrying ATF4 gene with the Flag-tag (from d to i). pWPI mouse brain sections were positive for GFP (green) (a) and negative for Flag-tag (blue) staining (b) in the hippocampus. ATF4 mouse brain sections were positive for both GFP (d) and Flag-tag staining (e) in the hippocampus. The merge in figure f shows that the Flag-tag was localized mainly in the cytoplasm and not in the cell nucleus. Figures g, h and i show the cortical area of the same brain section obtained from ATF4 overexpressed mice. Neurons in this brain area were not infected, so they were negative for both GFP (g) and Flag-tag (h) staining. 1 month after injection, the cells infected with the lentivirus carrying the ATF4 gene expressed both GFP and ATF4 protein detected by the presence of the Flag-tag cloned at the C-terminal of ATF4 gene (bar 5 μm).

3.4.2. BEHAVIORAL TESTS

To verify the involvement of ATF4 in learning and memory, ATF4 overexpressed and associated control mice were subjected to a variety of different behavioral tasks, able to assess different kinds of hippocampus-dependent memory. The following behavioral tests were performed: Morris Water Maze (MWM), Two-day Radial Arm Water Maze (RAWM) and Fear Conditioning (FC).

3.4.2.a. Morris Water Maze

MWM is a behavioral task widely used in behavioral neuroscience to study spatial learning and memory. It was developed by Richard G. Morris in 1981, who used it to show that hippocampal lesions impair spatial learning (Morris et al., 1982). ATF4 overexpressed and associated control mice were subjected to MWM test 4 weeks after lentiviral injection. The results of this behavioral test showed that during the standard training (Fig 3.10a), there was no significant difference between ATF4 overexpressed and control mice regarding the time taken by the mice to locate the hidden platform. Throughout the six sessions, both groups were comparable. At the end of training, during the last session, both groups of mice reached the platform in around 25s.

During the probe trial, both groups of mice spent more time exploring the target quadrant, where the platform was located, in comparison to the other three quadrants (Fig 3.10b1). Moreover, both groups spent less time exploring the opposite quadrant. Analyzing in detail, it can be noticed that ATF4 overexpressed mice spent on average around 37s in the target quadrant while the controls around 32s. This difference is not statistically significant. Other parameters were also considered: crossing frequency of the platform area (Fig 3.10b2) and time taken to reach the platform area for the first time in each probe trial (Fig 3.10b3) There were no statistically significant differences between the two groups while analyzing both parameters. Both groups crossed the platform area 2.5 times on average, in each 60s trial. ATF4 overexpressed mice reached the platform area for the first time in around 22s, while the control mice took a few seconds more, around 27s.

After MWM test, visible platform test was performed on the same mice where the aim was to locate a visible platform. The following parameters were analyzed during this test, time taken to reach the platform and mice's swimming speed. The aim of the test was to exclude those mice that did not locate the hidden platform, not because of cognitive impairment but because of visual and motor problems. The results of the visible platform test (Fig 3.10c) showed that all the mice enrolled in the experiment did not exhibit visual or motor deficits; hence none of them were excluded from the data analysis. The graph in Fig 3.10c1 shows that there was no significant difference between ATF4

overexpressed and control mice regarding the time taken by the mice to locate the visible platform during each session. During the first session, ATF4 overexpressed mice reached the visible platform in around 29s and the controls in 24s. During the last session, the average time was shorter; ATF4 overexpressed mice reached the platform in 24s and the controls in 17s. Regarding speed, both curves were identical (Fig 3.10c2). There was a small increase in speed during the sessions from 15cm/s in the first session to 20cm/s in the last one.

These results underline that ATF4 protein overexpression in the hippocampus of adult mice does not affect spatial learning and memory.

a) MWM: standard training

1

2   3

b) MWM: probe

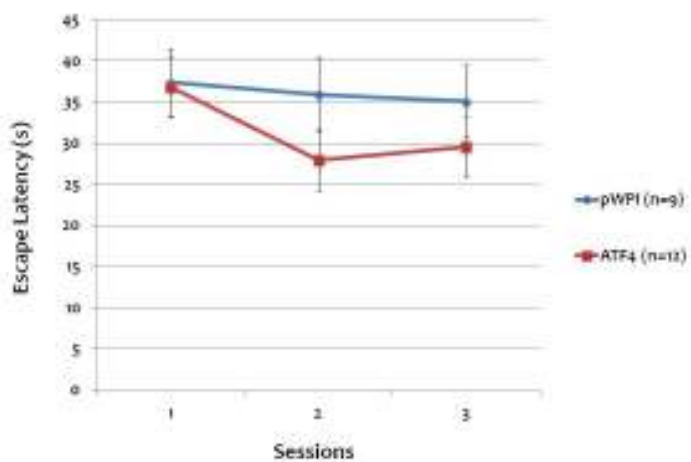1   2

c) visible platform test
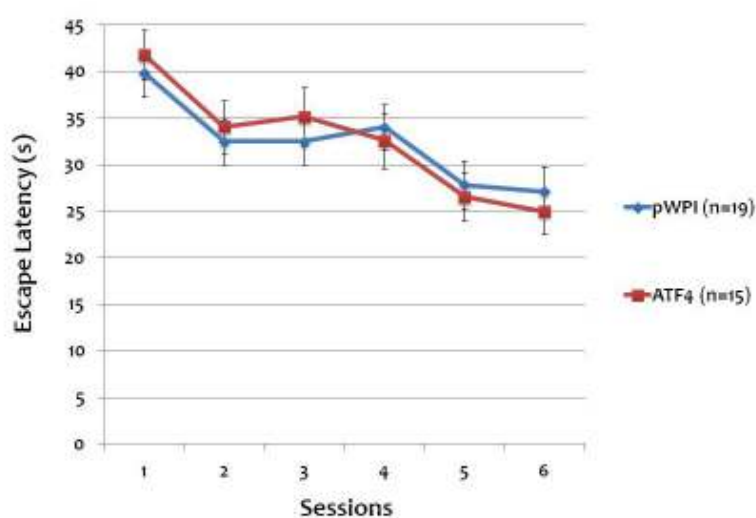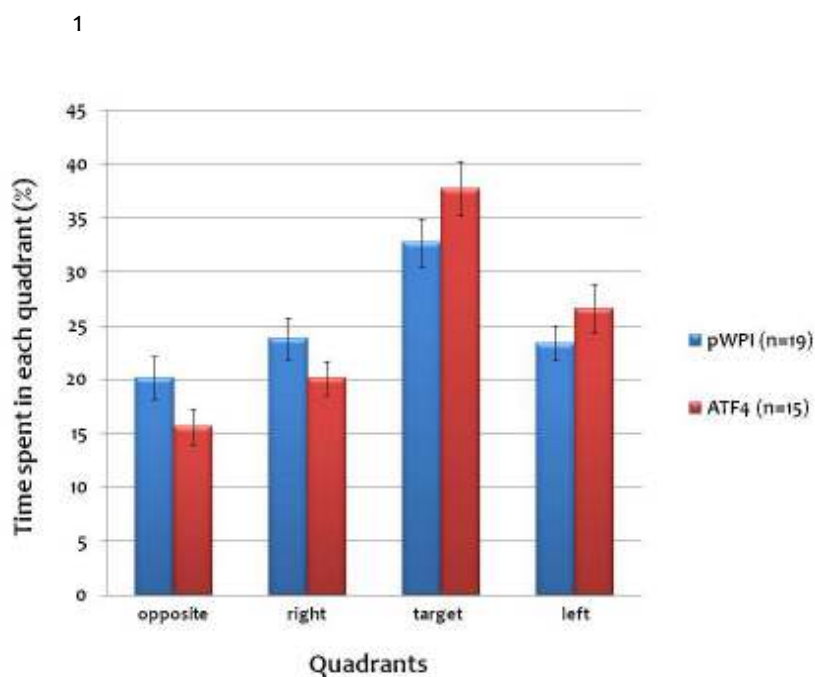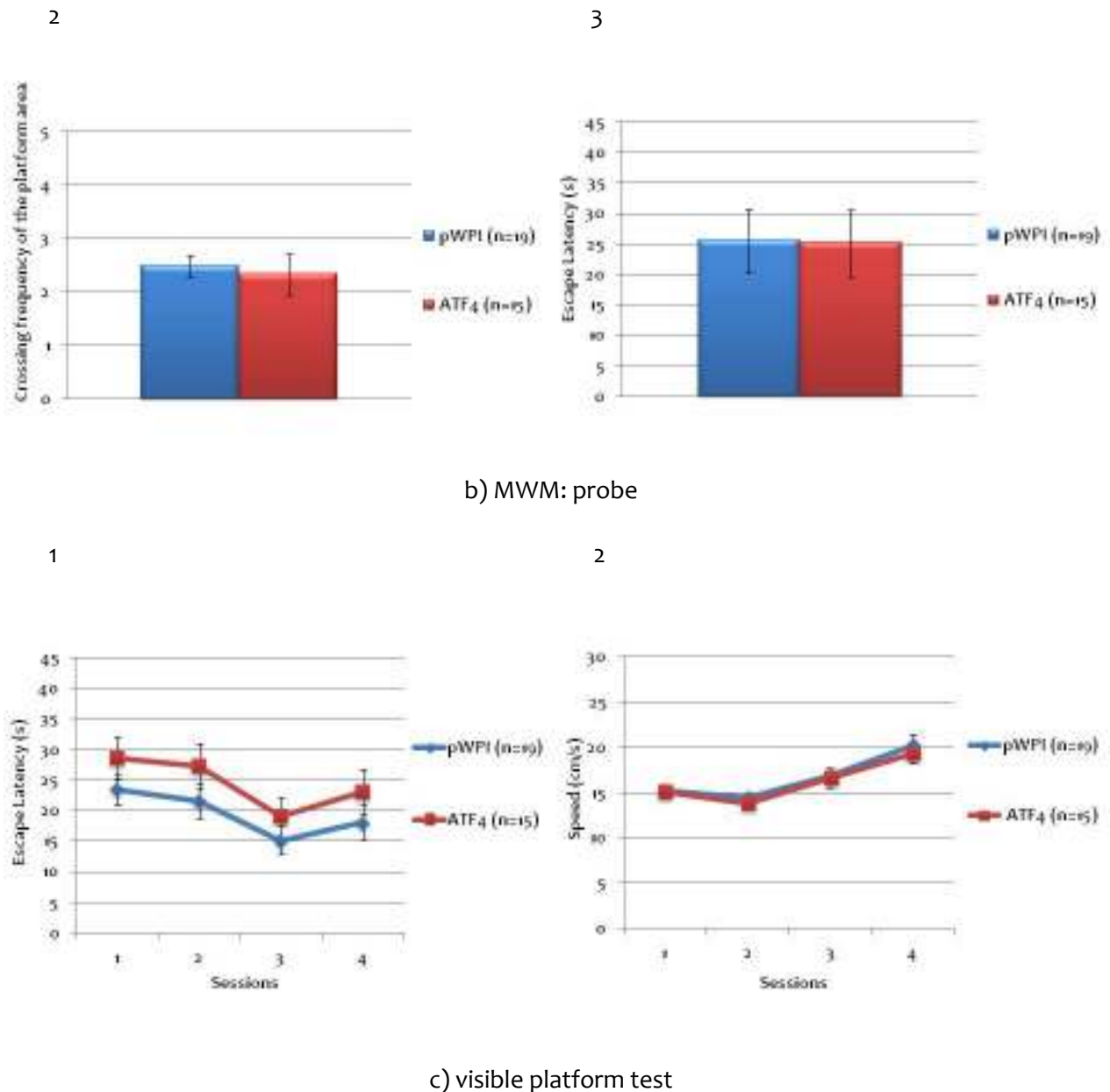
Fig 3.10. MWM. 4 weeks after lentiviral injection, ATF4 overexpressed mice and associated controls were subjected to MWM test. The graph in fig a shows that there was no significant difference between the two groups of mice during the standard training. Both groups learned to locate the hidden platform in around 27s. During the probe test, ATF4 overexpressed group spent around 37s while the control group around 32s exploring the target quadrant (b1). This difference is not statistically significant (t-test, p=0.13). There was not any statistically significant difference between the two groups while analyzing the crossing frequency of the platform area (b2) and the time taken to reach the platform area for the first time in each probe trial (b3). The results of visible platform test showed that there were no significant differences between ATF4 overexpressed mice and controls regarding the time taken by the mice to locate the visible platform during each session (c1) and the mice's speed (c2).

