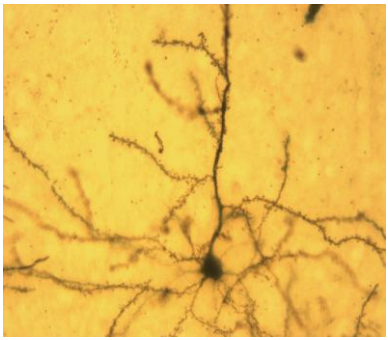
Università di Roma “La Sapienza”

Scuola di Dottorato in Neuroscienze

Dottorato di Ricerca in Psicobiologia e Psicofarmacologia

Spatial memory and plasticity: molecular mechanisms in the hippocampus and the ventral striatum



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Summary

Remerciements, Ringraziamenti, Acknowledgements

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« L'homme intelligent juge l'homme simple.

Mais si l'homme simple le jugeait, l'homme intelligent

deviendrait un simple homme »

Je dédie cette thèse à mes parents.

I. Introduction

I.1 Memory generality

Memory is defined as the organism capacity to store, retain and recall information and experiences. In cognitive-psychology, encoding, storage and recall are all components of "memory" (Sherry and Schacter, 1987).

There are five sensory modalities that can be used to acquire information from external environment (usually called allocentric information): vision, olfaction, taste, hearing, touch, and two important systems used to acquire information from our own body (usually called egocentric information) which are the proprioception and the vestibular system.

The brain uses all these information to integrate its own physical status with the external environment. The integration of these information leads to the understanding whether the environment is critical for life or it can be a source of food, as well as if other animals are potential predator or possible social partner. In this framework it is important to create memory of new information for its own survival. During the 60's, cognitive-psychologists studied the principles of memory and proposed the "modal model" of memory separating memory into three components, sensorial memory, short- and long-term memory (Atkinson and Schiffrin, 1968).

I.1.1 Sensory memory

"Sensory memory" is memory at sensory levels that treats information acquired from the external word. The sense most developed in the human is vision. Visual information is send to the occipital cortex and split in two ways. The dorsal way, from visual cortex to posterior parietal cortex (involved in the treatment of spatial information) and the ventral way, from visual cortex to inferotemporal cortex (involved in the recognition and identification of object) (Rousselet et al., 2004). A second sensory modality very much developed in mammals is olfaction. Sensorial organs of smell send information to the olfactory bulb, anterior olfactory nucleus, and piriform cortex and then to the entorhinal cortex. Hearing information is sent to the inferior colliculus via superior olivary complex and nuclei of lateral lemniscus to end in primary auditory cortex. Taste information is sent to the insula via the nucleus of the solitary track then via the thalamus. Touch information is sent to the primary somatic sensory cortex via the medial lemniscus then via the thalamus. Somatosensory system reacts to diverse stimuli as the proprioception, using different receptors: thermoreceptors, nociceptors, mechanoreceptors and chemoreceptors. Transmission of information from these receptors projects from the spinal cord to the central nervous system (CNS). Processing primarily occurs in the primary somatosensory area in the parietal lobe. Vestibular system, which contributes to balance in most mammals and to the spatial orientation, is the sensory system that provides the leading contribution to movement and sense of balance. This information is sent in the lateral vestibular nucleus to allow vestibule-ocular reflex and posture control. It is also sent to the brain to allow voluntary

control of the posture. All of these pathways are the initial input of the information to the brain.

The “sensory memory” is defined as the ability to retain sensory information after the original stimulus has ceased. It refers to items detected by the sensory receptors which are retained temporarily in the sensory register, which has a large capacity for unprocessed information but is only able to hold accurate images of sensory information momentarily. The two types of sensory memory that have been most explored are iconic memory and echoic memory but it’s also known the haptic memory (touch memory). The first account of this phenomenon was reported in 1740 by Johann Andreas Segner. Segner attached a glowing coal to a cartwheel and rotated the wheel at increasing speed until unbroken circle of light was perceived by the observer. He calculated that the glowing coal needed to make a complete circle in less than 100ms to achieve this effect. It was the first experiment calculating how persistent the sensory memory in human is. Sensory memory is still considered to operate within this approximate time frame (less than 1 second and no more than 2) and so is very short lived. It is also characterized for being outside of conscious control. Despite retaining information for a very short period of time, it is not to be confused with short term memory (which typically lasts 10-15 seconds, in the human, without rehearsal of the remembered material) and is so named to distinguish it from long term memory which can store information for as long as a lifetime.

Although it is usually referred to memory as the capacity to maintain information for a long time, it has been shown that exist different memories with different levels of retention time, retention capacity and complexity of information. The sensory memory has been

suggested to depend upon the electrical activity of the sensory system, and therefore this memory has the greatest capacity (because of the retention of all entrance information) but with a very short duration (less than 2 seconds).

I.1.2 Short term memory

In human, short-term memory (STM) is defined as the memory that allows recall for a period of several seconds to a minute without rehearsal and also called working memory (WM) such as the maintaining of phone number. The WM in the human is also defined by a longer period of recall (over the day), for example to recall several hours after where it was been parked your car. In the animal models, in general, has been hypothesised that STM/WM has a retention period of one hour. Its capacity is considered very limited. George A. Miller (1956), conducted experiments demonstrating that this memory has a span of 7±2 items in human. Modern estimates of the capacity of short-term memory are lower, typically of the order of 4–5 items. One item can be one letter as well as one word, or can be one figure as well as one number compose of more figures, or others. In fact there is a process that allows improvement of memory capacity called chunking. For example, in recalling a ten-digit telephone number, a person could chunk the digits into three groups: first, the area code (such as 215), then a three-digit chunk (123) and lastly a four-digit chunk (4567). This method of remembering telephone numbers is far more effective than attempting to remember a string of 10 digits; this is because we are able to chunk the information into meaningful groups of numbers. Herbert Simon

showed that the ideal size for chunking letters and numbers, meaningful or not, was three. Unlike sensory memory, short memory is a conscious memorization of the information that is used for a brief time. Then when is not any more utile, is canceled.

According to the models currently used this kind of memory has been suggested to depend upon changes in membrane properties leading to a change in synaptic efficacy (Lamprecht and Le Doux, 2004). In fact the literature suggests that the flow of important information can trigger a strong depolarization at the post-synaptic level. This strong depolarization increases synaptic efficacy thus leads to longer and higher activation of voltage dependent channel. This model would explain the retention of information during a short period.

I.1.3 Different long term memories: Explicit (declarative) and implicit (non declarative) memories

The conceptualization that memory is not a unitary system but composed of multiple systems, both at a psychological as well as from a neurobiologic point of view, establishes a fundamental point of view the modern neurosciences.

Using a qualitative classification criterion we distinguish two types of LTM: the declarative memory (or explicit) and the non-declarative memory (or implicit). The declarative memory includes all information memorized that recall fact and events (semantic and episodic memory), this type of memory is flexible and implies the capacity to create association among several information; moreover

its kind of memory type which is consciously available and is possible to recall verbally in humans. The non-declarative memory instead, does not require a conscious recall, includes procedural memory, the priming and the conditioning (Cohen e Squire, 1980; Graf e Schacter, 1985; Schacter, 1987; Squire e Zola, 1996).

The first theories proposing multiple systems of memory were elaborated thanks to studies analyzing the effect of human focal brain lesions. Old studies showed that lesion in a specific structure produced specific deficits (Zola-Morgan et al., 1986; Rempel-Clower et al., 1996). Fundamental findings were found with the patient H.M. Because of resistant epilepsy to all pharmacological treatments, it underwent neurosurgery with the ablation of large portion of the medial temporal lobe (MTL) in both hemispheres (Scoville and Milner, 1957). Following surgery, H.M. developed acute anterograde amnesia, but was able to remember information for short time. Deeper examination revealed specific amnesia for new information about fact and event: in fact he was able to learn new motor abilities, as it was shown in the "draw in mirror test", while he was unable to remember to have ever performed this task (Milner, 1968). Imaging studies in humans confirmed the specific role of MTL in declarative memory (Cohen and Squire, 1980 Squire and Zola, 1996). This discovery helped also to understand that non-declarative memory is using different neural substrates. Because of the lack of results from lesion studies in human showing an impairment for non-declarative memory, it was distinguished these memory types.

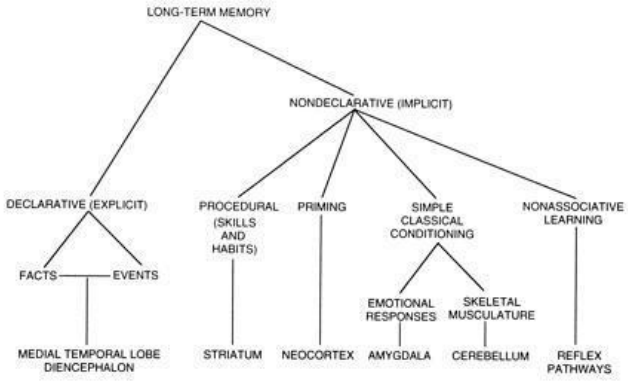


Figure 1: A taxonomy of long-term memory systems together with specific brain structures involved in each system (Squire and Zola, 1996)

Accordingly to the most used classification, memory system is separated in four principal characteristics. In term of psychological process underling the two kinds of memory, they are currents distinguished on the basis of four main characters: absence or presence consciousness; memory expression flexibility; type of information elaborated; neural structures involved (Squire, 2004). It should be mentioned that this classification, which is now the most used in the literature, can't be considered as definitive or "the absolute" model to explain the memory system.

I.2 Recording and stabilization of memory

Along with scientific researches, several theories emerged about molecular mechanisms necessary to acquire and maintain new information. There is a general accord in the literature that information processing can be divided into different steps that have been named: encoding, storage and retrieval. The behavioral theories however need to be adjusted to the biological constraint that experimental neurobiologist are suggesting as occurring after learning. Below are described some of the model most commonly used to explain the biological processes underlying memory.

I.2.1 Long term potentiation (LTP) model

On the basis of Ramon y Cajal observations and the Hebbian theory in 1949 it was been suggested that cells may grow new connections or undergo metabolic changes that enhance their ability to communicate. In 1966 Terje Lömo demonstrated in the hippocampus that the postsynaptic cells' response to single-pulse stimuli could be enhanced if it is first delivered a high-frequency train of stimuli to the presynaptic fibers. When such train of stimuli is applied, subsequent single-pulse stimuli elicited stronger, prolonged EPSPs in the postsynaptic cell population. This phenomenon, whereby a high-frequency stimulation could produce a long-lived enhancement in the postsynaptic cells' response to subsequent single-pulse stimuli, was initially called "long-lasting potentiation" (Bliss and Lömo, 1973).

Today, this mechanism is called “Long term potentiation” (LTP) and it has been observed *in vitro* as well as *in vivo*, in the hippocampus and

in others many brain structures such as the neocortex and the cerebellum. LTP studies show numerous analogies between this process and plastic and molecular mechanisms involved in learning and memory process (Lynch, 2004), but up to now there is no direct evidence that demonstrates LTP-induced learning.

The LTP is induced by a strong pre-synaptic stimulation that can be maintained for hours by intracellular molecular events. Experimental data suggested a separation between early LTP and a late LTP. The Early LTP is an early phase around seconds to minutes after induction and lasts for approximately three hours. During this phase, changes are observed in pre-existing protein and in membrane properties. The Late LTP occurs minutes to hours after induction. This phase is transcription dependent and protein synthesis dependent (Krug et al., 1984). The late phase can be divided in others two parts: the L-LTP2 that occurs with protein synthesis via mRNA pre-existent and maintains activity during several days; the L-LTP3 that occurs with ex novo synthesis of protein and maintains the activity during weeks.

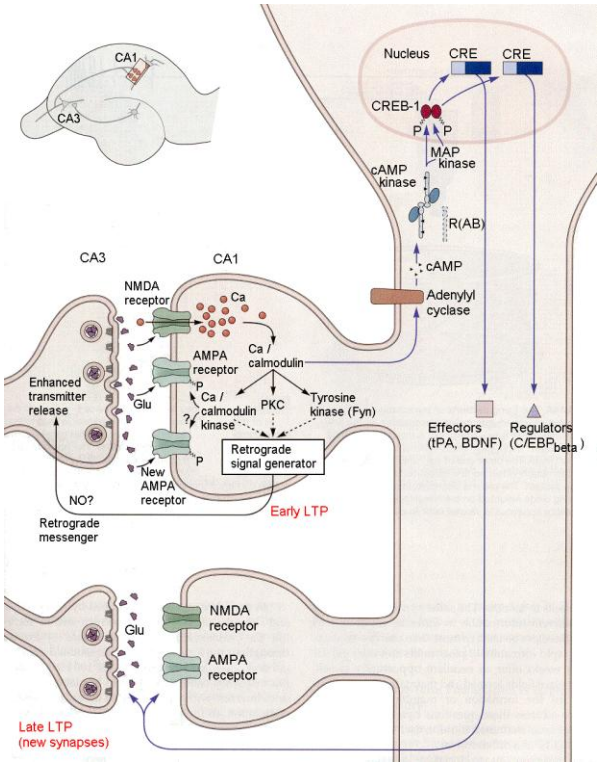


Figure2: molecular mechanisms between two neurons on the basis of Long term potentiation model (picture from the UNMC website).

The modifications occurred during early LTP has been well characterized and the most relevant event is post-synaptic phosphorilation. It occurs after the stimulation, and changes receptor activity and actives transcription factors. Different studies

demonstrated receptor changes in activities in particular the AMPAr (Lee et al., 2000; Oh et al., 2006). LTP induction allows changes in postsynaptic membrane properties, as increase of conductance as well as insertion of new AMPA receptors (Derkach et al., 1999; Song et al., 2006). Another consequence of LTP induction is activation of transcription factors such as CREB, which leads to transcription of new genes. CREB was demonstrated involved in plasticity and in different kinds of long-term memory (Bailey et Kandel, 1993; Lamprecht et Ledoux, 2004).

Examining the late phase of LPT, two different molecular mechanisms have been shown that allow the distinction of two phases: the Late LTP2 (L-LTP2) and the late LTP3 (L-LTP3). The L-LTP2 is gene transcriptions independent. During this phase there is translation of pre-existing mRNA that allow earlier changes at the synaptic level. The exact meaning of these changes and their functional roles however, is a less understood phenomenon.

During the second phase, L-LTP3 is associated to specific gene transcription, followed by an increase in protein synthesis (Krug et al., 1984). In this phase, mRNAs translated are originally from immediate early genes (IEG), which have a quick augmentation of transcription immediately after induction of LTP and turn down at basal level in a few hours. However it has been demonstrated that others genes defined as late- response genes (LRG) are induced and repressed with slower dynamics. The transcription of these gene changes starts after 2-3 hours and can maintained these changes until 48 hours after (Fazeli et al., 1993). Genes of the LRG are kinase, growth factors or proteases that could play a role in structural modification of synapses. From this model emerged the hypothesis of “synaptic tagging”, that implies that *de novo* transcript genes

should be transport and guided to the active synapse. In this new strategy, the translation of pre-existing mRNA at the single dendrite level should be able to individualize the single spine activated (Kelleher et al., 2004; Yu et al., 2004).

I.2.2 Synaptic system

The molecular and cellular mechanisms, which allow memory stabilization, are generally named synaptic consolidation (Dudai, 2004; Frankland et Bontempi 2005). It has been demonstrated that stabilization processes, in long term memory, are based at the beginning on receptor activation (Bliss et Collingridge, 1993) and among these of particular importance is the NMDA receptor, a subtype of glutamatergic receptor (Tsien et al., 1996; Rampon et al., 2000). Following NMDA activation, the Ca^{2}+kinase protein/ calmodulin dependent (CamKII) demonstrated to be necessary in maintaining and stabilization of memory (Silva et al., 1992; Mayford et al., 1995; Giese et al., 1998), and is activated together with others molecules. The CamKII can change the membrane properties at the post-synaptic level, for example increasing the excitatory post-synaptic potential (EPSPs) by AMPA receptor insertion and by increasing of the conductance in the same receptors (Nicoll et Malenka, 1999).

In the Ledoux's plasticity model (Lamprecht and Le Doux, 2004), it has been proposed an activation of new gene transcription and a structural remodeling at the synaptic level. During this phase it may be used mRNAs already present at the single activated synapse and translated in new protein for the early structural changes. Then this

remodeling is stabilized by the *de novo* protein synthesis, based on new gene transcription. Different studies showed that the transcription of new genes is necessary in the stabilization of long term memories (Alberini et al., 1994; Bailey et al., 1996; Guzowski et al., 1998), as CREB transcription factor (Bailey and Kandel, 1993; Lamprecht and Le Doux, 2004). The importance of new protein synthesis in LTM (Davis and Squire, 1984; Bourtchouladze et al., 1998; Artignand et al., 2004), has been demonstrated by the effects of inhibition of protein synthesis on memory performace (Bourtchouladze et al., 1998; Bailey, 1999; Bozon et al., 2002; Vazdarjanova et al., 2004; Artinian et al., 2007).

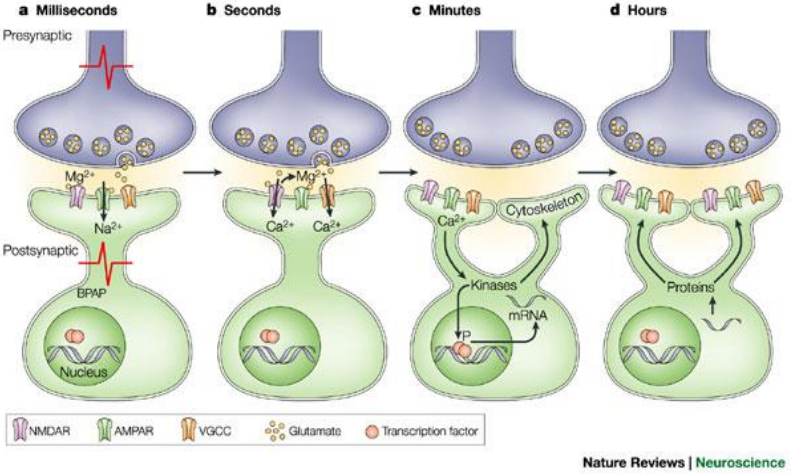


Figure 3: Plasticity model (Lamprecht and Le Doux, 2004)

At the functional level, newly synthesized proteins necessary for the LTM need to be specifically delivered to active synapse without affecting the synapses not involved in the process. Two hypotheses have been proposed: 1) The activated synapse triggers *de novo* genes and proteins synthesis, and those are transported by specific signal to the synapse activated: “synaptic tag” model (Frey and Morris, 1998). 2) Synaptic plasticity occurs by local synthesis of new proteins, at the synaptic level (Steward and Schuman, 2001). Among different molecular mechanisms of post-transcription regulation, microRNAs (miRs) have been suggested to play an important role in the fine and local regulation of mRNAs translation involved during the remodeling.

I.2.3 System consolidation

Recent evidence demonstrates that memories, may need weeks, months or years to be stabilized. This model of consolidation proposes that the molecular mechanisms involve slower dynamics and circuit reorganizations that need interaction among different cerebral areas: this model has been named “system consolidation” (Frankland and Bontempi, 2005).

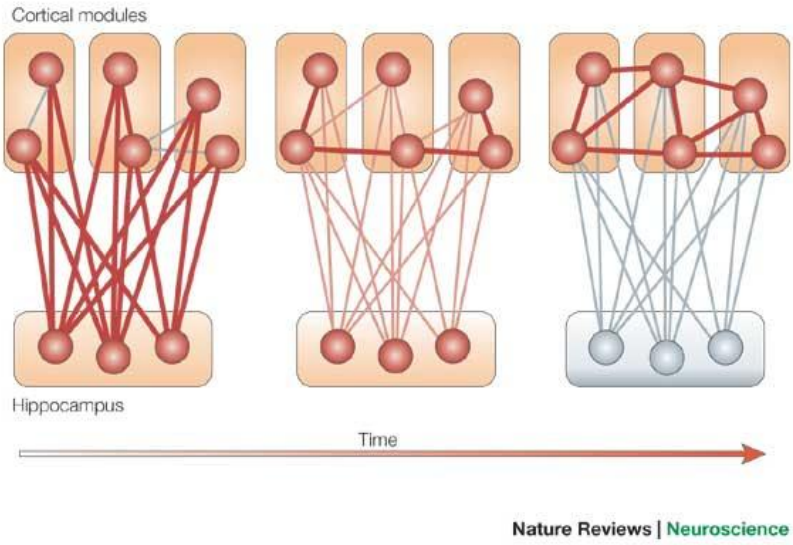


Figure 4: Synaptic consolidation model (Frankland and Bontempi, 2005).

The system consolidation theory suggests that the stabilization of memory, occurs during a short period after learning but can require several waves of repeated reactivation, also off-line, such as during sleep (Pennartz et al., 2004). The reactivation might help the plastic processes at the synaptic level involved in the processing of information (Wang et al., 2006). This hypothesis is called “synaptic reentry reinforcement (SRR) (Shimizu et al., 2000; Wittenberg and Tsien, 2002) and has been suggested to involve repeated activation of NMDA receptors.

I.3 microRNA

The microRNAs (miRs) were first discovered in C. Elegans in 1993 (Lee et al., 1993) but were recognized as a distinct class of biologic regulators with conserved functions only in 2001 (Lagos-Quintana et al., 2001). In 2002, miRs were demonstrated involved in negative post-transcriptional regulation (Lai, 2002). Different patterns of miRs expression are observed in mammal tissues (Lagos Quintana et al., 2002).

I.3.1 Definition

MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules, constituted by an average of only 22 nucleotides and are found in all eukaryotic cells, except fungi, algae, and marine plants. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression and gene silencing (Bartel, 2004; Bartel, 2009). The human genome may encode over 1000 miRNAs (Bentwich et al., 2005), which may target about 60% of mammalian genes (Friedman et al., 2009) and are abundant in many human cell types (Lim et al., 2003).

I.3.2 General roles

The precursors of miRNAs are transcribed from DNA. The processing of miRNAs from primary miRNA transcripts (pri-miRNA) into precursor miRNAs (pre-miRNA) and then into mature miRNAs is mediated by the enzyme, Drosha in the nucleus and Dicer in the cytoplasm. The mature miRNA will then associate with a complex called RNA Induced Silencing Complex (RISC). The figure 5 shows the main miRNA processing pathway. Recently, a dicer-independent pathway for maturation of miRNA has also been reported (Cheloufi et al., 2010).

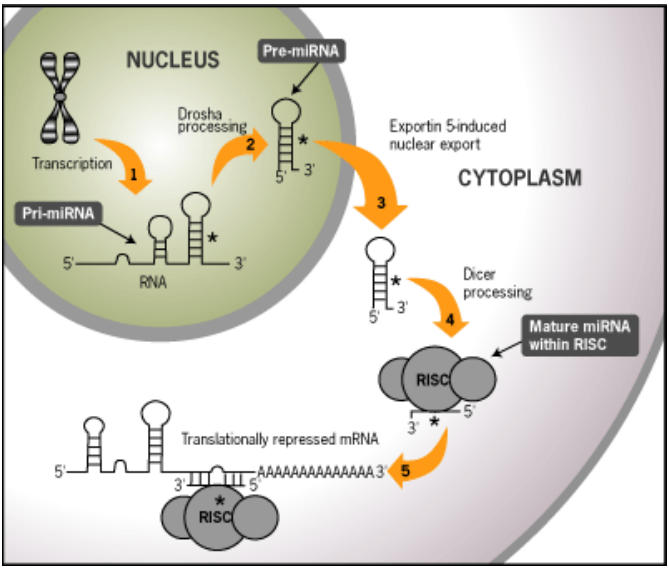


Figure 5: microRNA processing

Gene silencing may occur either via mRNA degradation or preventing mRNA from being translated. It has been demonstrated that if there is complete complementation between the miRNA and target mRNA sequence, Ago2 can cleave the mRNA and lead to direct mRNA degradation. Yet, if there isn't complete complementation the silencing is achieved by preventing translation (Lim et al., 2005).

The translational repression has been accepted as the main mechanism by which mature miRNAs contribute to the regulation of endogenous genes' activities. This is mainly via targeting specific region in the 3'untranslated regions (UTR) of messenger RNAs (mRNAs), which are usually partially complementary to miRNAs (Williams, 2008). There is evidence supporting the idea that miRNAs can also positively regulate protein expression. A part from their roles as posttranscriptional regulators, miRNAs have been shown to exert direct effects on the gene expression via histone modification and DNA methylation of target genes' promoters (Kawasaki and Taira, 2004). MicroRNAs may also indirectly regulate the transcriptional activation of a gene via targeting the related transcription factors and even coactivators (Hawkins et al., 2008; Tan et al., 2009).

miRs deregulations has been demonstrated involved in several diseases as lot of kinds of cancers (He et al., 2005; Mraz et al., 2009), as well as in cardiac development (Zhao et al., 2007) and in cardiomyopathies (Tatsuguchi et al., 2007).

I.3.3 Focus in plasticity

Role of miRs in the brain

miRNAs appear to regulate also nervous system function (Maes et al., 2009). Neural miRNAs are involved at various stages of synaptic development, including dendritogenesis (Edbauer et al., 2010), synapse formation and synapse maturation (Schratt, 2009). Some studies find altered miRNA expression in schizophrenia (Feng et al., 2009; Beveridge et al., 2009).

Several studies have demonstrated that exist specific pattern of miRs expressions in the different tissues. The brain has its own pattern of miRs expression (Hua et al., 2009), and also miRs might conserved (Kosik et al., 2005).

The first studies on neural specific miRs focused on the early stages of the development. These data showed that miRs are able to regulate progenitor cells (proliferation), neural tube development, growth cone (negative feedback in brain development) and cellular differentiation (Cao et al., 2007; Wulczyn et al., 2007; Brett et al., 2011; Miska et al., 2004; Sempere et al., 2004). miRs screening, during brain development, revealed specific miRs expression and also showed that the majority of these miRs were conserved through vertebrates and invertebrates, and in neuron development and function (Miska et al., 2004; Sempere et al., 2004). These findings suggest a fundamental role of miRs in transcriptional and post-transcriptional processes underlying regulation brain functions.

Role of miRs in plasticity

During postnatal development and in the adult, neural circuits are shaped by sensory experience. This process of experience-dependent plasticity, which occurs at the level of dendrites and synapses, underlies the brain's ability to adapt to changes in the environment (Kandel, 2001). At the molecular level, plasticity is orchestrated by sophisticated gene expression programs that ensure that environmental stimuli are converted into long-lasting alterations in synapse structure and function (Flavell et al., 2008). Among these mechanisms, the local control of mRNA translation in neuronal dendrites can account for the tight spatial regulation of plasticity at the level of individual dendrites or spines (Sutton and Schuman, 2006) suggesting a synaptic tagging in miRNAs regulation during synaptic plasticity (Smalheiser and Lugli, 2009).

The capacity to locally control the synthesis of protein at the dendrite level provides neurons with a specific response at subcellular level as the single dendrite as well as single synapse (Wang et al., 2009). The control of single spine plasticity seems an essential step in current models of memory. Accordingly, the hypothesis that miRs can regulate and control locally different aspects of protein synthesis, is particularly relevant for experience dependent plasticity. miRs-associated proteins as Dicer, Argonaute, FMRP or component of P-body, were found in a granular distribution in dendrite of mature neurons (figure 6), (Barbee et al., 2006; Lugli et al., 2005; Kye et al., 2007). Moreover recently it has been shown that miRs are mainly localized in dendrites and less in cell bodies (Kye et al., 2007) and that miRs co-localized with polyribosome in neurons at synaptic level (Kim et al., 2004). In this last study, Kim and coworkers demonstrated

specifically that two miRs, mir-324-5p and mir-191 weree localized at the synaptic level with polyribosome, in cortex cell cultures, where is the site of active translation. Consequently others studies were done in order to better understand miRs repertoire at dendrite level. First, Kye and co-workers used a multiplex reverse transcription PCR approach combined with laser capture microdissection to interrogate the expression of 187 candidate miRNAs in dendrites of cultured rat hippocampal neurons (Kye et al., 2007). This study detected differential expression between dendritic and somatic fractions for five different miRNAs. Subsequently, two studies used synaptosomes, a biological fraction of purified synaptic terminals, to identify miRNAs localized in the synaptodendritic compartment (Siegel et al., 2009; Lugli et al., 2008). Results from these studies extended the list of synaptically localized miRNAs to more than 20 (Schratt, 2009). It is reasonable to assume that this number is a large underestimate, and more sensitive methods, such as deep sequencing, should yield a more comprehensive picture of the synaptic miRNA repertoire in the near future.