65

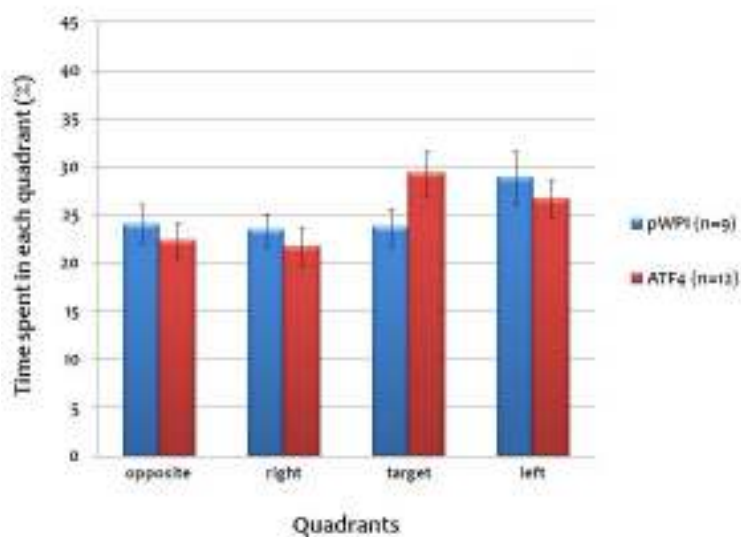3.4.2.b. Morris Water Maze: weak training protocol

ATF4 overexpressed and associated control mice were subjected to MWM test 4 weeks after lentiviral injection. During this behavioral test, mice were trained with a weak protocol (see experimental procedures) to verify if under this experimental condition there was a difference in spatial learning and memory between the two groups of mice. Fig 3.11a shows that during the 1st session, both groups of mice reached the hidden platform in around 37s, whereas during the 2nd and 3rd sessions, ATF4 overexpressed mice located the platform in a shorter time as compared to controls. These differences are not statistically significant. During the probe test (Fig 3.11b1), ATF4 overexpressed mice spent more time exploring the target quadrant, while controls spent more time exploring the left quadrant and equal time exploring the other 3 quadrants, meaning that they did not remember where the platform was located. However, the difference between ATF4 overexpressed and control mice regarding time spent to explore the target quadrant was not significant (t-test p =0,076). Analyzing the crossing frequency of the platform area (Fig 3.10c1), we found that ATF4 overexpressed mice crossed the platform area more times in a statistically significant manner in comparison to controls (t-test, p=0.011). Regarding the time taken to reach the platform area for the first time, ATF4 overexpressed group reached the platform area within 30s while the controls took around 45s, but the difference is not statistically significant (t-test, p=0.077). These results showed that under the weak training protocol, ATF4 overexpressed mice learned how to locate the hidden platform in a non significant shorter time as compared to controls. This non statistically significant learning improvement is reflected in a statistically significant memory enhancement. ATF4 overexpressed mice mapped more precisely the platform location inside the target quadrant, as compared to controls. After MWM test, mice were subjected to visible platform test. The results confirmed that none of the mice enrolled in the experiment exhibited visual or motor deficits; therefore no mouse was excluded from the data analysis. Fig 3.11c1 shows that there was no significant difference between ATF4 overexpressed mice and controls regarding the time taken by the mice to locate the visible platform during each session. During the first session, both groups of mice reached the visible platform within 25s while during the last one they located the platform within 10s. Regarding the speed, both curves were identical (Fig 3.11c2). There was a small increase in mice's speed during the sessions from 11cm/s in the first session to 15cm/s in the last one.
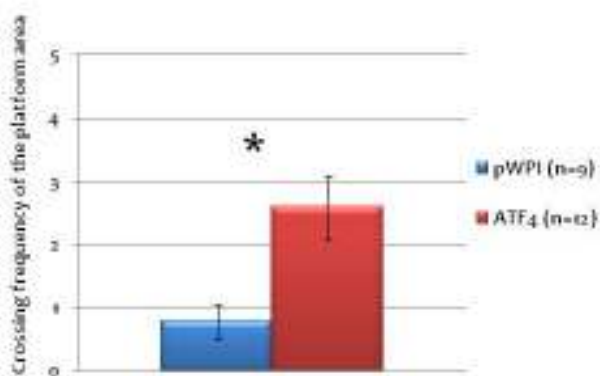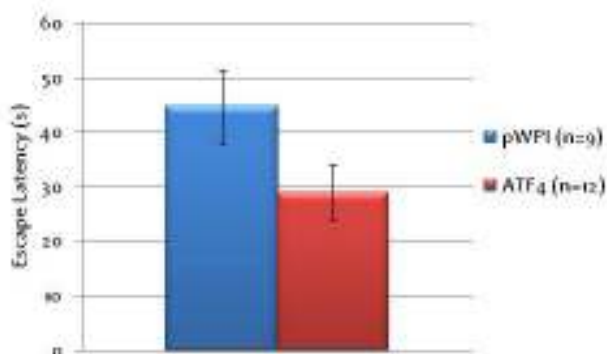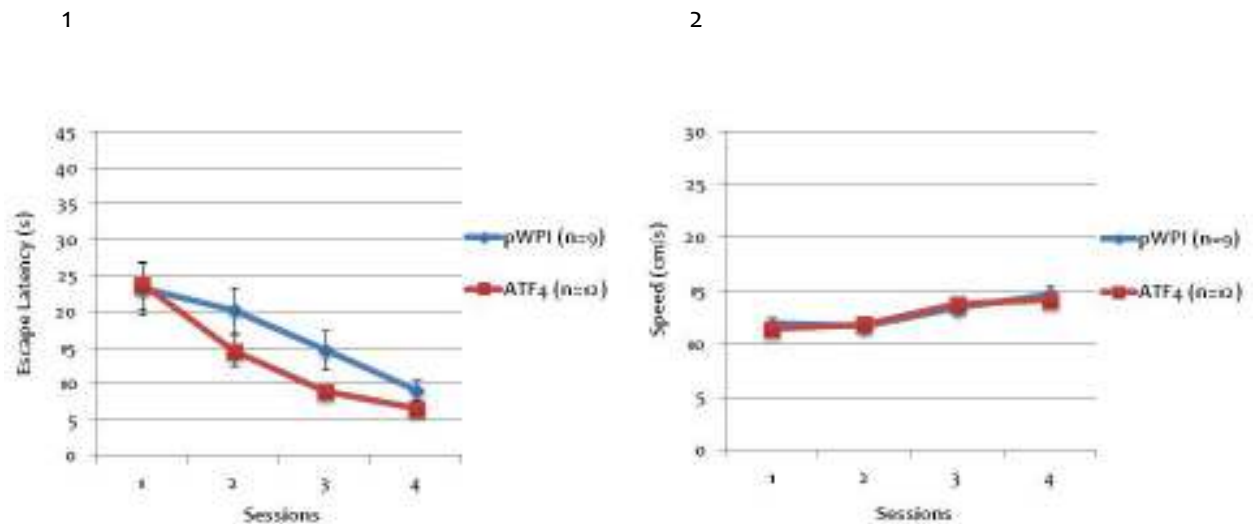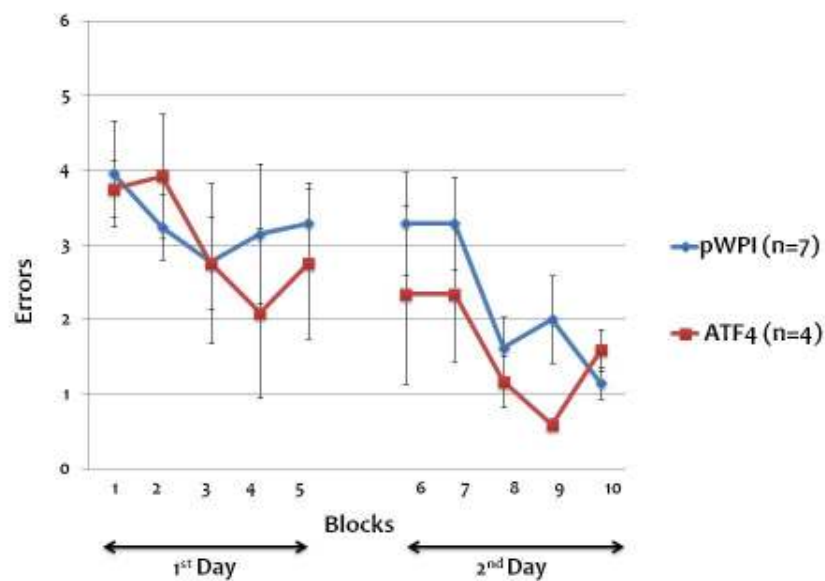
a) MWM: weak training

1

2 3

b) MWM: probe

1   2

c)   visible platform test

Fig 3.11. MWM: weak training protocol. 4 weeks after lentiviral injection ATF4 overexpressed mice and associated controls were subjected to MWM test, trained with the weak protocol. The graph in a shows that ATF4 overexpressed mice located the platform in a shorter time during the 2nd and the 3rd sessions in comparison with the control mice. These differences are not statistically significant. During the probe test ATF4 overexpressed group spent around 29s while the control group around 23s exploring the target quadrant (b1). This difference is not statistically significant (t-test, p=0.076). Graph b2 shows that ATF4 overexpressed mice crossed the platform area more times in a statistically significant manner in comparison to the controls (t-test, p=0.011).The graph in b3 shows the time taken to reach the platform arena. There were not significant differences between the two groups. ATF4 overexpressed group reached the platform within 30s while the controls took around 45s (t-test, p=0.077). The result of the visible test showed that there were not significant differences between ATF4 overexpressed mice and the controls regarding the time the mice took to locate the visible platform during each session (c1) and the mice's speed (c2).

3.4.2.c. Two-day Radial Arm Water Maze

ATF4 overexpressed and associated control mice were subjected to two-day RAWM 4 weeks after lentiviral injection. This behavioral test, like MWM test, analyzes spatial reference memory. This task is a hybrid of MWM and radial arm maze, with the benefit of scoring errors instead of time or proximity to platform location. It was found to be most reliable for detecting memory deficits in APP transgenic mice and to robustly discriminate between mice that learn well and those that learn poorly (Morgan et al., 2000; Wulcock et al., 2006). The result showed that there were no statistically significant differences between ATF4 overexpressed mice and controls regarding the task performance (Fig 3.12a). Analyzing the curves carefully, it is possible to notice that during the first day (blocks 1 to 5), both groups made the same number of errors on the 1st and the 3rd blocks (3 trials per block). During the 2nd block, ATF4 overexpressed mice performed worse than controls and in the last 2 blocks of the first day, ATF4 overexpressed mice made less errors compared to controls. On the 2nd day (blocks 6 to 10), during the first 4 blocks, ATF4 overexpressed mice made less errors in comparison to controls, particularly in the 9th block ATF4 overexpressed mice made less than 1 error on average while the controls made 2 errors (t-test, p=0.1). During the last block, both groups of mice performed the task equally well making less than 2 errors on average.