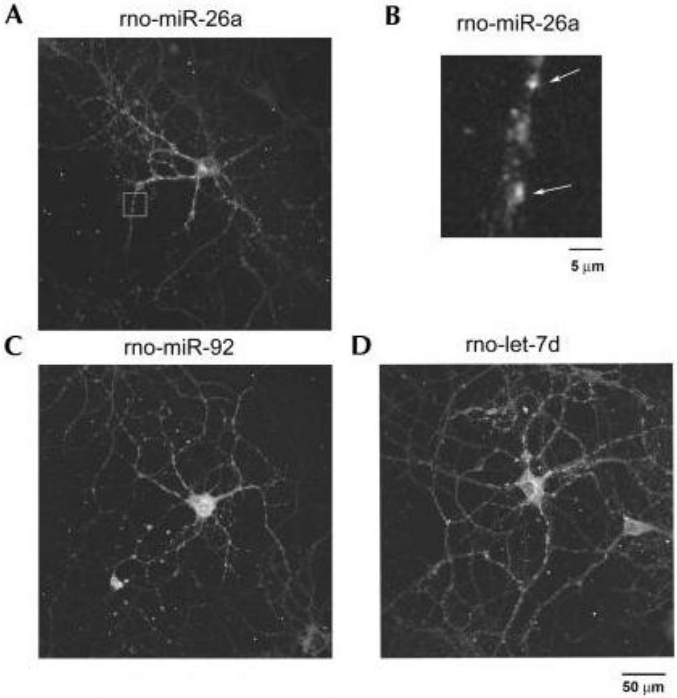


Figure 6: miRNAs appear granular by in situ hybridization in cultured hippocampal neurons (Kye et al., 2007). (*A*) rno-miR-26a. (*B*) High power of rno-miR-26a puncta; (*C*) rno-miR-92; (*D*) rno-let-7d.

The molecular mechanisms regulating dendrite growth and remodeling are highly relevant to our understanding of the development of neuronal circuits. Moreover new finding

demonstrating *de novo* protein synthesis for dendrite growth has been provided (Jaworski et al., 2005), thus the theory of miRs role in post-transcriptional regulation seems supported by experimental evidence. The first miRNA demonstrated involved in dendrite growth is mir-132 (figure 7), (Vo et al., 2005). This miRNA is activated by the Ca^{2}+-sensitive transcription factor CREB thus activity-dependent. Furthermore it has been shown that mir-132 regulates the p250GAP and Rac1-Pak actin remodeling pathway (Impey et al., 2010; Wayman et al., 2008). To the best of my knowledge, only one study shows *in vivo* the activity-dependent effect on mir-132 in the hippocampus, the striatum and olfactory bulb (Nudelman et al., 2010). This article reports that mir-132 is expressed in an activity-dependent manner in different brain structures after drug intake, fear conditioning and odor-exposure. These last findings demonstrate in vivo that miRNAs can be activity-dependent and thus their expression is "experience-dependent". Others miRNAs were demonstrated involved in CREB activity as mir-124 (Hansen et al., 2010, Figure 8) or also activity – dependent miRNAs expressed as mir-134 and mir-184, in this case (mir-184), have been shown to play an indirect role on DNA methylation (Wayman et al., 2008; Khudayberdiev et al., 2009; Nomura et al., 2008). Moreover, interestingly both mir-132 and mir-124 exert their growth-promoting effect by regulating the activity of Rho GTPases, which are crucial regulators of the dendritic actin cytoskeleton.

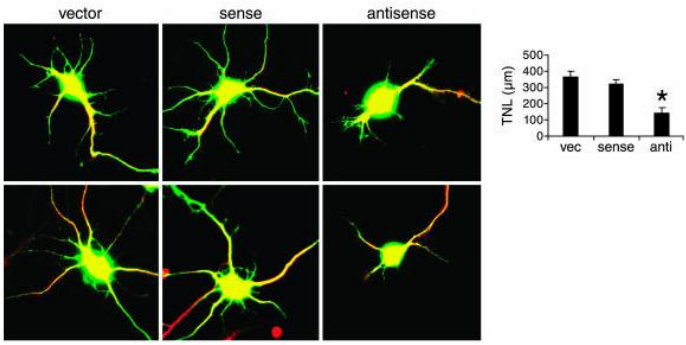


Figure 7: Transfection of a 2′*O*-methyl inhibitor of miR132 markedly attenuated neurite outgrowth (Vo et al., 2005). Cortical neurons were transfected with a GFP reporter (green) and cotransfected with empty vector or 2′*O*-methyl oligoribonucleotide directed against sense or antisense miR132. Cells were immunostained for the neuronal marker MAP2 (red).

In this framework the role of miRNAs in dendrite growth and plasticity mechanisms has been investigated using two different knockouts (KO): a total Dicer KO in mice; a conditional Dicer KO (a CRE-Lox Dicer construction). The total KO Dicer showed a severe reduction of dendritic branch elaboration (Davis et al., 2008; Cuellar et al., 2008). Whereas the conditional KO, showed a memory enhancement (Konopka et al., 2010). These two contradictory results provide two important insights. The total KO Dicer occurs at the beginning of the development of the brain and can affect the development by regulating neural stem cells and/or cellular differentiation and maturation, whereas the lack of Dicer only in adulthood mice showed an enhancement in memory. These two

Dicer knockouts demonstrate that miRNAs processes, during and after brain development, are different. The fact that the down regulation of miRNAs during brain development leads to reduction of dendritic branches elaboration but does not change the number of branches, demonstrate a specific involvement for dendrite elaborations, suggesting also that miRNA regulation is fundamental for the initial formation of synaptic contacts but crucial for their maturation (Davis et al., 2008). However, in the conditional KO Dicer, the general miRNAs down regulation improved memory in adult mice. This result converges more with the idea that down regulation of miRNAs could allow the increasing of the synthesis proteins necessary in long lasting changes. It is though that the result of total KO Dicer interacts with several others mechanisms during development thus so difficult yet to understand this effect.

Recently, miR-138 was identified in a functional screening for dendritic miRNAs that regulate spine morphogenesis (Siegel et al., 2009). Like miR-134, miR-138 acts as a negative regulator of spine size without affecting the total number of synapses. The key miR-138 target in spine regulation is APT1, an enzyme that catalyses depalmitoylation of proteins implicated in synapse biology, such as the RhoA activator Gα12/13. Therefore, a common theme is emerging whereby dendritic miRNAs control spine growth by tuning the activity of antagonistic signalling pathways that regulate the actin cytoskeleton in spines. Given the crucial role of the spine actin cytoskeleton in long-term potentiation (Fukazawa et al., 2003), it is tempting to speculate that activity-dependent regulation of these miRNA-related pathways might also contribute to long-lasting forms of synaptic plasticity. This suggestion is supported by the importance of miRNAs expression during sleep (Davis et al., 2007).

Much of our knowledge on miRNA function at synapses has come from studies on the fly neuromuscular junction (NMJ). Using classical genetic epistasis experiments, Jin and co-workers showed that the miRISC components Dicer-1, AGO1 and FMR1 functionally interact during synaptogenesis of the NMJ (Jin et al., 2004). Loss of FMR1 leads to excessive button formation and growth, and deletion of AGO1 exacerbates this effect. Although this study implicates the miRNA pathway in synaptic development, it does not distinguish the effects on synapse formation per se from those on stabilization and/or maturation of pre-existing synapses. More recent studies provide evidence that miRNAs might have a preferential role in the regulation of synapse growth and maturation. For example, let-7 family members are required for the maturation of the D. melanogaster NMJ but have no effect on NMJ formation (Caygill and Johnston, 2008).

Similar findings have been observed during spine development in cultured neurons. miR-134 localizes at dendrites of mature hippocampal neurons, where it inhibits the translation of LIMK1 encoding mRNA, a kinase that promotes actin polymerization and spine growth by phosphorylating the actin depolymerizing factor cofilin (Schratt et al., 2006). Importantly, the application of brain-derived neurotrophic factor shifts the inhibitory effect of miR-134 on Limk1 mRNA translation, suggesting that this interaction might be subject to regulation by neuronal activity and be relevant for plasticity. Recent findings revealed in transgenic mice, expressing excessive mir-132 in the forebrain, increased spine density in the hippocampus (figure 8). Moreover they showed that mir-132 up-regulation down-regulates MECP2, a transcription repressor through the DNA methylation (Hansen et al. 2010). They showed that miRNAs

are involved in plastic processes also in vivo. As Khudayberdiev and coworker demonstrated that miRNA-134 promotes dendritic growth by inhibiting translation of the mRNA encoding the translational regulator Pumilio2 (Pum2) (Khudayberdiev et al., 2009).

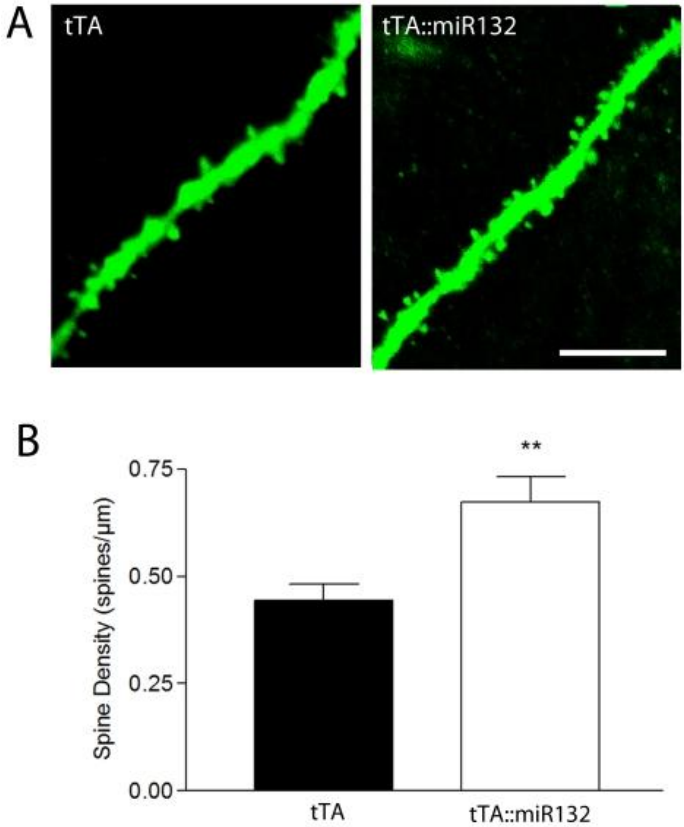


Figure 8: Transgenic miR132 affects neuronal morphology (Hansen et al., 2010). (A) Representative confocal images of CA1 pyramidal neuron basal dendrites from tTA::miR132 transgenic and tTA monotransgenic tissue. Note the increased spine density in the tTA::miR132 dendrite compared the tTA transgenic mouse. (B) Graphical representation of the mean ± SEM spine density. **P<0.01, two-tailed t-test, n = 6 animals for each group. bar: 10 µm.

Today there are few findings for a role of miRNAs in higher-order brain function in vivo. As it was presented before, several hypotheses were provided by the NMJ studies in fly, showing expression and efficacy changes at cholinergic or glutamatergic receptors level by miRNAs modulation (Simon et al., 2008; Karr et al., 2009). These results led to examine in vivo miRNAs effects on the behavior. Studies on drug addiction demonstrated that mir-212 is up-regulated in the dorsal striatum of rats with a history of extended access to cocaine. Conversely modulation of mir-212 in the dorsal striatum of rats reduces cocaine intake (Hollander et al., 2010; Im et al., 2010; Picciotto, 2010). Another field examined was the memory. The major part of the experiments on miRNA-induced memory was conducted by protein mutant, needed in memory, in mice. For example Gao and coworker demonstrated in Sirt1 mutant that mir-134 was up-regulated whereas BDNF and CREB were down-regulated and affect memory. In this study they evaluated whether up-regulation of mir-134, by virus infusion (with CRE-Lox construction) in WT mice, changed protein expression and affect memory. They showed that contextual fear conditioning response was impaired in mice infused with Lv-miR-134 and also a deficit in LTP in hippocampus (figure 9) (Gao et al., 2010). It has already been shown that LTP affect miRNAs expression in particular mature miRNAs (Park and Tang, 2009; Wibrand et al., 2010). A second study showed the behavioral alterations in mir-132 knockdown in mice. This study demonstrated a deficit in KO mice in novel object recognition correlated with neuronal spine density decrease (Hansen et al., 2010).

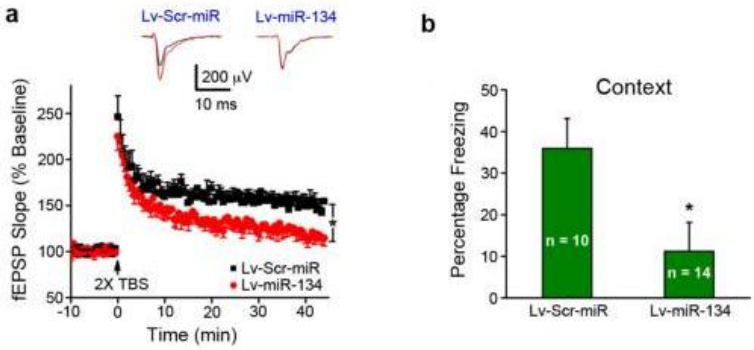


Figure 9: MiR-134 up-regulated rescues the LTP and memory impairments in WT mice (Gao et al., 2010). (**a**) LTP was measured in acute hippocampal slices of mice six weeks after injection with Lv-miR-134 or Lv-Scr-miR. (**b**) Lv-miR-134 and Lv-Scr-miR-injected mice were tested with a contextual fear conditioning task.

In conclusion, these two last experiments demonstrate the role of mir-134 and -132 in memory *in vivo*. It is still unknown however how many miRNAs are involved in memory processes and also the molecular mechanisms in brain structures involved in memory different from the hippocampus. However it has already been suggested that the miRNAs might be a new therapeutic tool in neurodegenerative disorders. Recently it has been shown in a mouse model of schizophrenia that these mice had impaired expression of mature miRNAs, including mir-134 (Stark et al., 2008). The behavioural defects in these mice coincide with abnormal dendrite and spine morphogenesis, further supporting a role for miRNAs in the regulation of neural connectivity. In the last year others two studies

supporting the possible role of miRNAs in neurological disorders have been published. The first showed that the regulation of NR2a 3'UTR by FMRP depends in part on mir-125b. Moreover the FMRP is known to be the original cause of the pathology of fragile X syndrome, in which the plasticity is altered (Edbauer et al., 2010). In the second study, it has been found that APP is a target of mir-16 and suggesting the hypothesis that an abnormally low expression of mir-16 could potentially lead to APP protein accumulation in Alzheimer Disease mice (Liu et al., 2010).

These accumulating findings suggest miRNAs as regulator of important molecular pathways involved in brain development, plasticity and behavior. Moreover their regulator capacity appears also as a good therapeutic tool in neurological pathologies presuming that miRNA may have a selective role in molecular processes.

II. Ventral Striatum versus Hippocampus:

II.1 Hippocampus

The hippocampus is one of the most studied brain region both in humans and others mammals. Historically it was hypothesized to be involved in olfaction. This interest led to several experiments that tended to demonstrate different synaptic connections between brain structures correlated with olfaction function and the hippocampus. Then it was studied the role of the hippocampus in olfactory memory. Now this structure is known to have a key role in short and long term memory and in spatial navigation. In rodents, the hippocampus has been studied extensively as part of a brain system responsible in behavioral inhibition and attention, spatial memory and navigation. It is generally thought today that the hippocampus is involved in the processing of different information and not only olfactive.