After RAWM test, mice were subjected to visible platform test. The result confirmed that none of the mice enrolled in the experiment exhibited visual or motor deficits; hence none of them were excluded from the data analysis. Fig 3.12b1 shows that there were no significant differences between ATF4 overexpressed mice and controls, regarding the time taken by the mice to locate the visible platform during each session. During the 1st session, ATF4 overexpressed mice reached the platform in around 8s while the controls took around 14s. During the last 3 sessions, both groups performed equally and at the end both groups of mice located the platform in 5s. Regarding the swimming speed, both curves were exactly the same (Fig 3.11b2). Both groups of mice swam at a speed of around 15cm/s on average throughout the 4 sessions.

a) Two-day RAWM

1   2
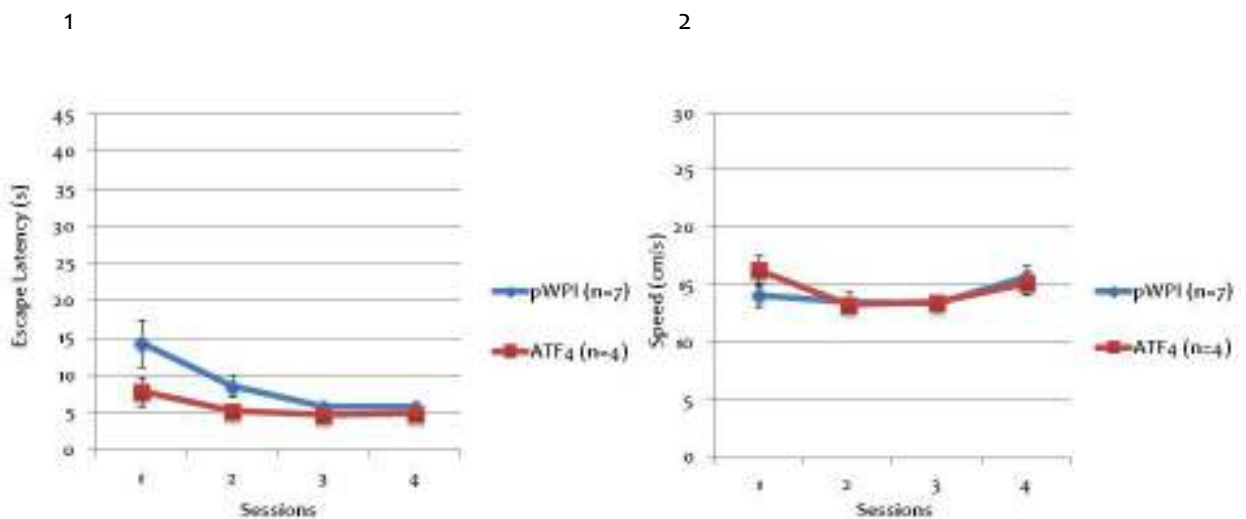
b) visible platform test

Fig 3.12. Two-day RAWM. 4 weeks after lentiviral injection, ATF4 overexpressed mice and associated controls were subjected to two-day RAWM test. The graph in fig a shows that there were no significant differences between ATF4 overexpressed mice and controls in terms of number of errors made to locate the platform. The result of visible platform test (b1 and 2) showed no significant difference between the two groups of mice regarding the time taken by the mice to locate the visible platform during each session (b1) and the mice's speed (b2).

3.4.2.d. Fear conditioning

Fear conditioning is a behavioral paradigm in which animals learn to predict aversive events. It is a form of learning in which an aversive stimulus, in this case an electrical shock, is associated with a particular neutral context, the chamber, or neutral stimulus, the tone, resulting in the expression of fear responses, freezing, to the originally neutral stimulus or context.

After completing the water tasks, ATF4 overexpressed and associated control mice were subjected to Fear Conditioning. The 24h Contextual result (Fig 3.13a) shows that the % of freezing during baseline, which corresponds to training, was exactly the same for both groups of mice, and it was around 1%, since the mice were naïve. During 24h Contextual, pWPI control mice froze around 20% while ATF4 overexpressed mice froze around 30% ($p<0.05$). This result underlines that ATF4 protein overexpression in the hippocampus improves associative memory.

On the contrary, there were no significant differences between the two groups during Cued testing (Fig 3.13b), performed 48h after training, where the % of freezing was around 35% for both groups. This result shows that the mice did not exhibit impairment in the basolateral amygdala, the brain region involved in cued fear conditioning, since we targeted only the hippocampal area. Moreover, the 48h Cued baseline showed that the % of freezing for both groups of mice was similar, 9% for controls and 10% for ATF4 overexpressed mice.
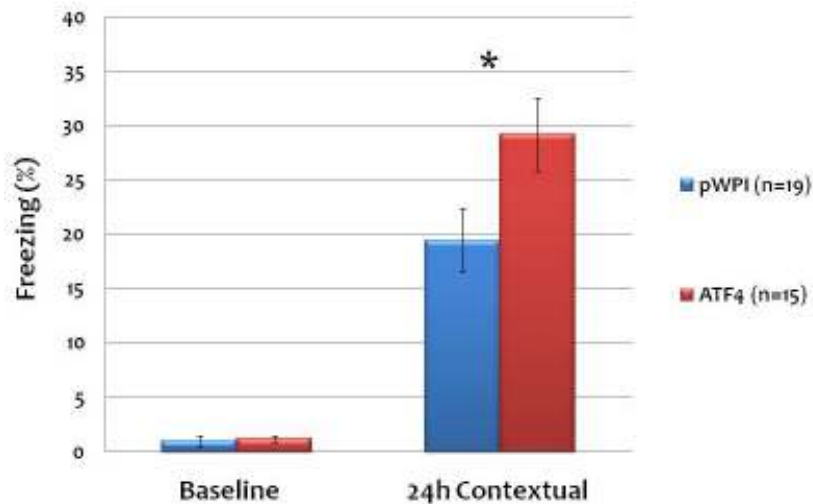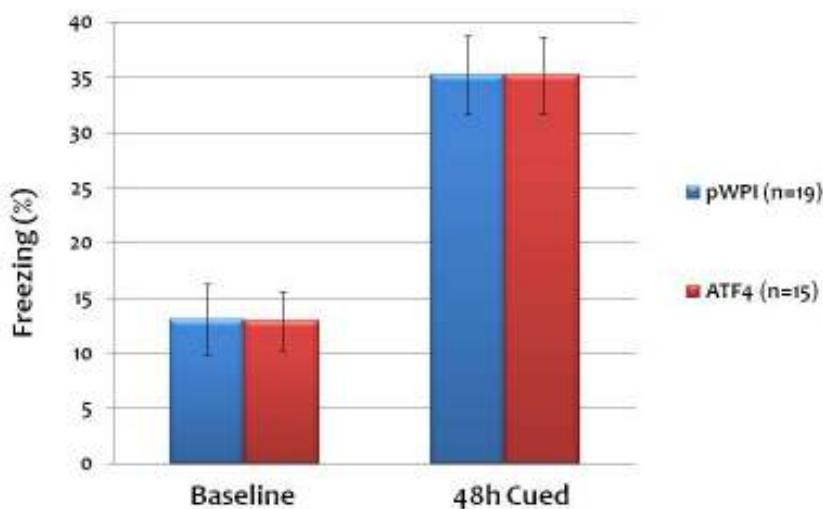
a) FC: 24h Contextual

b) FC: 48h Cued

Fig 3.13. FC. After finishing the water tasks, ATF4 overexpressed mice and associated controls were subjected to FC. The result of 24h Contextual (a) shows that ATF4 overexpressed mice froze more, about 30%, in a statistically significant manner ($p < 0.05$) as compared to controls which froze around 20%. The % of freezing during baseline was the same for both groups of mice and it was around 1%. The result of 48h Cued (b) shows that there was no difference between the two groups of mice which froze around 35% on average. The 48h Cued baseline also showed that there was no difference in the % of freezing between the two groups of mice.

3.4.3. ELECTROPHYSIOLOGICAL ANALYSIS

After completing the behavioral tests, the animals were sacrificed to verify if ATF4 overexpressed mice that exhibited a memory improvement in MWM and FC tests, also showed an enhancement in long term synaptic potentiation. LTP is a long lasting enhancement in neuronal transmission and is considered one of the mechanisms that underlies learning and memory. We compared LTP, evoked in the CA1 region of hippocampal slices, from ATF4 overexpressed mice (ATF4) and controls (pWPI). Unexpectedly, electrophysiological analysis performed around 2 months after lentiviral injection showed that theta burst stimulation elicited a robust LTP in the control slices while it produced only a moderate LTP in the ATF4 overexpressed slices (Fig 3.14). In particular, the decrease in fEPSP slope, in ATF4 overexpressed mice as compared to controls, was evident since the application of the theta burst stimulation and it was maintained throughout the recording session. The immediate reduction in field potentiation in ATF4 overexpressed mice suggests that ATF4 protein overexpression caused an impairment in E-LTP, maintained throughout the recording session also affecting L-LTP. The graph on the right shows the average of the last 10 recorded points (red circle). ATF4 overexpressed mice exhibited a statistically significant impairment in L-LTP as compared to controls (p<0.05).
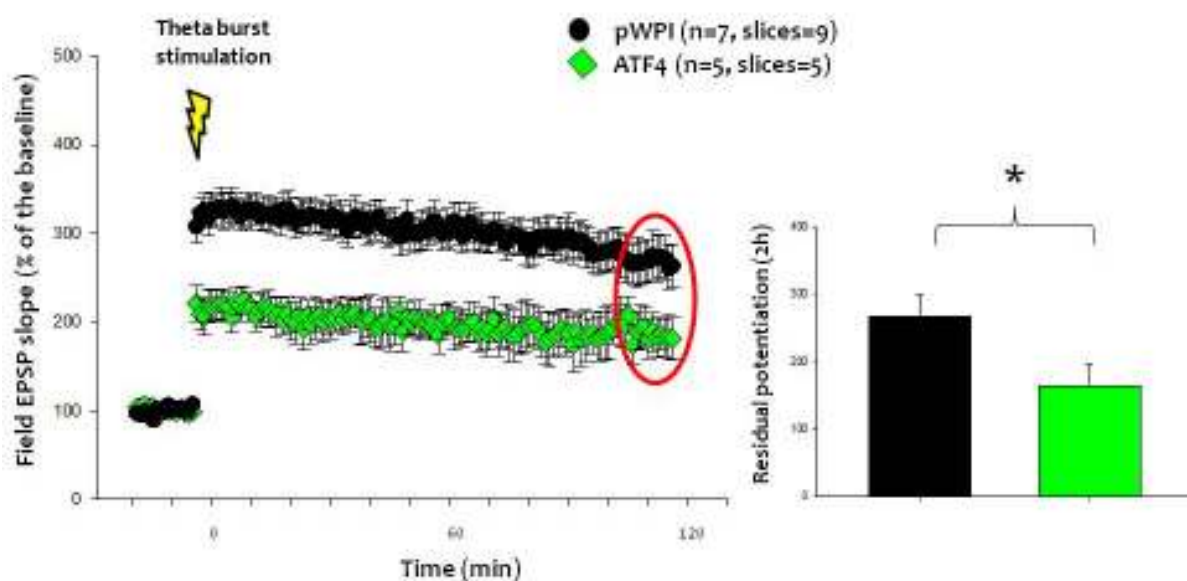
Fig 3.14. LTP. After behavioral tests, ATF4 overexpressed mice and associated controls were sacrificed to perform LTP recordings. Graph on the left: theta burst stimulation elicited a robust E-LTP in the control slices (pWPI), maintained throughout the recording session while it produced only a moderate E-LTP in the ATF4 overexpressed slices (ATF4). The plot of the last 10 recorded points, on the right, showed that 2h after stimulation, ATF4 overexpressed mice exhibited a statistically significant impairment in L-LTP as compared to controls (p<0.05).

3.4.4. SPINE ANALYSIS

After behavioral tests the morphology of hippocampal neurons infected with the lentivirus was examined, using DiOlistic Labeling technique, to verify if ATF4 protein overexpression can modify the density and morphology of dendritic spines. First the spine density between neurons infected with the control lentivirus and those with lentivirus carrying the ATF4 gene was compared. The graph in Fig 3.15a shows that the density of dendritic spines in both groups was around 1.2, meaning that the overexpression of ATF4 protein does not alter the spine number.

Regarding spine morphology (Fig 3.15b), ATF4 overexpression led to a small non significant increase in the number of mushroom spines. The percentage of mushroom spines in the controls was 24% and in ATF4 overexpressed group was around 27%. The increase in the % of mushroom spines in ATF4 overexpressed group was accompanied by a decrease in the % of stubby spines. The percentage of the stubby spines in the controls was 46% while in ATF4 overexpressed group was 43%. The percentage of thin spines was comparable between the two groups and was around 30%.