II.1.1 Anatomy

The experimental evidence in the last 60 years, demonstrated the important role of the hippocampus in mnestic processes.

The hippocampus is localized in the medial temporal lobe (MTL) surrounded by lateral ventriculun and connected to the subcortical nuclei via fornix fibers and to the neocortex via the parahippocampal region. Together with olfactory cortex, amygdala and cingulated

cortex form the limbic system. Anatomical studies identified the different regions which composed the MTL in the human, monkey and rodent, and it was suggested a hypothetic functional organization of the memory processes. The MTL can be divided in four parts: perirhinal cortex, parahippocampal cortex and entorhinal cortex, which compose the parahippocampal complex, and the hippocampus. Conventionally the hippocampus is separated in different anatomical regions: the *Cornu Ammonis* (for the ram's horn), which includes CA1, CA2, CA3, CA4 areas (distinguished on the basis of the cellular morphology), and the dentate girus, which together with subiculum and entorhinal cortex is called hippocampal complex.

The different structures, which constitute the hippocampus, are differentiated by the kind of cellular composition, their afferences and their efferences with the others structures. The *Cornu Ammonis* is constituted by large pyramidal cells. This substructure shows a laminar organization of seven layers. From the ventricular part we find: 1) the *alveo*, constituted by axons of the pyramidal cells that converge together to create the fornix; 2) the *oriens* layer constituted by the proximal part of these axons and by their basal dendrites; 3) the pyramidal layer constituted by pyramidal cells; 4) the *lucido* layer (only in the CA3) constituted by the mossy fibers; 5) the *radiato* layer constituted by the proximal part of the apical dendrites of the pyramidal cells; 6) the *lacunoso* layer constituted by Schaffer collaterals; 7) the molecular layer constituted by the distal part of the apical dendrites of pyramidal cells.

The connections in the hippocampal complex are organized in trisynaptic circuit (Andersen et al., 1971). Three important connections, which are the perforant path, the mossy fibers and the

Schaffer collaterals, allow the circulation of information within the complex. The entorhinal cortex (EC) is thought to be the main input of new information. The EC, located in the parahippocampal gyrus, is strongly and reciprocally connected with many other parts of the cerebral cortex. For example, the medial septal nucleus, the anterior nuclear complex and nucleus reuniens of the thalamus, and the supramammillary nucleus of the hypothalamus, as well as the raphe nuclei and locus coeruleus in the brainstem send axons to the EC. The main output pathway, the perforant path, (first described by Ramon y Cajal) of EC axons comes from the large stellate pyramidal cells in layer II that "perforate" the subiculum and project densely to the granule cells in the dentate gyrus, apical dendrites of CA3 get a less dense projection, and the apical dendrites of CA1 get a sparse projection. Thus, the perforant path establishes the EC as the main "interface" between the hippocampus and other parts of the cerebral cortex (figure 10). The dentate granule cell axons (called mossy fibers) pass on the information from the EC on thorny spines that exit from the proximal apical dendrite of CA3 pyramidal cells. Then, CA3 axons exit from the deep part of the cell body, and loop up into the region where the apical dendrites are located, then extend all the way back into the deep layers of the entorhinal cortex—the Shaffer collaterals completing the reciprocal circuit; field CA1 also sends axons back to the EC, but these are more sparse than the CA3 projection. Within the hippocampus, the flow of information from the EC is largely unidirectional, with signals propagating through a series of tightly packed cell layers, first to the dentate gyrus, then to the CA3 layer, then to the CA1 layer, then to the subiculum, then out of the hippocampus to the EC, mainly due to collateralization of the CA3 axons. Each of these layers also contains complex intrinsic circuitry and extensive longitudinal connections (Amaral et al., 2007).

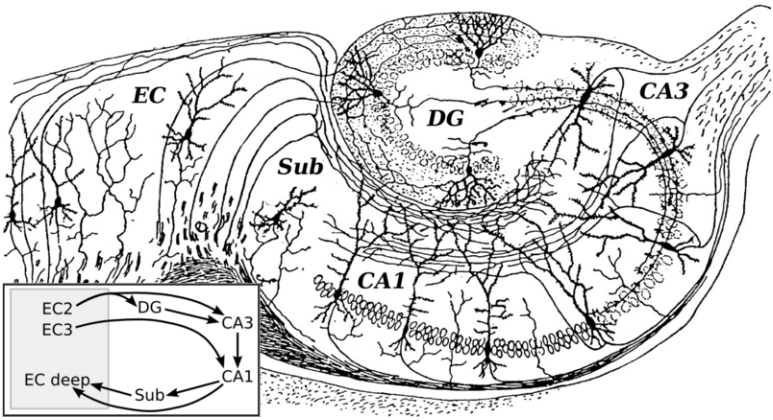


Figure 10: Basic circuit of the hippocampus, as drawn by Santiago Ramon y Cajal. DG: dentate gyrus. Sub: subiculum. EC: entorhinal cortex.

Several other connections play important roles in hippocampal function (Amaral et al., 2007). Beyond the output to the EC, additional output pathways go to other cortical areas including the prefrontal cortex. A very important large output goes to the lateral septal area and to the mammillary body of the hypothalamus. The CA1 with the ventral subiculum and the EC, which are the three important substructures of the hippocampal complex, were recently demonstrated sending glutamatergic axons into the ventral part of the nucleus accumbens (Voorn et al., 2004). These connections are thought to allow accessing to motor system needed for example in food and sexual partner search.

The hippocampus receives modulatory inputs from the serotoninergic, norepinephrinergic, and dopaminergic systems, and from nucleus reuniens of the thalamus to field CA1. A very important projection comes from the medial septal area, which sends cholinergic and GABAergic fibers to all parts of the hippocampus (Gorman et al., 1994). The inputs from the septal area play a key role in controlling the physiological state of the hippocampus: destruction of the septal area abolishes the hippocampal theta rhythm, and severely impairs certain types of memory (Winson, 1978). Moreover, it has been demonstrated that damage in both hippocampus and parahippocampus gives complete amnesia (Cohen et al., 1991). The hippocampus seems to be the general input to integrate all sensory information and can create memory trace.

II.2 The striatum

The striatal complex has been studied mostly in relation to the control of motor output. At the beginning of the 20th century the discovery of specific lesion in this structure leads to specific motor deficits (Vogt, 1911 in Denny-Brown, 1962). Then this hypothesis was confirmed by clinical phenomenology studies of patients with behavioral problems correlated to the basal ganglia nuclei (Marsden, 1982). Disease like Parkinson or Huntington diseases are now known as massed basal ganglia alteration with motor deficits that vary from hyperkinesia to hypokinesia (Albin et al., 1989; Wichmann et Delong, 1993). However it has been demonstrated that the striatal complex was also involved in learning and memory process. In fact this complex was shown necessary in habituation formation and also in

fear learning (Grafton et al., 1992; Knowlton et al., 1996; Packard et Knowlton, 2002; Schultz et al., 2003). In the last years, different studies have demonstrated the ventral part of the striatum as a crucial structure in spatial memory (Schacter et al., 1989; Sargolini et al., 2003).

II.2.1 Anatomy

The ventral striatum (VS) composed, mainly, by the nucleus accumbens (Nacc) is one of two parts that constitute the striatal complex. The second part is located at the dorsal level constituting a single entity closed and continuous. However anatomical studies suggest a further distinction in primate: the caudate nucleus and the putamen. This striatal complex is situated in the forebrain and has a central position. The striatum together with the globus pallidus, the subthalamic nucleus and the substantia nigra (subdivided in *pars compacta* and *pars reticola*) compose subcortical nuclei called basal ganglia nuclei.

The neuroanatomical position suggests that the striatum could have a role in integrating different information. This hypothesis seems to be confirmed by the morphologic characteristics of the major part of neurons which constitutes the structure. To understand, whether the striatum has an integrative role or whether it is a simple “channel” for transit information, it is necessary to see the kind of neurons present in the striatum and to see the pattern of projections convergence on this structure. The striatum is composed by projection neurons (golgi cells type I) and local interneurons (golgi

cells type II). The bigger part of afferences contacts neurons of medium dimension with lot of spine, called “medium spiny neurons” (Kemp et Powell, 1971). These neurons express GABA as neurotransmitter and include 96% of the striatal cellular population. Initially these neurons were considered as interneurons but different studies have shown them as projection neurons (Kemp et Powell, 1971; Somogyi et Smith, 1979). These neurons which can co-express neuroactive peptides as substance P, encephaline or dinorphine connect the cerebral cortex, the thalamus and some encephalic trunk nuclei. Interesting, cortical afferences from specific areas can connect the same cluster of medium spiny neurons in the striatum (Parent, 1990; Ramanathan et al., 2002). Structural data demonstrates that, on the same dendrites, it is possible to find juxtaposed cortical glutamatergic and substantia nigra *compars* (SNc) dopaminergic afferences (Kocsis et al., 1977), giving a central role to the medium spiny in the motor and motivational information integration.

Another kind of neuron present in the striatum is the interneuron, called aspiny neurons that likely provide a crucial role in information integration (Di Figlia et al., 1994; Izzo and Bolam, 1988). These neurons are characterized by the lack of spine, and represent the 4% of striatal cellular population (Bishop et al., 1982). The aspiny interneurons can be divided in two classes: 1) giant cholinergic neurons (2% of cellular population), 2) GABA-ergic interneurons (3-5% of cellular population) (Bishop et al., 1982).

Several studies have shown that the striatum is a brain region composed of substructures and can be divided on the basis of neuroanatomy, cytoarchitectony and function (Parent and Hazrati, 1995). Radioactive tracer injections for anterograde and retrograde transport have been provided a topographic map of structures which

connect the striatum. It is possible divided the striatum in three parts: 1) Dorso-lateral striatum (DLS), 2) Dorso-medial striatum (DMS), 3) Ventral striatum (VS) (Prenza et al., 2003).

Thanks to its key position in the brain. The striatum receives afferences from the whole cortex and from the limbic areas, the majority of which is glutamatergic, and sends efferences to thalamic nuclei through two ways: the indirect way and the direct way. Thus indirectly, through the thalamus, the striatum can affect the cortical activity in the regulation of complex behaviors. In addition, through direct projections to forebrain areas, which connect the middle spine, the striatum can also affect motor outputs (Joel, 2001; Parent et Hazrati, 1995). Moreover the striatum receives cortical dopaminergic afferences from the substantia nigra and tegmental ventral area, and noradrenergic afferences from the rafe nuclei (Bellomo et al., 1998; McLennan, 1980; Smith et Parent, 1987; Penney et al., 1981; Lavoie et Parent, 1990; Joel et Weiner, 2000), generally involved in motivational behaviors.

The VS receives information from the prefrontal cortex (median and ventral), from the amygdala and from the hippocampal complex (ventral subiculum, CA1 and entorhinal cortex), and the VS send axons through the ventral part of the globus pallidus either to motor nuclei of thalamus and to limbic nuclei, such as the dorsal median thalamus, and the hypothalamus (Groenewegen et al., 1980; Groenewegen et al., 1987). On the other hand, the dorsal striatum receives afferences from associative cortical areas as posterior parietal cortex, and also motor cortex (Selemon and Goldman-Rakic, 1985). Compared to the VS, the dorsal striatum shows different afferences, as previously described, and different efferences (Gerfen, 1992; Graybiel, 1990). In the case of the dorsal striatum, the majority

of the information is transmitted to the dorsal globus pallidus and then sends to motor thalamic nuclei (DeVito and Anderson, 1992; DeLong et al., 1983), suggesting a role more important in motor information integrations. On the contrary the afferences to the VS suggest a role of this structure memory processes.

Recently, on the basis of new behavior evidence, as well as neuroanatomical and neurophysiological studies, it has been proposed a model for the organization of the striatum that follows a dorso-lateral gradient. This is a result of the observation of the neuronal density gradient that populates the striatum (Haber et al., 2003). Further detailed studies of afferences, and behavior demonstrate that the striatum can be divided in two components: a part of the dorso lateral striatum (DLS) and a part that includes the dorso medial striatum (DMS) and the VS (Voorn et al., 2004).

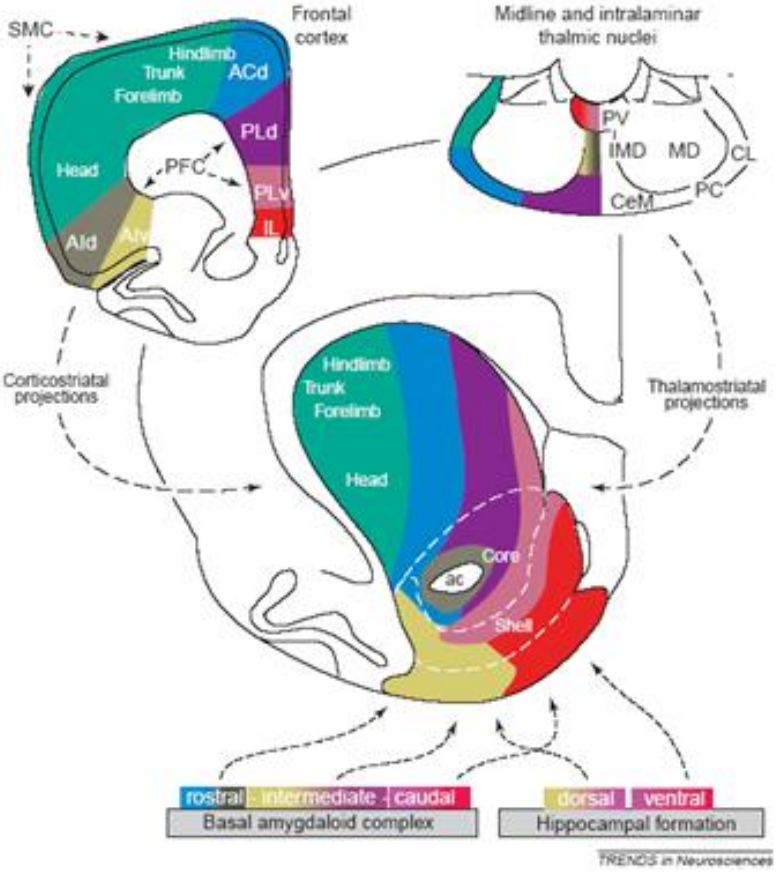


Figure 11: Dorso-lateral gradient representation of the striatum organization, on the basis of the glutamatergic afferences (Voorn et al., 2004).

The cortico-striatal organization is also another important characteristic to divide the striatum in substructures. In fact the cortico-striatal projections finish in cluster shape, and this distribution defines two striatal compartments: the striosomes and the extrastriosomal matrix identified on the basis of neurotransmitter distribution. The diversity of neurotransmitters in the basal ganglia nuclei can have a role in order to modulate sensory-motor behaviors, memory and associative behaviors received from neocortical and limbic system. The striosomes lack of acetylcholine whereas the matrix is full. Hystochimical and heterogenetic connection association put a light on functional differences between both compartments. In fact it is known that the matrix receives striatal afferences connected with sensory-motor processes thus controlling outputs from basal ganglia nuclei to cortex. On the contrary the striosomes, receive input from limbic system, specifically with the ventral subiculum, the hippocampus, the entorhinal cortex and the amygdala. The striosomes are necessary for the modulation and the functional integration of signals expresses from these structures (Brown et al., 2002). The evidence of segregated pathways strictly associated in the striatum could support the idea of communication between distinct functional pathways (Parent et al., 1995; Graybiel et al., 1978).

Others studies suggest that compartmental organization of the cortico-striatal inputs is in relation to the laminar origin and also cytoarchitectonic types. In fact each cortical area sends projections to both striosome and matrix. However the corticostriatal neurons from the infragranular layer send to the striosome whereas the neurons from the supragranular layer send to the matrix. Moreover the allocortical areas receive the highest concentration of

corticostriatal neurons into infragranular layers, whereas the neocortical areas receive a high number of corticostriatal neurons into the supragranular layer (Arikuni et al., 1986; Gerfen et al., 1990). This specific connection distribution suggests again distinct role of substructures in the striatum.

The ventral part of the striatum seems distinguish itself from the dorsal striatum. The ventral striatum composed by the nucleus accumbens. This region is divided in two substructures, the core and the shell of the nucleus accumbens. Dopamine release within the nucleus accumbens (NAcc) has been associated with both the rewarding and locomotor stimulant effects of abused drugs. The functions of the NAcc core and shell were investigated in mediating amphetamine potentiated conditioned reinforcement and locomotion.

Excitotoxic lesions selectively destroyed either the NAcc core or shell, and it has been assessed rats in a Pavlovian procedure after infusion intra-NAcc with D-amphetamine. Shell lesions affected neither Pavlovian nor instrumental conditioning but completely abolished the potentiative effect of intra-NAcc amphetamine on responding with CR. Core-lesioned animals were impaired during the Pavlovian retraining sessions but showed no deficit in the acquisition of responding with CR. However, the selectivity in stimulant-induced potentiation of the CR lever was reduced, as intra-NAcc amphetamine infusions dose-dependently increased responding on both the CR lever and a nonreinforced (control) lever. Shell lesions produced hypoactivity and attenuated amphetamine-induced activity. In contrast, core lesions resulted in hyperactivity and enhanced the locomotorstimulating effect of amphetamine. These results indicate a functional dissociation of subregions of the NAcc;

the shell is a critical site for stimulant effects underlying the enhancement of responding with CR and locomotion after intra-NAcc injections of amphetamine, whereas the core is implicated in mechanisms underlying the expression of CS–US associations.

Differences had also been shown during drug addiction experiment (Di Chiara, 2002) or during salience learning and impulsive-choice behaviour (Corbit et al., 2001; Pothuizen et al., 2005; Murphy et al., 2008). The double dissociations demonstrated that these studies supported a functional segregation between nucleus accumbens core and shell, and add weight to the hypothesis that the core, but not the shell, subregion of the nucleus accumbens is preferentially involved in the control of choice behaviour under delayed reinforcement conditions and in the inhibitory control of goal-directed behaviour. This function dissociation is studied in contextual drug addiction. It has been hypothesized that the decision making and the impulsivity dependent-nucleus accumbens, studied in drug addiction, could be selective for dorsolateral core in the nucleus accumbens.

Experimental evidence demonstrate anatomical and functional interactions between dopaminergic and glutamatergic system, also between cholinergic and dopaminergic system (Lehmann et Langer, 1983) and between serotoninergic, dopaminergic and cholinergic system (Azmitia et Gannon, 1986; De Simoni et al., 1987; Jackson et al., 1988). For example different reports show that the dopamine decreases the effect of the cortical stimulation and on the other hand the acetylcholine facilities the “medium spiny neurons” firing (Lehmann and Langer, 1983). These results demonstrate that also morphologic and structural characteristics support the hypothesis that the striatum can have both an active role in cortical and limbic

information elaboration, and in information processing from different cortical areas.

Interestingly the striatum receives: 1) cognitive information from a part of the cortical and limbic through glutamatergic afferences; 2) receives emotional/motivational and attentional information respectively through dopaminergic afferences and noradrenergic afferences (Reading et al., 1991; De Leonibus et al., 2001; Phillips et al., 1994). From a functional point of view, we can note that these different and specific connections seem to maintain a high segregation of information in the striatum. Another example is the direct projections from the hippocampal complex to the VS, suggesting a role of this structure in memory processes. In particular the projections from the hippocampus suggest a role of the VS also in spatial navigation. A study has demonstrated that the glutamatergic afferences within the VS are involved, *in vivo* , in memory (Sargolini et al., 2003; De Leonibus et al., 2003) and in spatial learning (Sargolini et al., 2003; ferretti et al., 2011).

II.3 Structures involved in declarative memory: spatial memory as a model of declarative memory.

II.3.1 Spatial memory

In cognitive psychology and neuroscience, spatial memory is the part of memory responsible for recording information about one's environment and its spatial orientation. For example, spatial memory is required in order to navigate around a familiar city, as well as to learn the location of food in its environment. It is often argued that in both humans and animals, spatial memories are summarized as a cognitive map. Researches indicate that there are specific areas of the brain associated with spatial memory.

Spatial memory is used as model for declarative memory. Moreover declarative memory is mainly studied through spatial memory ability. This ability, clearly defined in humans, was shown also easy to study in animals such as the rodents. First, because rodents need to pick-up food in huge environment and need to remember the position; Second, because they need to know the environment to escape behind the predator. Tasks have been built for rodents to analyze spatial memory such as the Morris water maze (were the animal is trained to remember position of an hidden platform in a swimming-pool and using external cues to localize it), the radial maze (were the animal is trained to remember each arm visited of the 8 arms of this

task using external cues), the cross maze (composed by 4 arms) and the open field (arena fills with different objects). These different tasks and others, well controlled because of the little space, allowed putting into light that rodents and human used the same cerebral structures (O'Keefe and Nadel, 1978) as well as that rodents learn quickly to associate contextual stimuli (Eichenbaum, 1999). Moreover there are superposition among processes and neuronal substrates responsible for spatial cognition and those responsible for learning and memory (Liu et al., 2007). These different findings have defined the rodent as good model to study spatial memory (as model of declarative memory).

Spatial memory has been differentiated on the basis of the information used to navigate. Redish in 1999 described five navigation strategies: 1) random navigation (without goal); 2) taxon navigation (stimulus-response S-R), for example the cue learning; 3) praxis navigation as motor sequence (S-R), for example the strategy response; 4) route navigation as sequence line of praxis navigation and taxon navigation, mainly considered S-R.; 5) local navigation as stimulus-stimulus learning (S-S), used to localize a goal position that it needs to go to different spatial reference points that lead to build a cognitive map (or spatial map). Learning with relation between multiple reference points of the ambient are processes more complex (Tolman, 1948; Poucet, 1993).

Numerous papers studied spatial memory in relation to the hippocampus, the most famous structure of the brain for encoding, acquisition and storage capacity of spatial information (Save et al., 1992; Riedel, 1999; Morris et al., 1991). However several others structures are well noted to participate during integration and memorization of spatial information as the ventral striatum (Roullet

et al., 2001; Sargolini et al., 2003; Ferretti et al., 2007). In this part, I will try to compare these two structures, both involved in the spatial memory circuitry, at different level, to determine whether these structures play a similar role or not and if it's possible to add others experiments to characterized the differences between the hippocampus and the ventral striatum.

II.3.2 Neuroanatomy of the spatial memory

Different brain structures have been involved in spatial navigation and spatial memory. It was shown a circuit starts from the structures processing sensory information (such as visual cortex or vestibular nucleus) followed by the perirhinal or postrhinal cortices, the limbic system (such as parahippocampal complex) to end in higher cortical areas (such as prefrontal cortex) and in specific region of the basal ganglia system (such as ventral striatum) (van Groen et Wyss, 1990; Groenwegewn et al., 1997; Totterdell et Smith, 1989; Walaas and Fonnum, 1979; Yang and Mogenson, 1984, 1985, 1986; Compton et al., 1997; Floresco et al., 1997; Kolb et al., 1994; Berendse et al., 1992). These different structures have been involved in spatial navigation thanks to lesion and electrophysiological studies. Other structures such as the amygdala and the tegmental ventral area are also able to modulate spatial performance possibly bringing motivational and emotional inputs (Tzschentke, 1998; Tzschentke and Schmidt, 2000; Burns et al., 1996).

The main structure involved in spatial learning processing since the 60's is the hippocampus. Fundamental findings were found with the

patient H.M., as described in the first chapter. Because of resistant epilepsy to all pharmacological treatments, it underwent neurosurgery with the ablation of large portion of the medial temporal lobe (MTL) in both hemispheres (Scoville and Milner, 1957). Following surgery, H.M. developed acute retrograde amnesia, but maintained the capacity to remember information during short time. Deeper examination revealed specific amnesia for new information about facts and events. More specific tests have shown that H.M. was unable to learn a journey for his new house after the trauma and he went back every day at his old house. The median temporal lobe (LTM) was the first part of the brain involved in spatial learning. More recently a study was on taxi driver from London have demonstrated that they had a bigger hippocampus than normal controls (Maguire et al., 2000). As it has been report in a study, D. merriami, a rodent which store food in different positions in a huge environment, has been found to have a bigger hippocampus than D. spectabilis, which store food in their own den (Jacobs and spencer, 1994). These findings were confirmed by bilateral lesion at the median temporal level in human and monkeys. Bilateral lesions at this level impaired utilization of multiple spatial references to resolve a spatial task (Feigenbaum and Morris, 2004; Lavenex et al., 2006). This lesion approach provides lot of information about the mechanisms of information integration during spatial learning. The same studies have been demonstrated the entorhinal cortex, the subiculum or the anterior cingulair cortex involved in spatial learning.

The first electrophysiological data, that has been shown specific cells involved in spatial navigation, were the unitary cell recording *in vivo.* In 1971 by O'Keefe and Dostrovsky discovered the "place cells". They demonstrated that a kind of cells could fire in a specific region of the

environment and only when the rat went through this specific region (O'Keefe and Dostrovsky, 1971). These place cells were defined to have a precision of 1 cm diameter to localize the mouse in the environment (Wilson and Mc Naughton, 1993) and can, after few minutes of exploration, encode a firing pattern for a specific position and maintain it during several mouths (Thompson et al., 1990). This kind of cell found before in the hippocampus and after in different others structures of the parahippocampal complex, showed important characteristics to encode different position in the environment and needed to remember a position already visited. But this place cell alone couldn't respond to the navigation question. Others scientists have done electrophysiological studies and have shown others different cells involved in spatial navigation. In rodent it has been described the "head direction cells", which fire for specific head direction (Taube et al., 1990), the "grid cells", which fire for different position as black cases of a chess board and hiding the whole field surface and the border cells, which fire for a specific wall orientation in the ambient (Hafting et al., 2005). These four kind of cells described above are the main cells but there are cells that can combine two types of cells, called the "conjunctive cells" (Sargolini et al., 2006), which can associate grid cells and head direction cells for example or place cells and reward correlates (Lavoie and Mizumori, 1994). Adding the properties of each kind of cell described above, it is possible to integrate position, direction and velocity information at the brain level. These cells are sufficient to allow the spatial navigation (Mc Naughton et al., 2006).

Studies based on rodent's behavior have been allowed to demonstrate that "cognitive map" is present and is codified in the brain (Tolman et al., 1948; Poucet et al., 1993). This cognitive map or

spatial map should allow the storage of spatial information of the environment. This learned cognitive map should give, to animal and human, the capacity to react face up to the lack of only one reference point in the environment (Ellen et Anschel, 1981) and also when it is added only one reference point more.

Along the years, these findings have been provided evidence for the mechanisms of spatial memory integration and storage. However the distribution of cells involved in spatial navigation and connection between structures from spatial brain circuitry seem show a specific role for each brain structure in spatial information integration. In this section I would describe two strong structures involved in spatial navigation that is the hippocampus and the ventral striatum, using current literature and all information previously described.

II.4 Anatomy differences between hippocampus and ventral striatum

The hippocampus and the ventral striatum are two structures with high anatomic differences.

First of all, these two structures have different positions in the brain. The hippocampus is localized into the medial temporal lobe (MTL) surrounded by lateral ventricular and connected to the subcortical nuclei via fornix fibers and to the neocortex via the parahippocampal region. The striatum is situated at the rostrocaudal level in the brain into the basal prosencephal. The specific region of interest is the ventral striatum (VS), the ventral part of the striatum composed by the accumbens nucleus (core and shell) and olfactory tubercule. The position for both structures seems to define roles totally different for spatial information treatment.

At the cellular level, both structures show different cellular composition. In the hippocampus we find granular cells and pyramidal cells and few interneurons whereas in the VS we find medium spiny neurons and others kind of aspiny neurons separated in two classes: cholinergic giant neurons and GABAergic interneurons. Moreover the cellular organization in the both structures is completely different. In the hippocampus there is a circuit for the information flow, probably required for the information analysis and treatment. In fact, some substructures of the hippocampus can be modulated by different neurotransmitters.

For example the CA1 receives modulatory input from the serotoninergic, norepinephrinergic and dopaminergic system. This circuit, in the parahippocampal complex, could carry out specific treatment of the information, which are then relayed to the distinct hippocampal substructure. This hypothesis is confirmed by partial lesion studies of the hippocampus showing impairment in long term memory and pharmacological studies showing that the dentate gyrus or in CA1 after glutamatergic receptors antagonists could enhanced or decreased performance in spatial long term memory (Clark et al., 2005a; Martin et al., 2005) Thus the hippocampus would seem more specific in treatment and analysis of spatial information. The VS is composed by 96% of medium spiny neurons and receives major part of afferences from cortex areas, such as from the prefrontal cortex, or from the limbic system, such as the hippocampus, subiculum and amygdala but also from other structure such as the ventral tegmental area (VTA). Medium spiny neurons can receive different kind of affences into the global structure (such as dopaminergic neurons or glutamatergic neurons) and from different structures and/or different kind of cells. Thus one medium spiny neuron seems to be done to receive different information suggesting a role in information integration. Moreover the VS, by its output position, in this spatial memory circuit, seems to be a key structure to coordinate spatial information and its motor execution.