The result of spine morphology analysis reveals that ATF4 protein results in a non significant increase in the number of mushroom spines also called as "memory spines" and a non significant decrease in the stubby ones, while there is no difference between the thin spines.

a

b
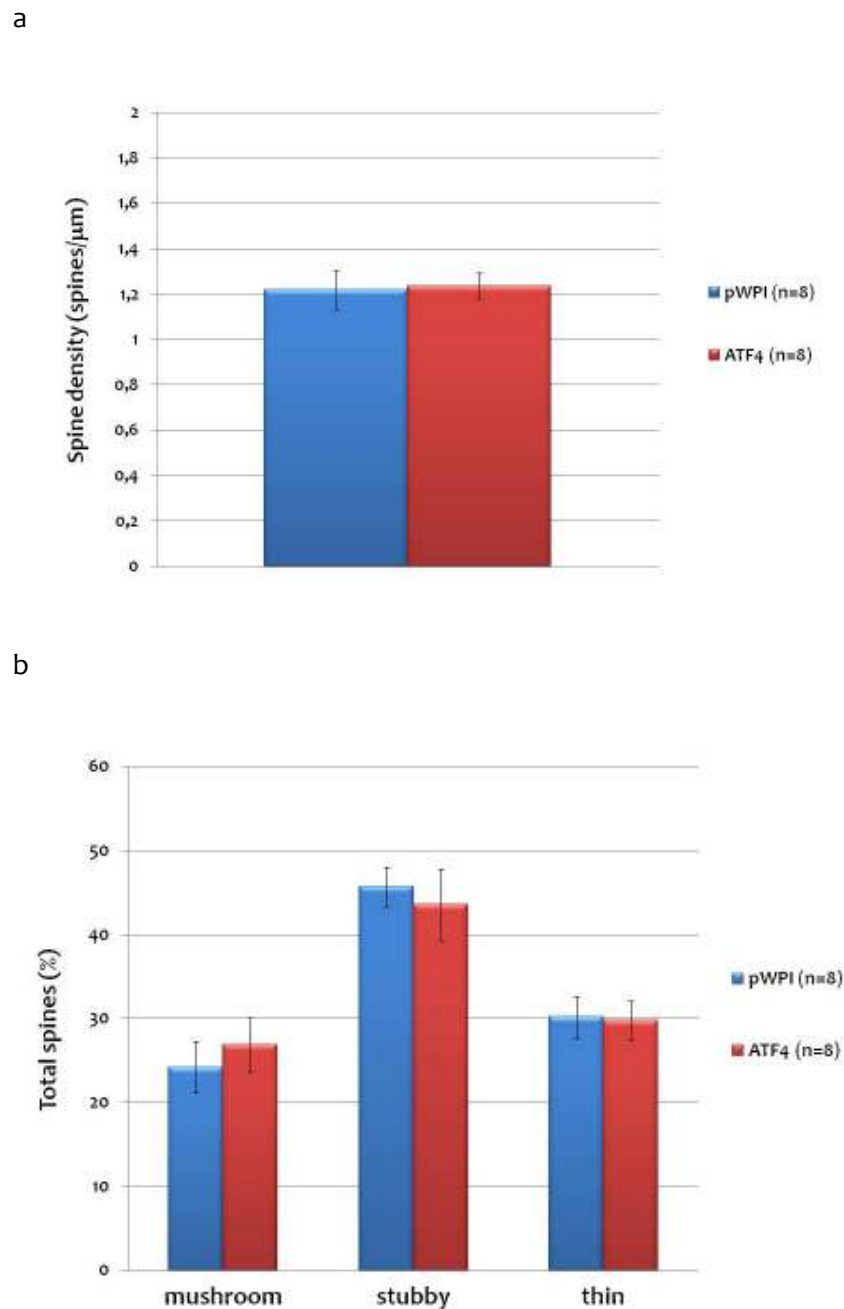
Fig 3.15. Spine density and morphology analysis. After behavioral tests, mouse brains were coronally sectioned and subsequently labeled to perform spine density and morphology analysis of infected neurons. The spine density in both groups of mice was around 1.2; therefore, the overexpression of ATF4 protein did not affect this parameter. Regarding spine morphology, ATF4 overexpressed mice showed a non significant increase in the percentage of mushroom spines, and a non significant decrease in the stubby ones as compared to controls. No variation in the percentage of thin spines was found between the two groups.

3.5. ATF4 DOWNREGULATION

After completing ATF4 overexpression studies, the same analysis on ATF4 downregulated mice were performed to verify if downregulation of this protein can affect learning and memory. ATF4 protein was downregulated in the mouse hippocampi by injecting lentiviral particles carrying shRNA against ATF4 mRNA.

3.5.1. DETECTION OF ATF4 mRNA DOWNREGULATION

To verify that injection of shATF4 lentivirus causes ATF4 downregulation a quantitative real-time PCR (qRT-PCR) was performed to assess the level of ATF4 mRNA in mouse hippocampi one month after injection. The level of ATF4 mRNA in the animals who received the shATF4 lentivirus was 0.58 as compared to controls (shMUT) whose ATF4 mRNA level was fixed at 1 (Fig 3.16) The injection of lentivirus carrying shRNA against ATF4 mRNA caused an ATF4 mRNA downregulation of approximately 40%, in the hippocampal cells.
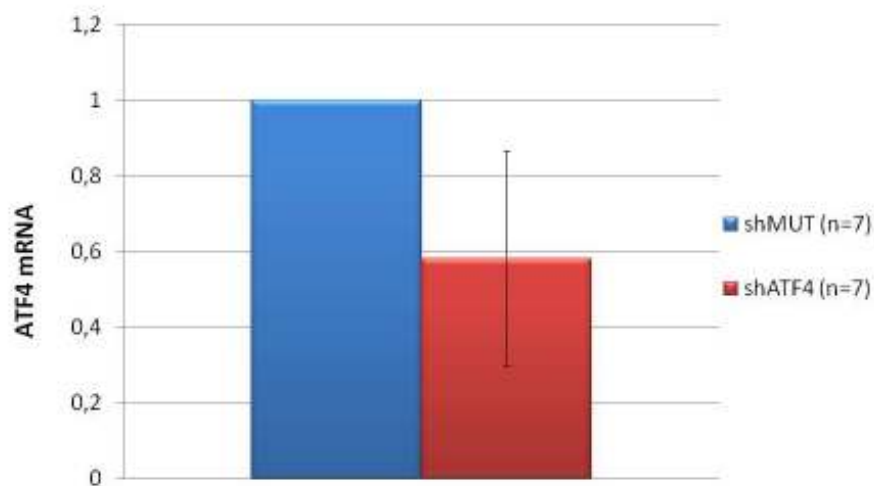

Fig 3.16. qRT-PCR to assess ATF4 mRNA level. One month after lentiviral injection, qRT-PCR was performed to detect ATF4 mRNA level in mouse hippocampi. shATF4 injected animals showed a reduction in ATF4 mRNA level of about 40% compared to control animals (shMUT) whose ATF4 mRNA level was fixed at 1.

3.5.2. BEHAVIORAL TESTS

3.5.2. a. Morris Water Maze

ATF4 downregulated and associated control mice were subjected to MWM test 4 weeks after lentiviral injection. The result showed that during training (Fig 3.17a), there were no significant differences between the two groups of mice regarding time taken to locate the hidden platform throughout the six sessions. During the 1st session, both groups took on average 35s to reach the platform; during the 2nd one, ATF4 downregulated mice took 24s while the controls around 28s and during the 3rd session, the average was 28s for both groups. During each of the last three sessions, ATF4 downregulated mice took on average 2 seconds less as compared to controls to locate the platform. These differences are not statistically significant. At the end of training, during the last session, both groups of mice reached the platform in around 26s. During the probe session, ATF4 downregulated mice spent less time, around 36s, exploring the target quadrant as compared to controls that explored the same quadrant for 44s ($p < 0.05$) (Fig 3.17b1). The control mice (shMUT) spent more time exploring the target quadrant in comparison to the other three ($p < 0.05$) while ATF4 downregulated group could not distinguish between the target and the right quadrants where they spent almost equal time. This result underlines that ATF4 downregulated mice could not remember exactly where the platform was located because they spent equal time exploring the right and the target quadrant as compared to controls that spent most of the time in the target quadrant. The spatial map generated from the control mice more closely resembled the platform area as compared to the one used by ATF4 downregulated mice, that could only remember in which half of the pool the platform was located. Regarding the crossing frequency of the platform area (Fig 3.17b2) and the time taken to reach the platform area for the first time in each probe trial (Fig 3.17b3), we did not find any statistically significant difference between the two groups. Both groups crossed the platform area 3 times on average in each 60s trial and both of them reached the platform area for the first time, on average, in around 25s. After completing MWM test, mice were subjected to the visible platform test. The result (Fig 3.17c) showed that all the mice enrolled in the experiment did not exhibit visual or motor deficits; hence none of them were excluded from the data analysis. The graph in Fig 3.17c1 shows that there were no significant differences between ATF4 downregulated mice and controls regarding the time taken to locate the visible platform during each session. Both curves are identical. During the 1st session, both groups of mice reached the visible platform in around 12s and during the last session, the average time was 10s. Also, regarding the speed we did not see any significant difference between the two groups. There was a small increase in speed during the sessions from 13cm/s in the 1st session to 16cm/s in the last one.

a) MWM: standard training.

1

2   3

b) MWM: probe

1   2
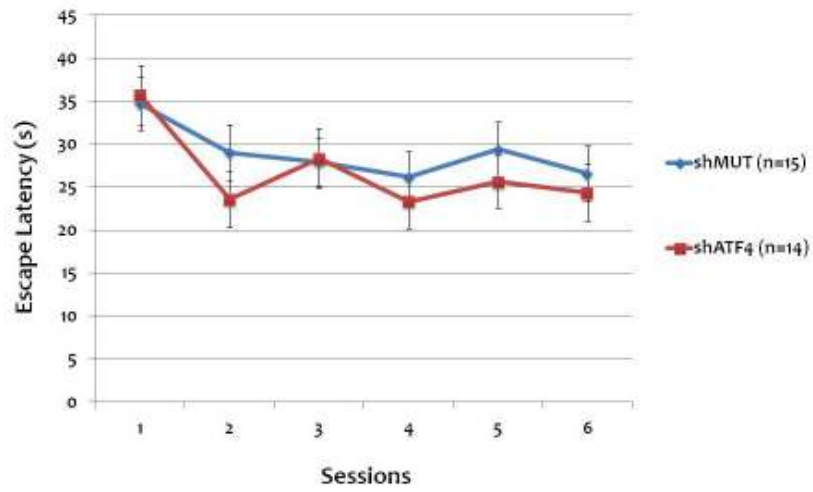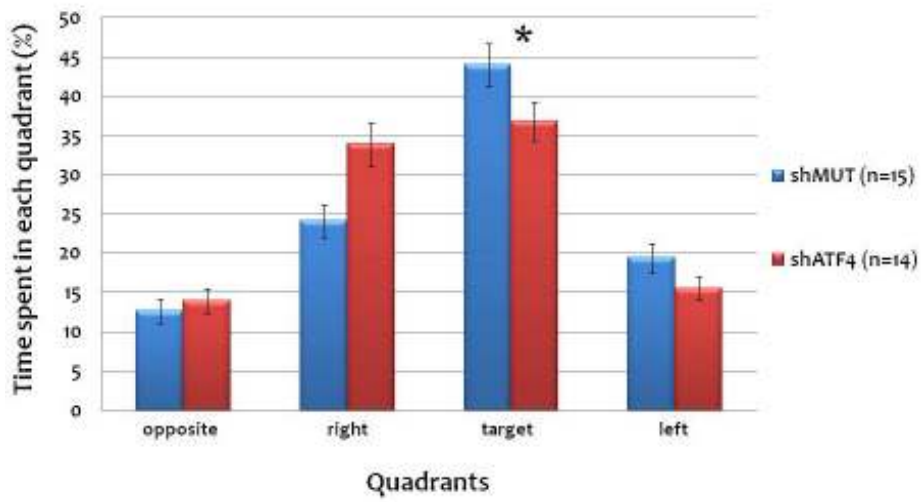
c) visible platform test

Fig 3.17. MWM. 4 weeks after lentiviral injection, ATF4 downregulated mice and associated controls were subjected to MWM test. The graph in fig a shows that there was no significant difference between the two groups of mice during the training. Both groups learned to locate the hidden platform in around 25s. During the probe test (b1), ATF4 downregulated mice spent less time exploring the target quadrant in comparison to the control group (t-test, $p<0.05$). We did not find any statistically significant difference between the two groups while analyzing the crossing frequency of the platform area (b2) and the time taken to reach the platform area for the first time in each probe trial (b3). The result of visible platform test showed that there were no significant differences between ATF4 downregulated mice and controls regarding the time taken to locate the visible platform during each session (c1) and the mice's speed (c2).

3.5.2. b. Morris Water Maze: weak training protocol

ATF4 downregulated and associated control mice were subjected to MWM test 4 weeks after lentiviral injection. During this behavioral test, mice were trained with a weak protocol to verify if under this experimental condition we could still see the difference in spatial learning and memory between ATF4 downregulated and control mice that we saw when the mice were trained with the standard protocol. Fig 3.18a shows the result for training, during which we did not find any difference between the two groups. During the 1st session, both groups of mice reached the hidden platform in around 45s. During the 2nd session, they located the platform in 37s and during the last one, ATF4 downregulated mice located the platform in around 27s and the controls in 31s, but the difference is not statistically significant. During the probe test, as shown in Fig 3.18b, there was no difference between ATF4 downregulated and control mice regarding the time spent exploring the target quadrant. The probe performance of ATF4 downregulated mice after the weak training protocol was the same as after the standard training, while the controls spent less time exploring the target quadrant. Moreover, the analysis of the crossing frequency of the platform area (Fig 3.18c1) and the time taken to reach the platform area for the first time did not show any difference between the two groups of mice. Both groups crossed the platform area 2 times on average, while regarding the time taken to reach the platform area for the first time, they took around 27s. The results of this behavioral test show that after the weak training protocol, the performance of ATF4 downregulated mice was similar to controls. This suggests that control mice (shMUT) can acquire spatial memory after an adequate training, while ATF4 downregulated mice failed to remember the platform location under both experimental conditions, standard and weak training protocols, meaning that the ATF4 protein plays a key role in spatial memory attainment. The result of the visible platform test confirmed that none of mice enrolled in the experiment exhibited visual or motor deficits; hence all of them were include in the data analysis. The graph in Fig 3.18c1 shows that there is no significant difference between ATF4 downregulated mice and controls regarding the time taken to locate the visible platform during each session. During the first session, ATF4 downregulated mice located the visible platform in 20s, while the controls took around 27s. During subsequent sessions, the difference between the two groups decreased until both reached the platform in 7s during the last session. Regarding the speed, both curves are equivalent (Fig 3.18c2). There was a small increase in speed during the sessions from 11cm/s in the first session to 15cm/s in the last one.

a) MWM: weak training
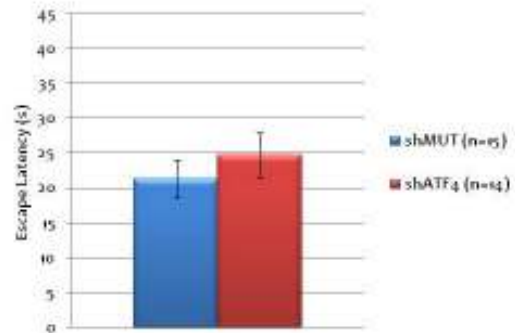
1

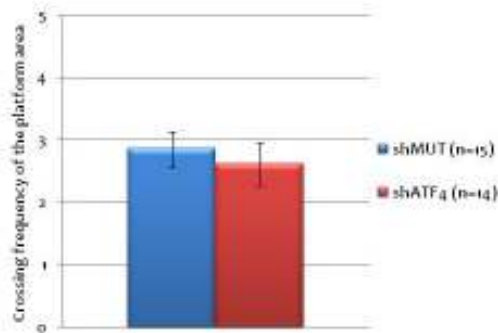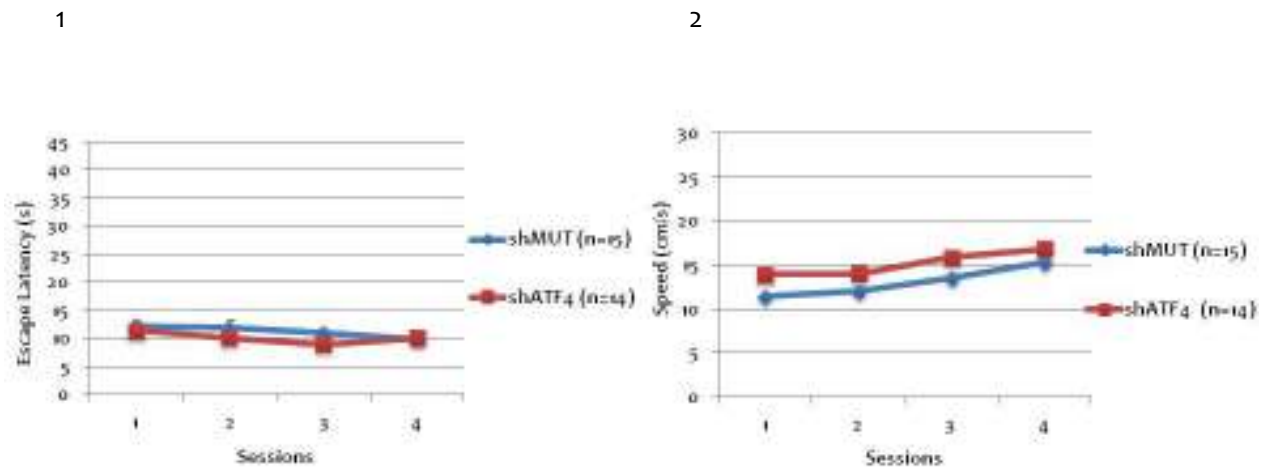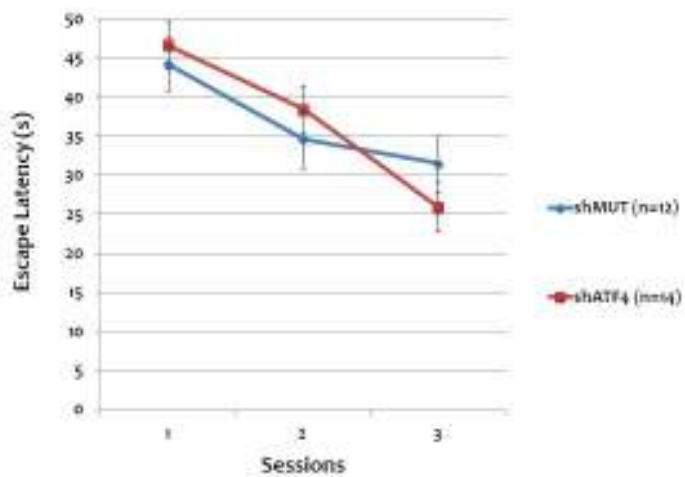2   3

b) MWM: probe

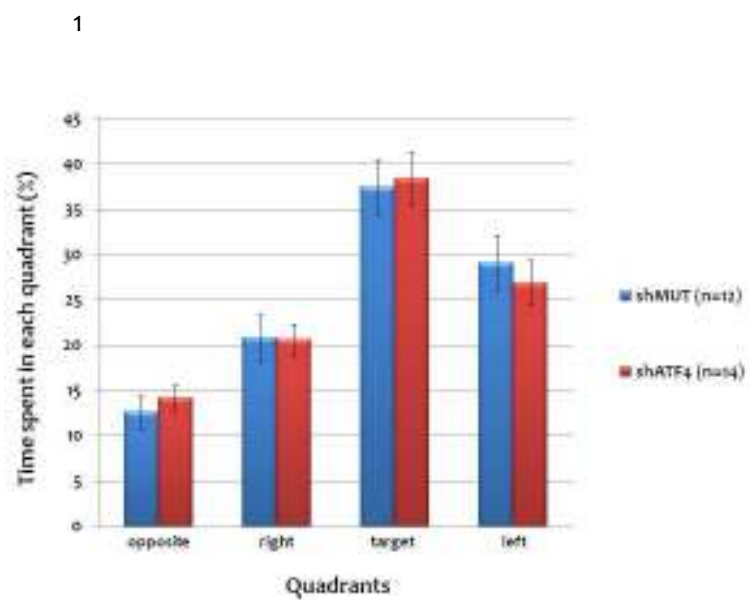1   2
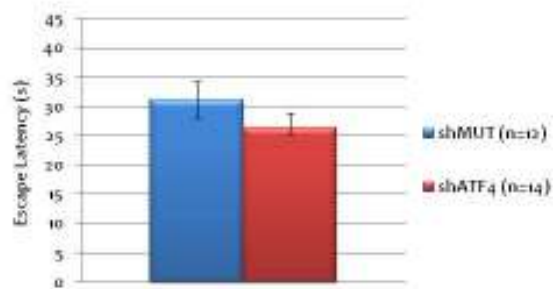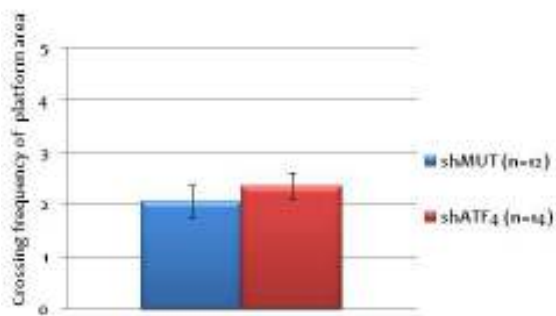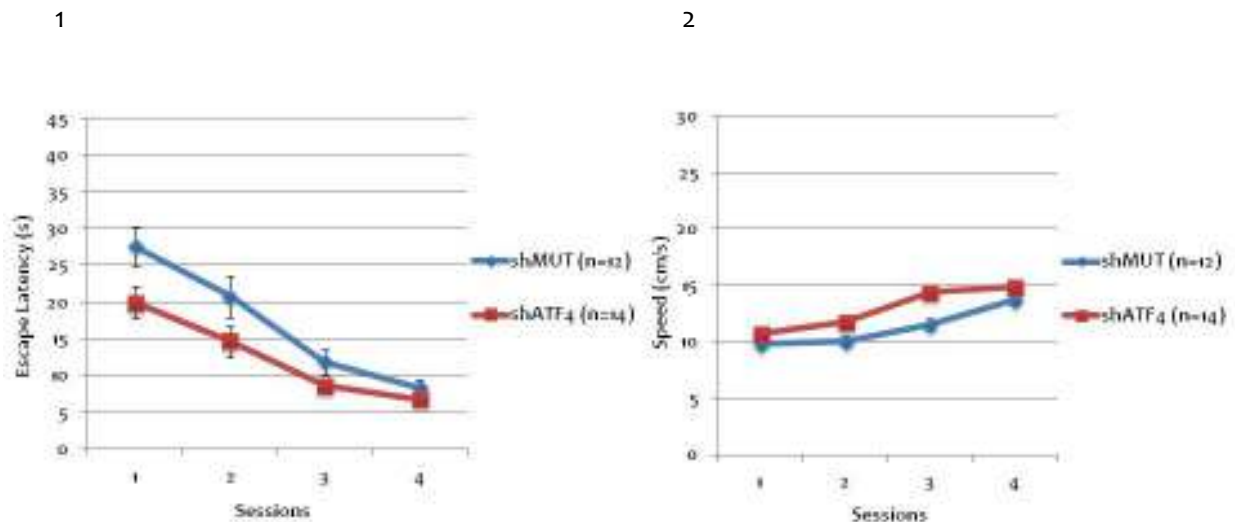
c) visible platform test

Fig 3.18. MWM: weak training protocol. 4 weeks after lentiviral injection, ATF4 downregulated mice and associated controls were subjected to MWM test, trained with the weak protocol. The graph in fig a shows that under this training protocol, there was no difference in learning between the two groups. During the 1st session, both groups located the platform in around 45s and during the last one, in around 30s. Also, during the probe test, as shown in the graph b1, we did not see any difference between the two groups. Both groups of mice spent more time exploring the target quadrant, around 37s, in comparison with the other three quadrants. Moreover, there was no statistically significant difference neither in the crossing frequency of the platform area (b2) nor the time taken to reach the platform area for the first time (b3). Both groups crossed the platform area 2 times on average and took between 25 and 30s, on average, to reach the platform area for the first time. The result of visible platform test showed that there were no significant differences between ATF4 downregulated mice and controls regarding the time taken to locate the visible platform during each session (c1) and the mice's speed (c2).