II.5 Differences in spatial learning

Hippocampus:

As described before the hippocampus was the first structure implicated in learning and memory, specifically in spatial memory processes and was revealed as a key structure in spatial learning. Main cell, in the hippocampus, involved in spatial learning today is "place cell". Place cell responses in rats and mice have been studied in hundreds of experiments over four decades, yielding a large quantity of information (Moser et al., 2008). Pyramidal cells from the ram's horn of the hippocampus proper and granule cells from the dentate gyrus have been shown place cells responses. These cells constitute the great majority of neurons in the densely packed hippocampal layers. There is little if any spatial topography in the representation: cells lying next to each other in the hippocampus generally have uncorrelated spatial firing patterns. The size of place fields varies in a gradient along the length of the hippocampus, with cells at the dorsal end showing the smallest fields, cells near the center showing larger fields, and cells at the ventral tip fields that cover the entire environment (Moser et al., 2008). In some cases, the firing rate of rat hippocampal cells depends not only on place but also on the head direction while the rat is moving, the destination toward which it is traveling, or other task-related variables (Smith and Mizumori, 2006).

The discovery of place cells in the 1970s led to a theory that the hippocampus might act as a cognitive map—a neural representation of the layout of the environment (O'Keefe and Nadel, 1971). Several lines of evidence support this hypothesis. It is a frequent observation

that without a fully functional hippocampus, humans may not remember where they have been and how to get where they are going: getting lost is one of the most common symptoms of amnesia (Chiu et al., 2004). Studies with animals have been shown that an intact hippocampus is required for initial learning and long-term retention of some spatial memory tasks, particularly ones that require finding the way to a hidden goal (Sutherland et al., 1982; Sutherland et al., 2001; Clark et al., 2005). The "cognitive map hypothesis" has been further advanced by recent discoveries of head direction cells, grid cells, and border cells (border cells are entorhinal neurons that are border-sensitive, reacting when a border is present in the proximal environment) in several parts of the rodent brain that are strongly connected to the hippocampus (Moser et al., 2008; Solstad et al., 2008).

The hippocampus receives information from a mass of structures which pick up all sensitive information. Therefore we can believe that the hippocampus should be involved in other kinds of memory. Damage to the hippocampus does not affect some types of memory, such as the ability to learn new motor or cognitive skills (playing a musical instrument, or solving certain types of puzzles, for example) but as it is the case for H.M. patient, it is impossible to encode new information thus unable to created new mnestic trace. Moreover the McNaughton theory suggest that cells like the grid cells, head direction cells, place cells, border cells and conjunctive cells, which most is localized in the parahippocampal complex, are able to calculate (through sensitive information) distance, time and velocity in order to to localize himself in his own environment. In summary, the hippocampus might be a key structure to encode and stabilize new information specifically for explicit and not implicit information

and the entire parahippocampal complex has the capacity to calculate and treat basic spatial information.

VS:

Studies focusing on the role of the VS in learning and memory were initiated by Mogenson during the 80's. At the beginning few papers had shown the mnestic role of the striatum (Kesner et Wilburn, 1974) then confirmed by infusion of agonistic and antagonistic treatment in the striatum in long term memory (Carr et White, 1984; Packard et White, 1989).

The major part of the studies in the literature is focused on the VS role during learning in reinforcement task. For example it has been demonstrated that the dopamine neurotransmitters, in the VS, mediate reinforcing properties during learning (Wise et Bozarth, 1982; Berke and Hyman, 2000; Grace et al., 2007). There are two types of reinforcement stimulus: the "primary stimulus" as food, water and addiction substances; the "secondary stimulus" which needs Pavlovian conditioning such as an odor or a noise. During several years it had been though that both the primary and secondary stimulus led to an instrumental learning through a common reward (Rescorla et Solomon, 1967; Bindra, 1974). Recently it has been described two kind of rewards: one represented by the value of instrumental response; the second represented by the reward itself (food or drug) or by pavlovien cues (noise or odor) (Balleine and Dickinson 1994; Balleine and Dickinson, 2000). Anatomical point of view, suggest that the reward itself or the reward value learning should be together mediated by the VS.

However it has been proposed that these two functions can be separated at the substructure level. A study has been shown that the core region is needed to codify the value of the instrumental response (Kelley and Swanson, 1997; Sokolowski and Salamone, 1998), and that the shell region was needed to mediate the excitatory effect of the reward and also to mediate stimulus information which helps its prediction (Johnson et al., 1995; Bassareo and DiChiara, 1997; Corbin et al., 2001).

Moreover recently studies have been demonstrated the VS involved in spatial learning. Electrolytic studies in the VS have shown, in rodent, selective inhibition of the capacity to resolve the spatial version of the Morris water maze created by Morris R.G.M. in 1984. In this task the rat had to localize specific position platform in a swimming-pool using links between platform and visual distal cues (Sutherland and Rodriguez, 1989). This data was confirmed by local infusion of an anesthetic drug, the lidocaine (Lidocaine alters signal conduction in neurons by blocking the fast voltage gated sodium (Na^+) channels in the neuronal cell membrane that are responsible for signal propagation). The same deficit was revealed in another spatial task called radial arm maze (Seamans et al., 1994). In this task rats were required, using visual distal cues, to visit all eight arms to pick up reward (one for each arm) without error (an error is counted when the rat turned again in one arm already visited). The same year a study focused on spatial learning assessed the participation of dopaminergic receptors mainly present in this structure. A local infusion of a dopaminergic receptor antagonist has been shown the same deficit in spatial learning (Ploeger et al., 1994). All of these data above had been controlled for their aspecificity in non spatial strategies such as locomotors components, motivational components

and sensorimotor coordination (Morris 1981, 1984). These findings clearly showed specific role of the VS in spatial learning and memory.

The VS was known for its capacity to integrate and to trigger a motor response. In this way, several researches focused on afferences to the VS. Afferent structures to the VS were showed involved in elaboration of different kinds of information. The main structures are the prefrontal cortex that is generally associated with integration of contextual information, and the hippocampus that allows connections between contextual information (Compton et al., 1997, Floresco et al., 1997, Kolb et al., 1994; Berendse et al., 1992). Also there are structures that modulate the VS by motivational and emotional information from environment stimulus, such as the amygdala and the VTA (Tzschentke, 1998; Burns et al., 1996). These afferent structures have been shown that were able to modulate the motor responses in the VS, thus modulate the motor output. The VS might provide the capacity to create motor sequences through emotional and motivational information during reinforcement tasks, such as water, food, sexual partner and drug (Yim et Mogenson, 1982; Mogenson et Nielson, 1984; Yang et Mogenson 1984). In this context the VS have really good place to integrate contextual information which will be necessary during spatial learning.

Lot of studies was effectuated at the anatomic level (van Groen et Wyss, 1990; Groenewegen et al., 1997; Totterdell and Smith, 1989), at the biochemical (Walaas and Fonnum, 1979), and at the electrophysiological level (Yang and Mogenson, 1984, 1985, 1986). These studies demonstrated numerous glutamatergic afferences to the VS such as afferences from the hippocampus. Then in 1991, Mogenson described for the first time the functional interface between limbic system and motor regions (Mogenson and Yang,

1991). Interestingly an electrophysiological study demonstrated that there are cells similar to the place cells in the VS but also able to modify its firing pattern respective to a specific position in the environment and to the position value. This position value could be associated to food position or everything that is needed at fixed position (Lavoie and Mizumori, 1994). Thank to this result they hypothesized that the VS should be responsible for the integration of contextual information and in the charge of the integration of the value of each object, such as reward position or dangerous position. In this case, connections and position suggest that the VS could be a key structure for spatial information integration and also a structure involved in final motor decision and excution.

Specific studies working on the spatial involvement of the VS have been shown that VS neurons showing position dependent activity might be modulated by hippocampic afferences, specifically from the CA1/subiculum to the shell of the accumbens nucleus (Groenewegen et al., 1987). This result had been sustained by study showing firing pattern of cells in the hippocampus 20 ms before the same pattern in the VS in rats moving in their environment (Tabuchi et al., 2000). This result seems to be the same in the entorhinal cortex (Brog et al., 1993), a structure involved in spatial information elaboration (Hafting et al., 2005). However similar modulations were not found in prefrontal cortex (Jung et al., 1998; Poucet et al., 1997), either in the amygdala (Pratt and Mizumori, 1998), even if there is connection between these structures. These findings seem demonstrated a specific communication between the VS and the hippocampus according to Mogenson's studies and probably two structures always activated together, even if the previous description of the hippocampus role suggested two different roles in treatment of

spatial information. According to this hypothesis, there is a difference between the similar place cells from the VS and the hippocampus about their time dependent activity. The hippocampal cells, which firing only during a specific position of the animal (Trullier et al., 1999), maintain the same firing pattern also during repetitive sessions in the same context. This cell kind in the VS, during repetitive sessions, showed response variations. To explain, the VS cells which had fired for spatial position in the first session, could fire with a different pattern and for different stimuli (for example the reward anticipation) during successive sessions (Shibata et al., 2001). This result suggests VS processes depend of different afference input from key structures in spatial learning, and /or an intrinsic process that changes firing pattern during sessions. The scientists have been hypothesized that the response variations in the VS might be due to subpopulation of neurons and could be elaborated alternative representation and should be selected following competitive process. This hypothesis is supported by several lesion studies. These works suggested a subregion in the VS able to elaborate spatial information but to have also a switching behavior role (Dubois et al., 1991), a role in flexibility (Readind and Dunette, 1991) and a role in decision making (Albertin, 2000; Annett et al., 1989; Floresco et al., 1996). Finally this structure receives spatial information from different key structures, has a key role in motor output and is involved in different behavioral role.

Glutamatergic transmission integrated to spatial learning:

Several researches have been demonstrated that the glutamatergic transmission was involved in spatial memory process, specifically

AMPA receptor and NMDA receptors (Romanides et al., 1999; Stephens and Cole, 1996; Pin et al., 1995). For example, two old studies had demonstrated that systemic NBQX, an AMPA antagonist, can block acquisition of spatial information in Morris water maze (Flood et al., 1992; Zivkovic et al., 1995). In both structures the hippocampus and the VS are present glutamatergic receptors as such AMPA receptors and NMDA receptors involved in memory stabilization.

In the hippocampus, the memory modifications are visible in a range from some minutes to some hours after the codification phase (Mc Gaugh et al., 2000; Kandel et al., 2001; Dudai et al., 2004). Moreover the hippocampus receives lot of glutamatergic projections from external structures and also intrinsic to the hippocampus. It though that glutamatergic transmission in this structure is predominant to transmit the information flow and the others neurotransmitters as a role of modulator such as the cholinergic system associated to the concentration during acquisition (Deiana et al., 2011). Therefore several studies have been examined whether the hippocampus can be a storage place of new information, particularly pharmacological studies. Interesting studies on the hippocampus come from Morris's laboratory during 90's. They have demonstrated that the AMPA receptors blocking, at different time point before or after learning, impaired mice memory during the retrieval. For example Riedel and collaborators have shown that the inhibition of AMPA receptors during or after training created deficit in long term memory in the spatial version of the Morris water maze (Riedel et al., 1995, 1999). During the same period it was demonstrated that ionotropic AMPA receptors blocked before training decrease the capacity to acquire relevant information for spatial map (Maldonado-Irizzarry et Kelley,

1995; Usiello et al., 1998; Smith-Roe et al., 1999). Later AMPA receptor activities were studied to know activation phase during memory processes. In an experiment with micro-pumps, which liberated LY326325 (an AMPA/Kainate antagonist) during several days, mice were unable to use AMPA receptors localized in the hippocampus during definite period. In this study they showed that AMPA receptors are needed during spatial training but also several days after acquisition (Riedel et al., 1999). Thus the studies above described confirmed that the hippocampus is involved in spatial learning but also that specific receptors are involved during acquisition and in the stabilization after training. In addition to the AMPA receptor activities in memory processes it has been studied NMDA receptors, demonstrated in LTP to be activated after a strong depolarization of AMPA receptors. The NMDA receptor antagonist infusion caused a selective impairment of place learning, without affecting visual discrimination learning (Morris et al., 1986). It was the first work to involved NMDA in spatial learning. Behavioral analysis of a specifically NMDAR1 knock out in hippocampal subregion, the CA1, has confirmed that spatial information stabilization needed NMDA receptors activation but also showed that the CA1 of the hippocampus is necessary in spatial memory processes (Tsien et al., 1996). Another study showed that the NMDA receptor is needed until 2 hours after learning in the hippocampus and sufficient to impaired long term memory (Packard and Teather, 1997). Conversely to LPT idea, these findings showed that windows activation of NMDA receptor began after AMPA receptor activation but finished before the end of AMPA activation.

The hypothesis that has been done suggested that the AMPA receptors were needed for others processes but not for the

stabilization of recent memory. In fact, after stabilization of recent memory there is a phase called system consolidation (Franckland and Bontempi, 2005). During this phase the hypothesis is that consolidated information are sent in cortical structures and stored for a longer time. Based on metabolic activity, lesion and pharmacological studies it has suggested that the hippocampus is needed, for 5 days after learning for retrieval (Bontempi et al., 1999; Vann et al., 2000; Takerara et al., 2003; Squire et al., 2004; Ramos, 1998; Kubie et al., 1999). Recently evidence confirmed that the hippocampus should be send information in cortical structures. After learning in fear conditioning, a task where animal receives electrical shock in a context and are tested later in the same context without shock assessing freezing, it has been analyzed whether at different time points changed dendritic spine growth in the Anterior cingulate cortex (Acc) and in the hippocampus. 48 hours after training they found dendritic spine growth in the hippocampus but not in Acc inversely they found 37 days after dendritic spine growth in the Acc and a relative reduction in the hippocampus (Restivo et al., 2009). Today more studies are neededto provide further support with the system consolidation hypothesis.

In summary the hippocampus is key structure for the acquisition, stabilization and retrieval of spatial information. However this structure seems to be involved only during recent memory and not remote memory that is no more than 5-7 days. We can hypothesize that the hippocampus is the first step of information analysis and acquisition. Therefore rapidly the hippocampus discharges this memory in different structures in order to free space for the following information. But if it is almost established today that the

hippocampus sends information into the Acc, it is probable that there are others storage sites.

In the VS, results showing glutamatergic afferences in the VS suggested a role in spatial memory process. Therefore studies have been recently used glutamatergic manipulations, for different receptor classes and at different time of the integration process of spatial information. Interestingly in the striatum there is a dorso-ventral AMPA receptor gradient higher in the nucleus accumbens (Albin et al., 1992; Dure, 1995). Pharmacological modulation in the VS showed an involvement in spatial learning (Setlow and McGauth, 1998; Roullet et al., 2001; Sargolini et al., 2003; Ferretti et al., 2007). Studies on focal post-training NMDA antagonist (AP-5) injection were assessed in the VS, in a Morris water maze task and tested 24h after training. The results demonstrated deficit in spatial memory stabilization (Sargolini et al., 2003). The same experience was been done in a different task. Using mice, the authors trained it in an open field with object (object recognition task). Mice were trained during several sessions and habituated to the open field during the first sessions, and then were introduced five different objects with particular disposition. The test consists to change position of two objects, if the mouse learned the object positions then it will spend more time to explore the displaced object. This task uses mice curiosity and reduces the motivational component. In this study the authors obtained the same results observed in the Morris water maze, demonstrating that the spatial memory storage wasn't dependent of the emotional component but clearly that the VS played a key role in modulation of spatial learning (Roullet et al., 2001). Interestingly identical studies were done blocking dopaminergic receptors and have been shown the same implication

of the VS in spatial memory stabilization (Setlow and Mc Gaugh, 1998; Mele et al., 2004). All of these studies have never been shown deficit in non spatial version for AMPA antagonist, NMDA antagonist and Dopamine antagonist. These findings prove the VS role in spatial consolidation.

Researchers also tried to determinate windows activation of AMPA and NMDA receptors during spatial learning. Spatial tasks as object recognition and Morris water maze have been used. The blocking of AMPA receptor showed effects on short term memory (STM) and long term memory (LTM) when the antagonist has been infused immediately before the training. But didn't shown any effect when has been infused after training. AMPA receptors are active during training phase but not after in the VS and are necessary to spatial memory consolidation. Moreover AMPA antagonist injection just before testing showed a deficit to recall spatial memory 24 hours after training. Thus the VS is needed also for the recall of spatial memory. Finally AMPA receptor is needed during training and during testing for recall spatial information. However NMDA receptor blocking showed effects in STM and in LTM but in STM only when the antagonist was infused pre-training and in LTM only when the antagonist was infused immediately after training. The NMDA receptor is needed during AMPA active phase (training) but also after (Roullet et al., 2001; Sargolini et al., 2003; Ferretti et al., 2007).

These results showed the high level of involvement of the VS in spatial memory consolidation and also showed the importance of AMPA receptors in the encoding during training and during retrieval. In summary, the VS is involved in memory processes, specifically for spatial information, and in consolidation processes of the mnesic trace as it is described for the hippocampus. Memory consolidation

seems to have the same mechanisms in both structures but it isn't still shown whether the VS can store spatial information during several days.

Cellular and molecular mechanisms associated to spatial memory processes:

Memory processes has been demonstrated starting by receptor activation at the cellular level (Bliss and Collingridge, 1993). Also as it has been described before, they found crucial role of glutamatergic receptors in long term memory, such as AMPA and NMDA receptors (Tsien et al., 1996; Rampon et al., 2000). Between the different pathway activated after NMDA receptor activation, an interesting protein might be play an important role in memory modulation (Silva et al., 1992; Mayford et al., 1995; Giese et al., 1998) which is the protein kinase $Ca+^{2}$/Calmodulin dependent (CAMKII). This kinase presents at the synaptic post density (PSD) level, might determinate the excitatory post synaptic potential (EPSP), increasing, by phosphorilation, quantity of AMPA receptors insertion within the synapse and the channel conductance themselves (Nicoll and Malenka, 1999). Studies on single structure interest has been shown that bilateral infusion of an inhibitor of CAMKII in the hippocampus impaired spatial learning (Abel et al., 1997) and has been also impaired in the VS (Perri et al., 2011 submitted). This pathway containing the CAMKII is associated to c-AMP responsive element-binding protein (CREB) (Vianna et al., 2000) and might be responsible to lead transcriptional and post-transcriptional modification into the neuron in order to stabilize information for longer time (Izquierdo et al., 1998) without impaired short term memory (Bailey and Kandel,

1993; Lamprecht and Le Doux, 2004). Again the CREB protein has been demonstrated important for spatial learning in the hippocampus (Mizuno et al., 2002; Colombo et al., 2003; Florian et al., 2006) and also in the VS (Ferretti et al., 2011). Further the pathway containing cAMP-dependent protein kinase (PKA) associated with ERKs/MAPKK (mitogen-activated protein kinase kinase) activation, which might be more associated with molecular changes leading to the maintaining for lesser time (Vianna et al., 2000), has been also demonstrated involved in spatial learning. Bilateral PKA inhibition in the hippocampus impaired spatial memory (Abel et al., 1997) and also in the VS (Perri et al., 2011 submitted). These important proteins, i.e. plasticity markers, seem have the same role in both structures.

It has been demonstrated that mainly determinant factors for long term memory formation are those which start and process the new gene transcription (Alberini et al., 1994; Bailey et al. 1996; Guzowski and Mc Gaugh., 1997; Guzowski et al., 2001) and the new protein synthesis (Davis and Squire, 1984; Bourtchouladze et al., 1998) as it has been seen after anisomycin injection (Bourtchouladze et al., 1998; Bailey et al., 1999; Bozon et al., 2002; Vazdarjanova et al., 2004). This new protein synthesis inhibition selectively blocked long term memory formation and not short term memory. Doing focal injection of anisomycin into the hippocampus (Artinian et al., 2008) or into the VS (Kelley et al., 1997, 2004; Ferretti et al., 2011) they showed impairment in spatial long term memory in both structures. These results suggest that memory consolidation processes are the same in both structures.

Consequently to this molecular cascade, transcription and new protein synthesis there is plastic reorganization and consolidation for

new synaptic interactions. Moser and collaborators demonstrated after spatial learning that there was an increased of dendritic spine growth in CA1 of the hippocampus (Moser et al., 1994). In the VS few studies on plasticity mechanisms are available. However plasticity markers have been demonstrated involved just before in spatial long term memory and studies on drug addiction have been shown increasing of spine density in the VS (Robinson and Kolb, 1999), providing indirect evidence in a possible role of storage of the VS. Recently in my laboratory it has been correlated a dendritic spine growth in the VS after spatial learning and negatively correlated whether Ser845 phosphrylation of AMPA receptors in the VS were blocked, avoiding the activation of intracellular molecular cascade (Perri et al., 2011 submitted). Thus the VS, as the hippocampus, might be able to store information, specifically spatial information.

At functional level, new protein from new synthesis are necessary to long term memory stabilization and might have a specific role in the activated synapse without affect others synapses around. It has been proposed two model possibilities: 1) synaptic activation should allow synthesis of genes and proteins, which might be transported to the activated synapse, tagged by specific signals also called “synaptic tag” (Frey and Morris, 1998, figure 12); 2) in addition or instead of the first possibility, the synaptic plasticity could be mediated by local production, at the synaptic level, of new proteins (Steward and Schuman, 2001) via post-transcriptional regulation mechanisms such as microRNAs.

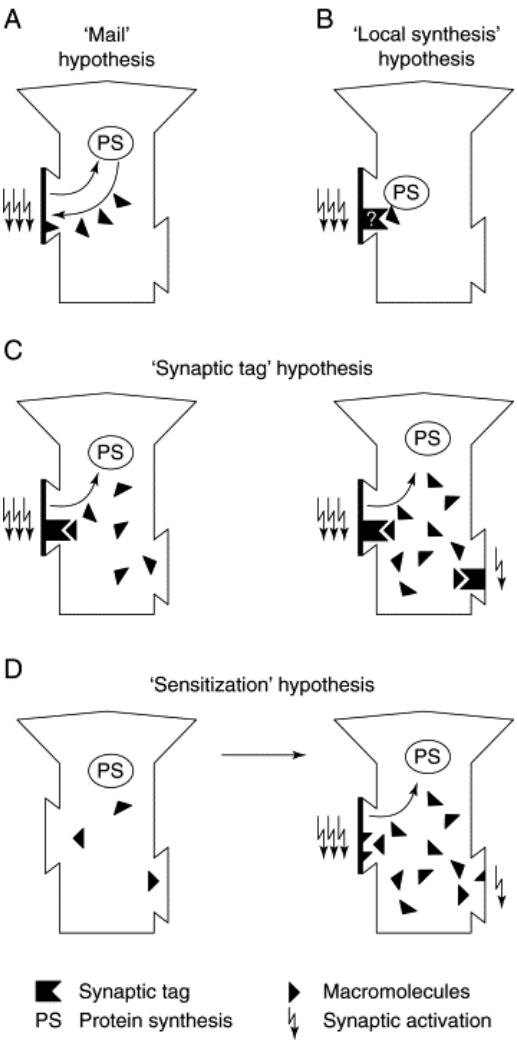


Figure 12: Four ways in which the synapse specificity of late LTP could be achieved. (Frey and Morris, 1998). (A) The `mail' hypothesis involves elaborate intracellular protein trafficking, where proteins, at the time of their synthesis, are given a `synaptic address' to which they are then sent (indicated by curved arrows). (B) The `local synthesis' hypothesis asserts that the relevant protein synthetic machinery is present at, and only activated by, stimulation of nearby synapses. Input specificity is a straightforward consequence of this cellular architecture. However, as protein synthesis does not take place in single synaptic spines but in the nearby dendritic area and, there might yet be a need for a supplementary tagging mechanism to guarantee input specificity illustrated by the `tag' symbol. Recent

findings by Kang and Schuman support local dendritic protein synthesis by neurotrophin-induced potentiation, but this form of plasticity is input-nonspecific. (C) The `synaptic tag' hypothesis involves setting, at activated synapses, a `tag' whose job is to sequester selected proteins. This tag obviates the need for elaborate protein trafficking. Plasticity-related proteins can be synthesized in the soma (or in the dendrites) and then distributed throughout the dendritic tree of a cell relatively diffusely. In this view, the proteins have no address to go to, and thus are only used when captured. (D) The `sensitization' hypothesis entails distribution of plasticity-related macromolecules to every synapse of the cell. These would have the effect of altering the threshold at which synaptic activation (or Ca^{2+} influx) gives rise to lasting synaptic changes. When few of these macromolecules are available, a high threshold prevails, and tetanization usually induces early LTP only; when many macromolecules are available, it is much easier for late LTP to be induced

This consolidation model, that I have described, was defined “a single cascade hypothesis” (figure 13). It provides structural reorganization from a single event of NMDA receptor activation, thus activating intracellular cascade where it is involved CAMKII, PKC, PKA, new gene expression (Arc, tPA, BDNF) and new protein synthesis. This involved mechanisms with limited time, few hours after initial stimulus. The previous results described seem show the same molecular mechanisms in both structures but it has never been examined what specific genes are transcribed, what specific molecules regulates transcriptional and post transcriptional mechanisms in the VS and the hippocampus at the single synapse level.

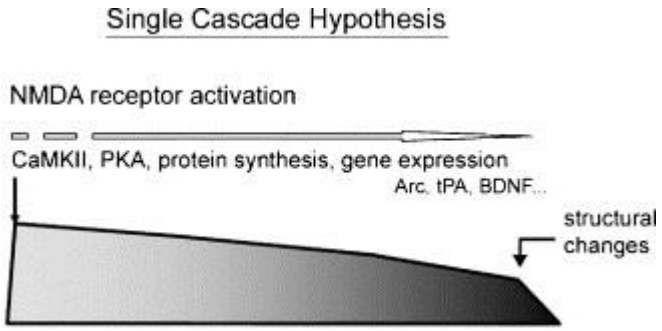


Figure 13: "A single cascade hypothesis" for memory consolidation and storage (Wang et al., 2006). The traditional view of memory formation is represented by the "single cascade hypothesis." Learning activates NMDA receptor and various kinases such as CaMKII and PKA, followed with new protein synthesis, and gene expression. This molecular cascade has been postulated to lead structural changes underlying long-term memory consolidation and storage, but faces many problems such as unmatched time courses with systems-level consolidation as well as the failure to consider the metabolic turnovers of synaptic proteins, etc.

In a last part, numerous publications have been described that long term memory can need more than hours but weeks to years in order to be stabilized. This consolidation system defined by Frankland in 2005, report only studies in the hippocampus and high cortex areas (Ramos, 1998; Kubie et al., 1999; Bontempi et al., 1999; Takerahara et al., 2003; Frankland and Bontempi, 2005), but never into the VS. However evidence let think that the VS can have a role in long term memory storage. It will be interesting to see whether the VS is involved during the same windows activation of the hippocampus or whether the VS is a structure of information storage later as the Acc

or whether the VS is an independent structure in the spatial memory circuit. Some evidence showed that the hippocampus and the VS were needed during the acquisition and consolidation (Ferretti et al., 2007; Micheau et al., 2004) and some evidence showed that these two structures communicated after the end of the training that is "off-line". A study on disconnection between these three structures has been shown direct connection between hippocampus and prefrontal cortex (Floresco et al., 1997). Therefore we can hypothesize that the VS is independent to the hippocampus/prefrontal cortex interaction. Moreover information integrated in the interaction hippocampus/prefrontal cortex might be different from information integrated in the interaction hippocampus/VS. Spatial map information should be memorized in the prefrontal cortex and spatial point value information thought to be stored in the VS. In fact the VS should be able to extract the position value information thanks to specific neurons able to associated reward position and value of this reward. However the prefrontal cortex might receive information already treated and integrated by the hippocampus. Then it's just required to the prefrontal cortex to store the information previously integrated.

II.6 Conclusions

In summary, the experimental findings suggest that the hippocampus is central in extracting, treating and integrating spatial information and possesses molecular mechanism able consolidate and stabilized memory. The VS as the hippocampus is able to consolidate and stabilize spatial information but at the difference collects also information form numerous other afferences and seems less involved in the treatment of spatial information. Although molecular mechanisms of plasticity seem the same between the two structures fine regulation of molecular mechanisms of plasticity such as transcriptional and post-transcriptional regulation can be specific for each structure.

Moreover after memory stabilization the hippocampus is able to send its own information into the prefrontal cortex five-seven days later. In the VS it has never been demonstrated but now we know that spatial information are transferred from the hippocampus to the VS and extracted both reward position and reward value information and these two structure work together during the training and also in off-line just after learning. We also know that correlational evidence suggests that the VS might able to store spatial information in long term memory but it is unknown for remote memory yet.

In this model, probably the VS maintains spatial information longer time than the hippocampus because the VS, to stabilize new information, must integrate also information of motivational and emotional. Finally to do the parallel with the hippocampus, likely the VS sends to the prefrontal cortex, as well as the hippocampus in the Acc, to store position value information there, to allow a quicker

motor response adaptation when it is needed in a known environment.

III. Experiment part

III.1 Research goal

The formation of a long term memory is a dynamic process that consists in the creation of mental representation of the reality, encoded into cerebral circuits. The term "consolidation" (Müller and Pilzecker, 1900) was coined for pointing out that temporal window, immediately following to the phase of learning, in which the necessary molecular processes to the stabilization of the learned information would happen (Duncan et al., 1949; Gerard et al., 1949; Mc Gaugh 2000).

A crucial matter in the study of the memory is the location of the cerebral regions in which the different types of memory are stored. Evidences provided by clinical cases (Scoville and Milner, 1957; Zola-Morgan et al., 1986; Rempler-Clower et al., 1996) and from rodent studies (O'Keefe and Nadel, 1978; Packard and McGaugh 1996), in which lesions located of cerebral regions have induced deficit in specific types of memory, and have brought to the development of theoretical models that demonstrate a clean separation among the different structures in the elaboration of specific functions. According to this model, the hippocampus is the structure of election appointed to the elaboration of declaratory memories (such as spatial memory), and the striatal complex is responsible for the elaboration of procedural memories.