3.5.2. c. Two-day Radial Arm Water Maze

ATF4 downregulated and associated control mice were subjected to the two-day RAWM test 4
weeks after lentiviral injection, which showed that there were no statistically significant differences
between ATF4 downregulated mice and the controls regarding the task performance (Fig 3.19).
During the 1st block of 3 trials, shATF4 group made almost 5 errors while the controls 5.5. During the
2nd block, both groups made 3 errors, while during the 3rd and 4th blocks, the control mice made
fewer errors as compared to the shATF4 mice and during the last block of the first day, both groups
performed exactly the same way making 1.2 errors on average. During the first block of the 2nd day
(block 6), ATF4 downregulated mice made fewer errors, 1.5, with respect to the controls who made
2.5, but during the following 4 blocks, shATF4 mice made almost the same number of mistakes
starting from 1.5 errors on the 6th block to 1 error on the last one, while the control mice at the
beginning of the second day started from 2.5 mistakes until 0.5 errors at the end of the task. After
RAWM test, mice were subjected to visible platform test. The result of visible platform test is
described in the MWM standard training protocol section (Fig 3.18c).
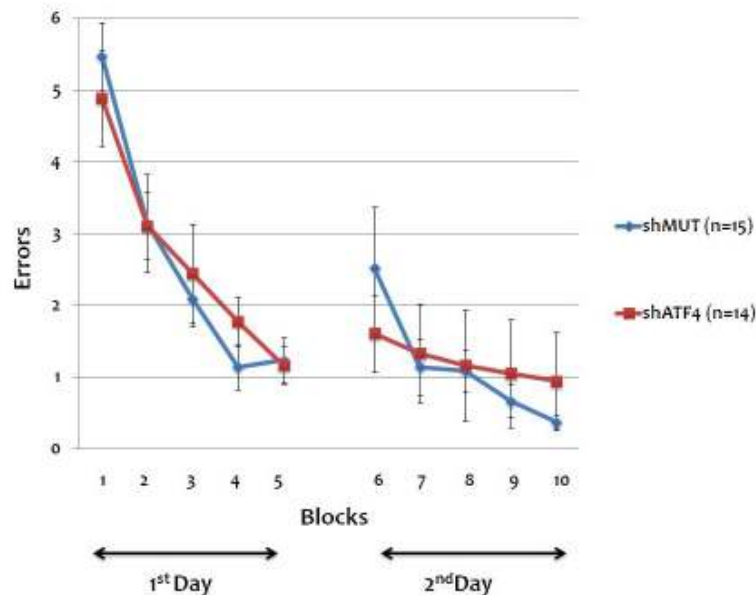
Fig 3.19. RAWM. 4 weeks after lentiviral injection, ATF4 downregulated mice and associated controls
were subjected to the two-day RAWM test. There were no significant differences between the two
groups of mice regarding the number of errors made before locating the platform during each block.

3.5.2. d. Fear Conditioning

After completing the water tasks, ATF4 downregulated and associated control mice were subjected to the Fear Conditioning test. The 24h Contextual result showed that the % of freezing during baseline, which corresponds to training , was exactly the same for both groups of mice, and was around 1.5%, since the mice were naïve. During 24h Contextual, we did not find any significant difference between shATF4 mice, which froze around 20%, and the controls that froze around 19% (Fig 3.20a). This result shows that downregulation of ATF4 protein in the hippocampus does not affect associative memory.

Moreover, we did not find any statistically significant difference in freezing between the two groups of mice during the 48h Cued test (Fig 3.20b). During pre-Cued, used as baseline, both groups of mice froze around 8%, while during the 48h Cued test, shATF4 mice froze around 35% and the controls around 40%. This difference is not significant, meaning that the basolateral amygdala, the brain area involved in cued fear conditioning, was not impaired.

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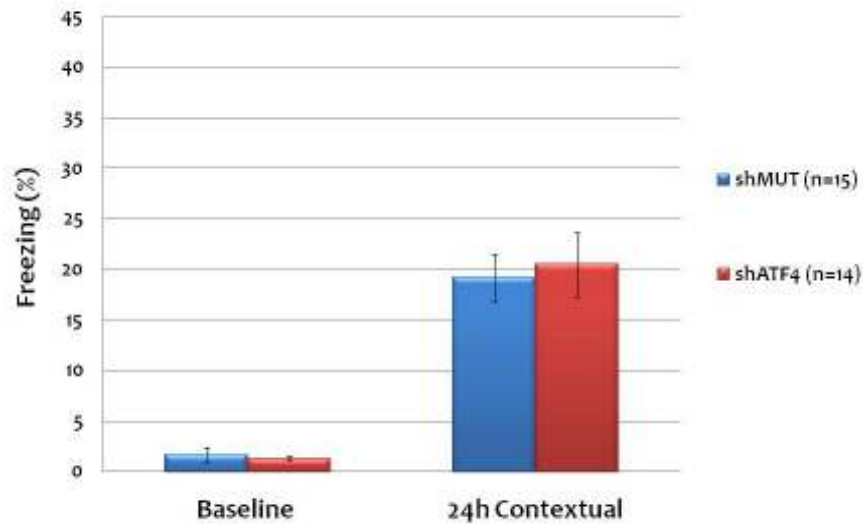
a) FC: 24h Contextual
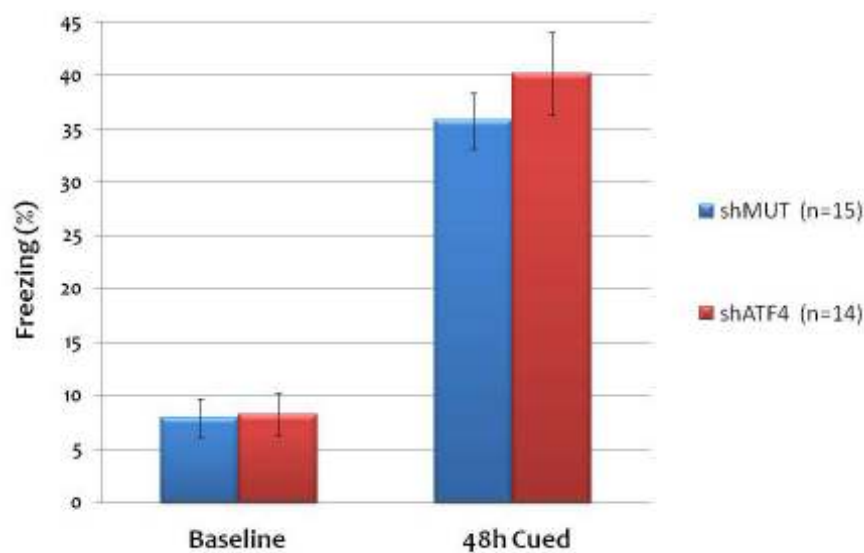
b) FC: 48h Cued

Fig 3.20. FC. ATF4 downregulated mice and associated controls were subjected to FC after the water tasks. The 24h Contextual result (a) showed that there was no difference in freezing between the two groups of mice, meaning that downregulation of ATF4 protein does not affect associative memory of these animals. The % of freezing during baseline was the same for both groups of mice, and was about 1.5%. The result of 48h Cued (b) showed that there was no difference between the two groups of mice. ATF4 downregulated mice froze around 40%, while the controls around 35%, meaning that the amygdala was not impaired. The 48h Cued baseline also showed no difference in the % of freezing between the two groups of mice, which froze around 8%.

3.5.3. ELECTROPHYSIOLOGICAL ANALYSIS

After the cognitive evaluation, the animals were sacrificed for electrophysiological analysis, and LTP evoked in the CA1 region of hippocampal slices was compared between ATF4 downregulated mice (shATF4) and controls (shMUT). In the control slices, theta burst stimulation elicited an E-LTP that was around 250% compared to the baseline and remained constant throughout the recording session. Interestingly, when compared to control mice, the induction of E-LTP was significantly reduced in ATF4 downregulated mice and further decreased after 1 hour (Fig 3.21). The average of the last 10 recorded points (graph on the right) showed that shATF4 slices exhibited a statistically significant reduction in L-LTP compared to the controls (p<0.05). Accordingly with results obtained in the MWM test under the standard training protocol, our data clearly show that ATF4 downregulation produces a significant reduction of LTP induction and a dramatic drop of L-LTP. This suggests that ATF4 protein plays a key role in the mechanisms underlying LTP. Furthermore, such impairment of LTP can explain the long term spatial memory deficit showed by the ATF4 downregulated mice during the MWM task performance.
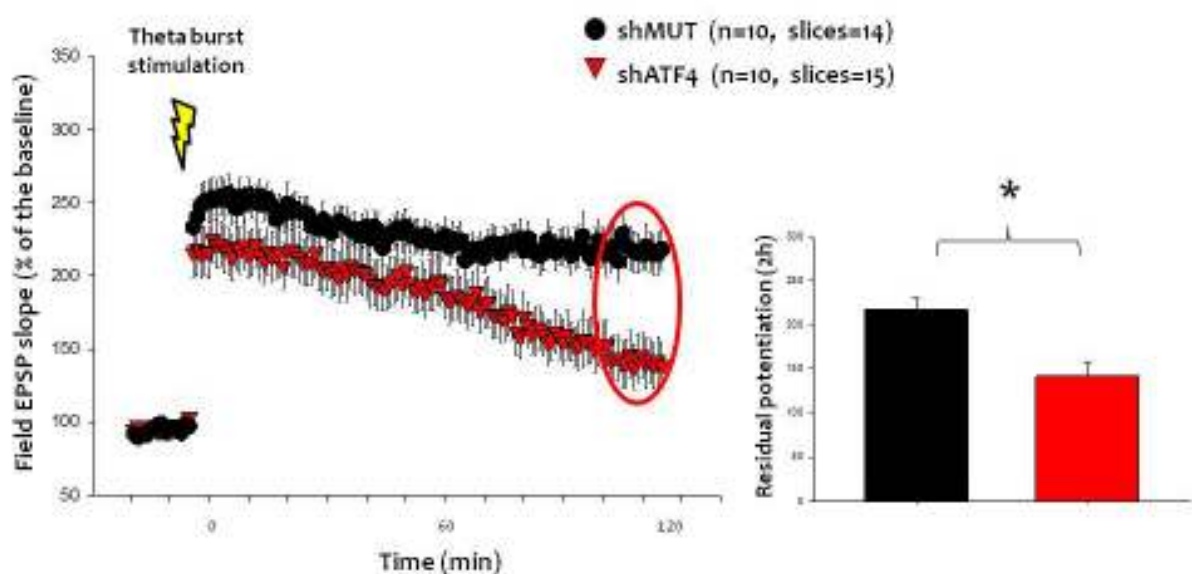
Fig 3.21. LTP. After behavioral tests, ATF4 downregulated mice and associated controls were sacrificed to perform LTP recordings. The graph on the left shows that theta burst stimulation elicited a lower E-LTP in shATF4 slices when compared to control slices (shMUT) and a dramatic drop as regards with the L-LTP. On the right, the plot of the last 10 recorded points shows that 2h after the stimulation, ATF4 downregulated mice exhibited a statistically significant impairment in L-LTP compared to the controls (p<0.05).

3.5.4. SPINE ANALYSIS

After behavioral tests the morphology of hippocampal neurons infected with the lentivirus, was examined using DiOlistic Labeling technique, to verify if ATF4 downregulation can alter spine density and morphology. The spine density between neurons infected with the control lentivirus (shMUT) and those with the lentivirus carrying shATF4 was compared. The graph in Fig 3.22a shows that there was no statistically significant difference in the spine density between the two groups. The spine density in shATF4 mice was 1.4 and in controls 1.2. This result implies that ATF4 downregulation does not affect the density of the dendritic spines.

Regarding spine morphology (Fig 3.22b), ATF4 downregulation led to a non significant reduction in the number of mushroom spines (t-test, p=0.109). The percentage of mushroom spines in the controls was 21% and in ATF4 downregulated group was around 15%. The reduction in the % of mushroom spines in the shATF4 group was accompanied by a non significant increase in the % of stubby spines (p=0.148). The percentage of the stubby spines in the controls was 44%, while in ATF4 downregulated group was 51%. The percentage of thin spines was identical between the two groups and was around 34%. The spine morphology result underlines that ATF4 downregulation leads to a non significant decrease in the number of mushroom spines also called as "memory spines" and a non significant increase in the stubby ones, while there is no difference in the thin spines.

The spine analysis results obtained from ATF4 overexpressed mice combine with those of ATF4 downregulated mice, show that there is no statistically significant difference in the density of the dendritic spines between the two groups (Fig 3.23a). However, ATF4 downregulated mice displayed a significant reduction in the % of mushroom spines (15%) compared to the overexpressed ones (26%) (t-test, p<0.05). This modification in the % of mushroom spines was accompanied by an opposite variation in the % of stubby ones; the % of stubby spines in ATF4 overexpressed mice was 43% and in shATF4 group was 51%. This difference is not statistically significant (t-test, p=0.2).

This result suggests that ATF4 is involved in spine morphology modification by promoting the formation of mushroom spines.

a

b
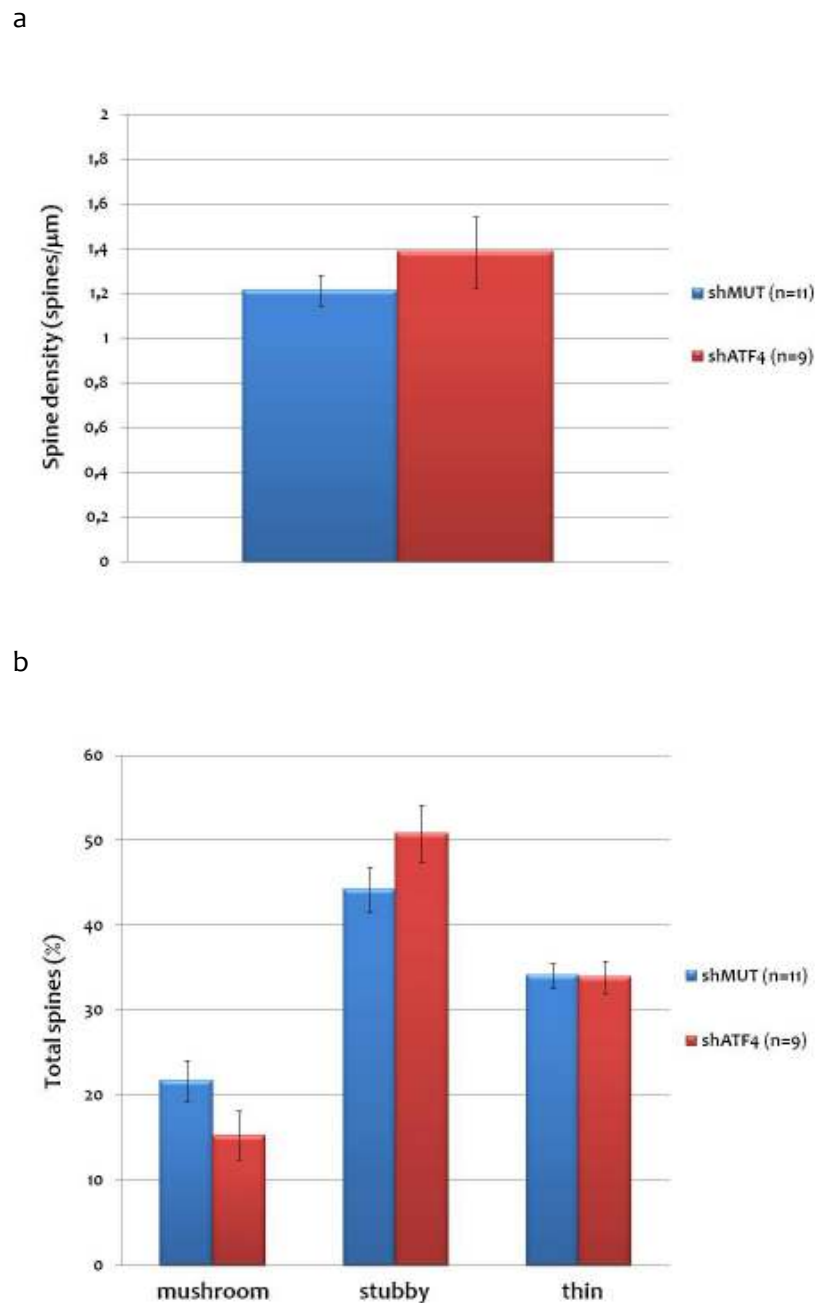
Fig 3.22. Spine density and morphology analysis. After behavioral tests, mouse brains were coronally sectioned and subsequently labeled to analyze spine density and morphology of infected neurons. Fig a shows the result of spine density, which is 1.2 for the controls and 1.4 for the shATF4 group. This difference is not statistically significant, meaning that the downregulation of ATF4 protein does not alter the density of the dendritic spines in the hippocampal neurons. Regarding spine morphology (b), ATF4 downregulated mice showed a non significant reduction in the % of mushroom spines , and a non significant increase in the stubby ones. No variation in the percentage of thin spines was found between the two groups.

a
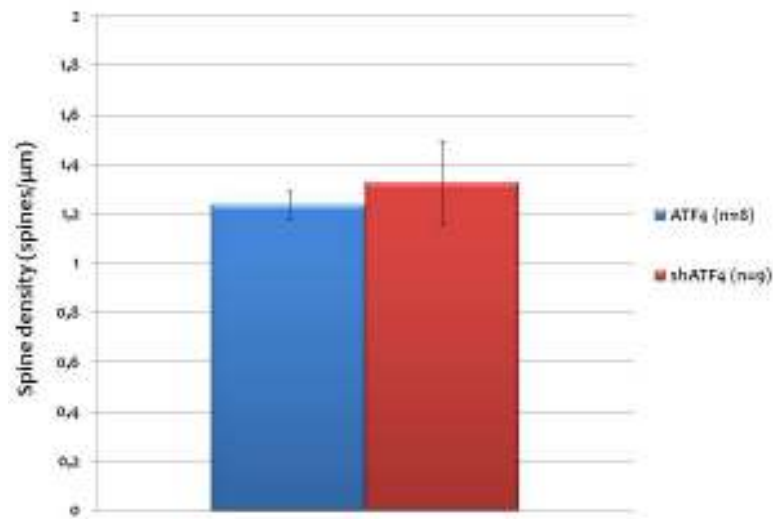
b
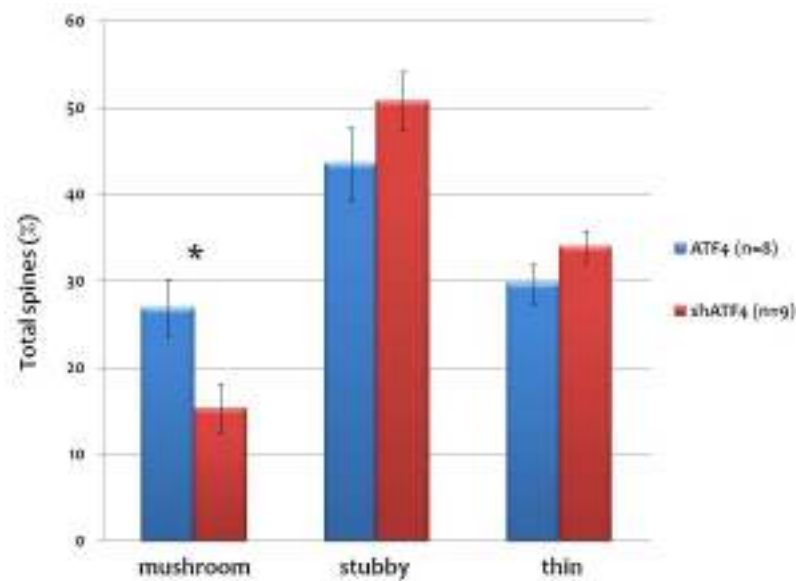
Fig 3.23. Spine density and morphology analysis: combined data. Considering the spine analysis data obtained from ATF4 overexpression and downregulation experiments, no difference in the spine density was found between the two groups, which was around 1.3 (a). On the contrary, a statistically significant reduction in the % of mushroom spines, connected to learning and memory,was found in the neurons where ATF4 was downregulated as compared to those in which the protein was overexpressed.

CHAPTER IV: DISCUSSION

Several approaches can be used to study the function of a gene. These include the administration of pharmacological agents like agonists and antagonists or the generation of knockout mice, a genetically engineered mice in which the gene of interest is replaced with a related sequence that has been modified to contain a mutation that causes the loss of gene activity. Unfortunately the production of knockout mice is not always possible because the gene of interest can be fundamental for normal mouse development (Copp et al., 1995). Researchers overcame this problem through the use of conditional knockouts, which provide a temporal and tissue-specific gene knockout control, while allowing the mouse to develop under the normal operating role of the gene of interest (Lobe et al., 1998). Another tool is the employment of a different kinds of viruses, such as adenovirus or lentivirus, as vectors to stably introduce or silence genes into cells (Naldini et al., 1996; Dodart et al., 2004; Ahmed et al., 2004; Singer et al., 2005; Kanninen et al., 2009).

To study the role of ATF4 in learning and memory, the consequence of its gain-and loss-of-function in mouse hippocampus was evaluated by the performance of standard behavioral tests such as Water Maze tests and Fear Conditioning, widely used to asses spatial and associative memory respectively (Sharma et al., 2010; Maren and Holt, 2000). For the-loss-of-function studies, ATF4 null mice could not be used because they exhibit visual and skeletal problems that interfere with the completion of the behavioral tasks (Tanaka et al., 1998; Yang et al., 2004). Indeed, ATF4 is fundamental for lens fiber differentiation (Tanaka et al., 1998) and is a crucial factor promoting osteoblast maturation (Dobreva *et al.,* 2006). Therefore the viral-based tool for gene delivery was used to modify *in vivo* gene expression with neuroanatomical selectivity and without the cost and the difficulty of generating conditional knockouts. Lentiviral particles were inoculated into mouse hippocampi to modulate ATF4 gene expression. Lentivirus carrying ATF4 gene, for the gain-of-function studies, and lentivirus with shATF4 for the loss-of-function analysis, were inoculated into mouse hippocampi. Lentivirus is one of the most suitable virus for gene transfer, capable of great potential. Lentiviral infection has the advantage of high-efficiency infection of terminally differentiated primary cells, including neurons, because the viral genome can penetrate through an intact nuclear membrane to access the cellular genome. It ensures long-term stable expression of a transgene, and low immunogenicity (Lever, 2000; Durand and Cimarelli, 2011). Unfortunately, it has some drawbacks, like its random integration that can be with or without desired consequences. Several efforts are now focusing on achieving site directed viral integration, but the results are still unsatisfactory. A

different strategy to overcome the negative effects of integration is the non-integrated lentivirus that remains stable in the host non-dividing cells as episomes (Saenz et al., 2004).

The employment of viral-based tool for gene delivery is widely used in neuroscience to target neurons in specific brain area such as the hippocampus and the cerebellum, etc. (Alisky et al., 2000; Ohashi et al., 2011; Grillo et al., 2007). Through this techniques several authors have been able to clarify the role of specific genes in brain functions; particularly, this approach has been widely use to study the involvement of various genes in learning and memory (Kanninen et al., 2009; Caccamo et al., 2010).

The role of ATF4 in memory storage and synaptic plasticity has already been investigated, in rodents, by Kandel's group through the generation of transgenic mice expressing, in the forebrain, a broad dominant-negative inhibitor of C/EBP proteins, AZIP, under a tetracycline-regulated system. This approach, though extremely sophisticate, is not specific, because the AZIP expression causes the downregulation of not only ATF4 protein, but also of C/EBPβ protein. Moreover they showed that the transcript profile of AZIP transgenic mice is altered compared to the control mice without doxycycline treatment. They presented a list of genes that are upregulated as a result of the inhibitor expression, such as NFX1, Carbonic anhydrase IV, squamnous cell carcinoma antigen 2 and others (Chen et al., 2003). Our method for the ATF4 loss-of-function studies involves the employment of small hairpin RNAs designed to specifically target ATF4 mRNA, leading to a downregulation of ATF4 gene and subsequently a reduction of the ATF4 protein level only. Moreover using the same approach the consequence of ATF4 overexpression was eveluated after the inoculation of lentivirus carrying ATF4 gene.

However, in vivo lentiviral delivery has its own issues regarding the extent and the magnitude of virus spreading and infection. Lentiviral constructs were tested, *in vitro,* by infecting dissociated primary hippocampal neurons before inoculating them in mouse hippocampi. 1 week after infection, more than 90% of the cells expressed GFP protein (data not shown) and ATF4 protein level in ATF4 lentivirus infected neurons was higher compared to the controls (pWPI) and in shATF4 lentivirus infected neurons was lower compared to the controls (shMUT). Afterwards lentiviral constructs were injected into mouse hippocampi and a time course analysis of the lentiviral spreading was performed. The number of infected neurons in the same hippocampal area increased with each passing day after the injection. Moreover 1 and 2 weeks after injection the lentivirus infected only the Dentate Gyrus and the CA3 while, 4 weeks after injection, the lentivirus also spread to the CA1 region. This lentiviral spread can be explained by the fact that the lentivirus takes more than 2 weeks to spread and infect neurons from the DG to CA1 region or in the DG, the brain area known to be site of adult neurogenesis (Abrous et al., 2005), lentivirus infected neuronal progenitors underwent cell division, neuronal differentiation and migration. Regarding the infection efficacy, 1 month after

lentiviral infection around 55% of the cells in the DG were GFP positive. Though the lentiviral infection did not involved the whole hippocampus, there were a statistically significant differences in the performance of behavioral tests. Moreover the analysis of ATF4 mRNA level in the whole hippocampus revealed that ATF4 downregulated mice showed a 50% reduction in ATF4 mRNA level compared to the controls. This implies that perhaps the lentivirus only needs to infect a relatively small proportion of the hippocampus in order to be functionally significant, as reported in other studies conducted in other regions of the brain (White et al., 2008, Burnet et al., 2011).

The behavioral test results support the hypothesis that ATF4 plays a positive role in learning and memory. In particular ATF4 protein overexpression enhances spatial memory, under the weak training paradigm in the Morris Water Maze test, and associative memory while ATF4 downregulation impairs spatial memory under the standard training condition without affecting associative memory. The fact that associative memory is not impaired in ATF4 downregulated mice can be due to the ATF4 downregulation level. The injection of shATF4 lentivirus caused a 50% reduction in ATF4 mRNA level in the hippocampus, which maybe is not sufficient to cause an associative memory impairment.

These results showing ATF4 as a positive players in learning and memory are opposite to those present in literature which describe ATF4 as a repressor of CREB mediated gene transcription, hence a memory repressor (Karpinski at al., 1992; Bartsch et al., 1995). ATF4 is a stress response gene activated by several stressors such as metabolic deficits, oxidative stress and ER stress. These stress signals activate different kinases that in turn phosphorylate the α subunit of eIF2. The phosphorylation of this subunit results in the inhibition of translation initiation by impairing the eIF2B-catalyzed guanine nucleotide exchange reaction (Moldave, 1985) leading to a global protein synthesis inhibition, including CREB protein, and a concurrent expression of genes that function to alleviate stress damage in cells. One of these stress response genes is ATF4 which is strongly induced after eIF2α phosphorylation. Long term synaptic plasticity and memory require new protein synthesis, so eIF2α is a key factor for the translational control involved in these processes (Costa Mattioli et al., 2007). Modification of proteins involved in this pathway, located upstream of ATF4, alters synaptic plasticity and memory formation. It has been demonstrated that GCN2 null mice, and mice homozygous for eIF2α $^{+/S51A}$ mutation, in which the phosphorylation of the α subunit of eIF2 is reduced, have a reduction in the level of ATF4 and an increase in CREB activity. These mice showed a decreased threshold for eliciting L-LTP and an enhancement in Morris Water Maze performance after a weak training protocol. On the contrary, strong stimulation and standard training protocol caused an impairment in LTP and memory formation in GCN2 null mice (Costa Mattioli et al., 2005) and an

enhancement in eIF2α $^{+/S51A}$ mutant mice (Costa Mattioli et al., 2007). The proposed mechanism for the synaptic plasticity and memory enhancement, in both cases, was ascribed to ATF4 downregulation as consequence of eIF2α phosphorylation reduction, excluding the possibility of the involvement of other eIF2α downstream genes (Fernandez et al., 2003; Zhou et al., 2008; Sikalidis et al., 2011; Palam et al., 2011). No study has ever investigated the way in which synaptic plasticy and memory can be affected by a direct and specific modulation of ATF4, to confirm its role in these processes.

Recent studies have shown that ATF4 can also act as transcriptional activator. It can induce different kinds of genes such as receptor activator of nuclear factor-kappa B ligand (RANKL) (Elefteriou et al., 2005), osteocalcin (Yang & Karsenty, 2004), E-selectin etc. Moreover, ATF4 can upregulate genes involved in mitochondrial function and amino acid metabolism (Harding et al., 2003). Most importantly, ATF4 was found to act as a prosurvival factor, able to preserve cells from ER stress-induced apoptosis through the upregulation of Parkin protein (Bouman et al., 2010).

The long term memory impairment of ATF4 downregulated mice is supported by electrophysiological analysis in which the L-LTP was found impaired in ATF4 downregulated slices compared to the controls. LTP is widely considered one of the major cellular mechanisms that underlies learning and memory in the central nervous system. A lot of studies have shown that the presence of cognitive deficits, assessed with behavioral tests, are associated with impairment of synaptic potentiation (Liu et al., 2008; Tamayev et al., 2011) or the opposite, where an improvement in the LTM is supported by a restoration of LTP (Tinchese et al., 2008; Puzzo et al., 2009). However, hippocampal LTP does not necessarily positively correlate with learning and memory (Migaud et al., 1998; Gu et al., 2002). In ATF4 overexpressed mice, the spatial and associative memory enhancement was not accompanied by an improvement in synaptic potentiation. On the contrary an impairment in E-LTP was found in ATF4 overexpressed mice compared to the controls. One hypothesis that can explain this contradictory result is that the ATF4 overexpressing lentiviral vector lead to a strong upregulation of ATF4 protein that in turn, over the course of weeks, lead to neuronal damage. It has been shown that the upregulation of ATF4 protein, up to 3 weeks, in dissociated primary hippocampal neurons causes neuronal death (Personal communication). Any sign of neuronal degeneration was observed in the hippocampus of ATF4 overexpressed mice until 6 weeks after injection, time point at which spine analysis was performed. Electrophysiological studies were performed from 6 until 9 weeks after lentiviral injection so it is possible that ATF4 protein may have started to cause neuronal degeneration at the time of these studies. Anyway It is premature to speculate as to the reason for these differences. A time course evaluation of ATF4 protein overexpression and LTP will be necessary to understand the consequence of ATF4 overexpression in long term plasticity.

Eric Kandel, in 2001, described the long term memory process as a "dialog between genes and synapses"(Kandel, 2001) where new gene expression and protein synthesis are required for the formation and morphological changes in spines. Spines represent the substrate upon which LTM can be established (Engert and Bonhoeffer, 1999). Therefore it has been verified if the cognitive differences observed after the ATF4 up and downregulation can be due to spine density and morphology modification, since morphological differences between spines are known to be correlated with functional differences. Changes in ATF4 level does not affect spine density but ATF4 overexpression causes a significant increase in the percentage of mushroom spines in comparison to that found after ATF4 downregulation. It has been demonstrated that 4 months old J20 mice, an Alzheimer's disease mouse model, which show long term memory deficits, have a modification in spine morphology without any alteration in hippocampal neuronal spine density compared to the wildtype. In particular J20 mice present a significant reduction in the % of mushroom spines and an increase in the stubby spines, the same modification found in our ATF4 downregulated mice (Personal communication). Moreover, transgenic mice overexpressing human tau protein, whose accumulation and hyperphosphorylation cause NeuroFibrillary Tangle (NFTs), a histopathological hallmark of Alzheimer's disease, have less mushroom and more thin spines in both apical and basal dendrites as a consequence of human tau accumulation (Dickstein et al., 2010). Our results in agreement with the literature data support the idea that spine morphology modification and in particular the reduction in the mushroom spines are linked to cognitive deficits and that spine morphology modification is not always accompanied by spine density modification. Spines are small membranous protrusions arising from dendrites and represent the postsynaptic component of most excitatory synapses in the brain. Spine head is a local compartment where all the molecules and proteins of the postsynaptic machinery are concentrated. Mushroom spines with a big head have larger and more complex PSD and higher density of glutamate receptors. (Bourne and Harris, 2008). It has been hypothesized that spines with a large head such as mushroom spines are more stable and contribute to strong synaptic connection; hence they represent the "memory spines" (Bourne and Harris, 2007). The improvement in cognitive function following ATF4 overexpression as well as the memory deficit in ATF4 downregulated mice can be due, in part, to an effect of ATF4 in spine morphology modification. Another in vitro study confirmed the involvement of ATF4 in the modulation of spine morphology showing that knocking down ATF4 in cultered rat hippocampal and cortical neurons reduces the proportion of mushroom spines and increases the proportion of thin, filopodia-like dendritic spines, without affecting spine density (Jin et al., 2011). In our in vivo model, the reduction of mushroom spines was not followed by a significant increase in the proportion of the thin spines, maybe due to the differences in the experimental condition. They also found that the cytoskeletal regulator RhoA and its active form RhoA-GTP are downregulated by ATF4 knock-down,

so it plausible that, also in our in vivo model, ATF4 can affect spine morphology through the modulation of Rho GTPases proteins involved in the regulation of actin polymerization. The Rho family of GTPase includes Rho A, Rac 1 and Cdc42. These proteins, with several other factors, cooperate in a complicated network to modulate the polymerization and depolymerization of cytoscheletal actin, highly enriched in spines, to determine spine shape and activity (Murakoshi et al., 2011). Uncovering the mechanisms and the players involved in spine modification is fundamental because abnormalities in spine number and shape are strongly correlate with several psychiatric and neurological diseases.

ATF4 protein, in addition to its DNA binding site, has a leucine zipper domain though witch it can dimerize, generating homodimers and heterodimers, with a variety of proteins including members of the AP-1 and C/EBP family like Fos (Hai & Curran, 1991) and Jun (Chevray & Nathans, 1992), p300, RSK2, (Yang et al., 2004), Satb2 (Dobreva et al., 2006), Nf2(Cullian & Diehl, 2006) and others.

ATF4 was also found to be partner of Gamma Amino Butyric Acid$_B$ receptor (GABA$_B$). GABA receptors are a class of receptors that respond to the inhibitory neurotransmitter GABA in the nervous system. There are two classes of GABA receptors: the ionotropic ligand-gated ion channel (GABA$_A$) and the metabotropic G-protein coupled receptors (GABA$_B$). ATF4 through its bZIP domain interacts with the C-terminal tail of the GABA$_B$ receptors. Functional GABA$_B$ receptors are heterodimers made by the interaction of two subunits GABA$_B$ R1 and R2 (Kuriyama et al., 1993). A direct and specific interaction has been demonstrated between ATF4 and GABA$_B$ R1 subtype in the neuronal cytoplasm and along the neuronal processes (Nehring et al., 2000). Our analysis in mouse hippocampi after lentiviral infection showed a neuronal cytoplasmatic localization of overexpressed ATF4 protein. It is possible that, also in our model, overexpressed ATF4 protein in hippocampal neurons interacts with the GABA receptors modulating its functionality. The consequences of this interaction are largely unknown. It can facilitate the translocation of ATF4 from the cytoplasm into the nucleus (White et al., 2000) leading to a modification in transcription mediated by ATF4 (Helm et al., 2005). This suggests a new neuronal signaling pathway through which activation of metabotropic receptors can direct modulate gene transcription.

ATF4 can also, totally independent from its role as transcriptional modulator, promote or prevent some mechanisms by acting as a binding protein. ATF4 binding to GABA$_B$ R1 also promote ATF4 translocation in the opposite direction, from the nucleus to the cytoplasm (Vernon et al., 2001). It has been speculated that ATF4 is a trafficking partner of GABA$_B$ R1 and regulates the heteromeric assembly of the receptor. Moreover, it has been shown that ATF4 controls the GABA$_B$ R1 subunit gene expression and during post natal mouse brain development, it can bind both GABA$_B$ R1 and R2 subunits (Ritter et al.,2004).

The role of GABA$_B$ receptor in LTP is dual. Several papers have demonstrated a positive role of GABA$_B$ in the LTP. It is already well established that LTP, which occurs in CA1 region, requires the activation of NMDA receptors. During high frequency stimulation, GABA inhibits its own release by acting on GABA$_B$ autoreceptors which allows sufficient NMDA receptor activation for LTP induction (Davies 1991, Mott and Lewis, 1991). The same result is supported by another study in which GABA$_B$, localized at the dendritic spines of hippocampal CA1 pyramidal neurons, contributes to the induction of LTP with stimulation in the range of theta rhythm (Huang et al., 2005). On the contrary, it has been shown that phaclofen and CGP35348, two selective GABA$_B$ receptor blockers, facilitate the induction of LTP (Olpe and Karrison, 1990).

Concluding, the results described in this thesis enable us to assert that, contrary to the literature, ATF4 plays a positive role in the cognitive processes of learning and memory. Further study needs to be done to outline, in detail, the mechanisms through which this transcription factor acts. Some of these mechanisms have been proposed on the basis of the results obtained in this study and those found in literature. These mechanisms includes: the involvement of ATF4 in spine morphology modification and the involvement in synaptic plasticity through its possible interaction with GABA$_B$ receptors. However, these hypothesis still have to be confirmed. Absolutely one of the first thing to clarify is if the role of ATF4 is mediated by its capacity to modulate gene expression. To address this question ATF4 protein with a mutation in its DNA binding site, that prevents its ability to bind DNA and modulate gene transcription, will be express in mouse hippocampus.

Moreover ATF4 has been found to play a role in synaptic plasticity. After clarifying the role of ATF4 overexpression in the LTP, it is necessary to perform further electrophysiological analysis to understand if ATF4 affects the pre or post synaptic functions or both. These analysis includes: pair pulse facilitation, vescicle cycling and mEPSPs to assess the pre-synaptic function and mEPSC amplitude, amplitude of AMPAR/NMDAR evoked EPSCs and AMPAR trafficking and phosphorylation for the post-synaptic activity.

CHAPTER V: BIBLIOGRAPHY

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