From the molecular point of view, the research of neural substrates of different memory kinds has underlined the existence of key processes at the base of the long term memories such as the activation of transcription factors and immediate early genes, the synthesis of new proteins and morphological modifications at the synaptic level, in distinct regions, particularly in the hippocampus (Guzowski and McGaugh, 1997; Davis and Squire, 1984; Bourtchouladze et al.,1998; Moser et al., 1994). Different data ponting out the involvement of different structures in the elaboration of specific forms of memory (Whishaw et al., 1997; Yin and Knowlton, 2004, Sargolini et al., 2003; Lavoie and Mizumori 1994), suggested that memory systems can't be perfectly distinguishable, but that more structures, or cerebral circuit, can participate to complex memories.

From molecular point of view, classical studies have been direct toward a narrow number of key molecules and mechanisms. An example of this approach is provided by the cAMP response element-binding (CREB), whose role has been studied and confirmed in different structures and in different forms of memory (Guzowski and McGaugh 1997; Lamprecht et al., 1997). This approach is due to the idea, now outdated, that few molecules can be enough to explain the formation of memory, and to the possibilities offered by the available techniques.

Nevertheless we are able to aknowledge that complex molecular processes are involved in plasticity and thus the involvement of an indefinite number of molecules and systems in this processes. Very recent molecular techniques, that allow contemporary the analysis of the expression levels of a high number of genes, have recently been applied to memory studies (Kelley et al., 2005; Cavallaro et al., 2001;

Cavallaro et al., 2002; Di Agatha and Cavallaro 2003; Mei et al., 2005; Miyashita et al., 2007). These systems of analysis, paralleled by bioinformatic analysis, have the advantage to increase the number information available relative to the number and the types of genes that participates in these processes. Thus they seem appropriate to provide a more complete view of the complex molecular mechanisms involved in memory.

During my thesis I tried to use an approach based on the assumption that the memorization of spatial information could involve the hippocampus, but also other brain regions. In particular I focused my research on the role of the ventral striatum (VS), a subcortical structure, traditionally involved in reinforcement learning (Kesner and Wilburn, 1974). Based on nauroanatomical evidence, lesional studies (Sutherland and Rodriguez, 1989), pharmacological manipulation (McGaugh et al., 1998; Sargolini et al., 2003; Roullet et al., 2001), and correlative studies relating dendritic growth and CREB activation (Ferretti et al., 2010; Perri et al., 2011 submitted) with memory a role has been suggested for the VS in spatiallearning and memory.

In order to shade light on molecular processes underlying such stabilization in this study we performed a large scale screening of microRNAs (miRs) and mRNAs expression in two brain regions, the hippocampus and the VS, after spatial learning in CD1 mice. As described before, several molecular plastic mechanisms are conserved in both structures and we could observe the same variation of miRs and mRNAs after learning. However fine regulatory mechanisms such as transcriptional and post-transcriptional regulation, suggesting possible molecular differences between the hippocampus and the VS, even if the same information are processed

and stored in the two structures. To verify this we analyzed significant miRs and mRNAs variation specifically selected for spatial learning and we effectuated bioinformatic analysis.

The second part of this study was aimed at investigating possible causal relationship between miRs variation plasticity and spatial learning *in vitro* and *in vivo*. In a first phase we assessed a miR varied in common in the two structures. In a second phase, we examined whether miRs varied in one and not in the second structure could be region specific.

III.2 Materials and methods

Animals

CD1 male mice (Charles River) were used in the present study. Upon arrival, animals were housed in groups of four or five in standard breeding cages (21 x 21 x 12) placed in a rearing room at a constant temperature (22 ± 1 °C) under diurnal conditions, with food and water ad libitum. At the time of the behavioral procedure, they were 10 to 12 weeks old and their weights ranged from 35 to 40 g. All experiments were run during the light period (between 09.00 a.m. and 5.00 p.m.). Every possible effort was made to minimize animal suffering and all procedures were conducted according to Italian and European laws and regulations on the use of animals in research and National Institutes of Health guidelines on animal care.

Behavior task

Morris Water maze

The circular swimming pool (110 cm in diameter and 30 cm in height) was made of ivory-colored PVC, filled with water (24± 1 °C) made black with non-toxic painting, to 15 cm below the edge of the wall. Four start positions were located equidistantly around the edge of the maze, dividing it into four equal quadrants. During training, a circular goal platform painted black had a rough textile surface

providing sufficient grip for the animals to climb on top of it. The apparatus was placed in a separate room and surrounded by white curtains, 100 cm away from the pool, containing several bi- and three- dimensional extra-maze cues. It was illuminated by a white light (60 W) that diffused behind the curtains and had a video camera placed overhead and connected to a video recorder and monitor.

The general procedure consists of three different phases: a familiarization phase, a training phase, and a probe test. On the first day, mice are individually submitted to a single familiarization session of three trials, with the platform immerged 2 cm over the surface of the water. The session starts with the mouse standing on the platform for 30 sec. At the beginning of each trial, mice are introduced in the maze facing the wall at one of the four designated starting points (N, S, E, W), and allowed to swim freely until they reached the platform. Mice failing to find the platform within a fixed period of 60 seconds are gently guided by hand to the platform, and a maximum escape latency of 60 sec is recorded. After the animals climbed to the platform, they are allowed to remain on it for an additional 30 sec, and are subsequently replaced in the maze from a different starting position.

Training starts the next day. Mice are submitted to six consecutive sessions of three trials, with an intersession delay of 15–20 min during which they return to their home cage with a hot infrared light overhanging the cage. The starting positions were determined in a pseudorandom order, such that each position was only used once in a single session. The procedure is the same as in the familiarization phase, except for the platform, which is submerged 0.5 cm beneath the surface of the water. 24 h after the last training session, mice are

submitted to a single trial of the probe test. The platform is removed, and mice, starting from the center of the pool, are allowed a 60-sec search for the platform.

Two different versions of the water maze were used.

In the **spatial version**, several visual cues, 50–100 cm away from the pool, are attached to the curtains surrounding the apparatus. Mice are required to navigate to the invisible platform using the external spatial cues available in the room. The platform is located always in the same quadrant during familiarization and training phases but the platform location was changed between familiarization (EST place) and training (NORD place).

In the **pseudo spatial version**, the mice were exposed to the same context and the same training with two modifications. The white platform is immerged 2-3cm above the surface of the water during the training. The position of the platform changes across sessions, to prevent animals from using spatial bias.

Mice used for microarray analysis were sacrificed 1H after the training without the test phase.

RNA extraction

Immediately after sacrifice, brains were rapidly removed, and the hippocampus and the VS dissected with punching protocol in sterile ambient. Samples were collected in Trizol (Invitrogen, Italy) and frozen in liquid nitrogen. Total RNA was isolated from mouse brain tissues using Trizol (Invitrogen) following the manufacturer's

recommendations. RNA quantification was made by Nanodrop ND1000 spectrophotometer. RNA quality was assessed by gel electrophoresis. *We have utilized three different pools for miRNA experiment (First pool, Trained n=15, Pseudo-trained n=15, naïve n=30; Second and third pool, Trained n=12, Pseudo-trained n=12, Naïve n=24).*

Microarray analysis

We performed miRs and mRNAs microarray analysis on total RNA obtained from the hippocampus and VS of naive, spatial and pseudo-spatial mice trained, in order to identify candidate miRs and mRNAs regulated by spatial learning. The microarray was run in six technical replicates disperse on 3 biological replications for each group. We assessed also mRNA microarray analysis on the same total RNA. This microarray was run in five or three technical replicates on the first biological pool.

Synthesis of amplified mRNA and labeling

1 µg of total RNA from each sample was amplified using the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit (AM1753) based on the RNA amplification protocol developed in the Eberwine laboratory (Van Gelder et al. 1990). Amino allyl UTP is incorporated during the transcription step to produce amino allyl modified amplified RNA (aRNA). The aRNA is ready for coupling to the NHS ester label (Cy3 and Cy5).

Hybridization and image acquisition

The microarrays used in this study contains spotted 70-mer oligonucleotides from the Array-Ready Oligo Set for the mouse genome v3.0 set from OPERON, spotted onto glass slides at the microarray facility service of the Norwegian Microarray Consortium. Slides were pre-hybridized at 42°C for at least 45 min in a solution containing, 5× SSC, 0.1% SDS and 0.1% BSA. The labeled aRNAs (Cy3 sample and Cy5 sample mixed) were added to 50 µl of hybridization buffer containing 50% formamide, 10× SSC, 0.2% SDS pre-heated at 95°C for 3 min. Hybridization was carried out for 16 h at 42°C and unbound DNA was washed off using 3 steps with solutions containing: I. 1XSSC 0.2% SDS pre-heated at 42°C; II. 0.1X SSC 0.2 % SDS; III. two times 0.1X SSC.

A ScanArray Lite Microarray Scanner (Packard Bioscience) was used to acquire images, and GenePix Pro 6.1 software and ScanArray Express software were used to quantify hybridization signals. Absent and marginal spots were flagged automatically by software and subsequently each slide was inspected manually.

Locked Nucleic Acid (LNA) based miRNA microarray

Microarray experiments were performed using miRCURY^{TM} LNA microRNA Array Power Labeling Kit (Exiqon, Vedbaek, Denmark). The miRCURY microarray is dualcolored in order to accommodate parallel hybridization of a reference sample; 3µg of total RNA reference sample (1 naïve mouse) was labeled with a Hy3-conjugated RNA-linker and 3µg of RNA experimental sample (spatial trained/pseudo-spatial trained mice) was labeled with a Hy5-conjugated RNA-linker following the Exiqon manufacturers protocol.

Hy3 and Hy5 labeled RNAs were mixed together and precipitated adding 2,5µL of RNase free Sodium acetate (3M, pH5.3) and 75µL of 99,9% ethanol in order to remove unincorporated dyes. Sample was incubated at -80°C for 20 min and then centrifugated at 12.000 rpm for 20 min in a cooled centrifuge. A second wash was performed adding 125µL of 75% ethanol; sample is spun for 10 min at 12.000 rpm, at 4°C. Supernatant was discarded and pellet was briefly air-dried at R.T. Subsequently pellet was dissolved in 20µL of RNase free water and 20µL Hybridization Buffer (Exiqon). Sample was denatured for 2 min at 95°C and than hybridized on homemade slides, containing LNA modified microRNA capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.1. Each slide was placed in a slide chamber (Ambion Cat.#10040) and hybridized in a water bath for 16 hrs at 56°C. After hybridization, slides are washed as reported in Exiqon washing protocol (www.exiqon.com). A ScanArray Lite Microarray Scanner (Packard Bioscience) was used to acquire images, and GenePix Pro 6.1 software was used to quantify hybridization signals. Absent and marginal spots were flagged automatically by software and subsequently each slide was inspected manually. Microarray images were processed and analyzed using Genepix Pro. 6.1, Excel and TIGR Multiexperiment viewer version 4.0 software. The data was pre-processed and normalized using spike-in capture probes spotted onto slides and different positive control capture probes. The resulting generalized log2 ratio values were used in further data analysis.

Rt-PCR

To validate microarray data, the real-time RT-PCR was used. The pools of control, Trained and Pseudo-trained, from those used for microarray analysis, were used.

Quantitative Real-Time PCR on microRNA

Ten nanogram of total RNA from trained, pseudo-trained and naïve pools (2^{nd} and 3^{rd} pool), was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using specific miRNA primers from the TaqMan MicroRNA Assays (Applied Biosystems) following manufacturer's instructions. The cDNA served as template for subsequent Real Time PCR reactions that were set up in duplicate for each sample using the TaqMan Universal PCR Master Mix, No UNG (Applied Biosystems) using an Applied Biosystems Prism 7300 Sequence Detector. The reaction mixtures were kept at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The level of transcripts was evaluated by primers and labeled (FAM fluorophores) probes of the TaqMan MicroRNA Assays system (Applied Biosystems).

Fluorescence output was analyzed using Sequence Detection Software, version 1.2 (Applied Biosystems). Relative quantification was carried out with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the abundance of snoRNA 202 as endogenous house-keeping control. Data were statistically analyzed by Student's t-test.

Quantitative Real-Time PCR on mRNA

One microgram of total RNA, from different single mouse in the three different experimental groups (on the firs biological pool), was reverse transcribed using RNA to cDNA Kit (Applied Biosystems) following manufacturer's instructions. The cDNA served as template for subsequent Real Time PCR reactions that were set up in duplicate for each sample using the TaqMan Universal PCR Master Mix, No UNG (Applied Biosystems) using an Applied Biosystems Prism 7300 Sequence Detector. The reaction mixtures were kept at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The level of transcripts was evaluated by primers and labeled (FAM or VIC fluorophores) probes of the TaqMan Gene Expression Assays system (Applied Biosystems).

Fluorescence output was analyzed using Sequence Detection Software, version 1.2 (Applied Biosystems). Relative quantification was carried out with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the abundance of GAPDH mRNA as endogenous house-keeping control. Data were statistically analyzed by Student's t-test.

Mir-335-5p infusion

Surgery: hippo/VS/ICV

Mice underwent surgery 1 week after their arrival. They were anesthetized with an i.p. injection of chloral hydrate (500 mg/kg; Fluka) and placed in a stereotaxic frame (David Kopf Instruments). The head skin was cut longitudinally and bilateral guide cannulae (7 mm in length, 0.5 mm in diameter) were fixed on the calvarium with

dental acrylic (Shofu). The following coordinates were usedfor the injection: Ventral striatum coordinates: anterior to bregma, +1.7 mm; lateral to midline, ±1 mm; ventral from the dura, –4.3 mm; Hippocampus coordinates: anterior to bregma, –1.7 mm; lateral to midline, ±1.5 mm; ventral from the dura, –1.8 mm; ICV coordinates: anterior to bregma, –0.0 mm; lateral to midline, ±1 mm; ventral from the dura, –2.2 mm according to the Mouse Atlas (Franklin BJ and Paxinos G., 1997). Mice were left in their home cages for at least 1 week before all behavioral tests.

Drugs infusion

Two sequences of microRNA were used. The sequences of the miscript mmu-mir-335-5p sense (mimic335) and the miscript mmu-mir324-5p sense (mimic324-5p) and a scramble Allstar negative control, producted by Qiagen. The microRNA sequence used was: mmu-mir-335-5p 5'- ucaagagcaauaacgaaaaaugu -3' and mmu-mir-324-5p 5'- cgcauccccuagggcauuggugu -3'. The miR senses and the scramble molecule were dissolved in free water (1nmol/µl) and 10 minutes before the injection were add to Hiperfect reagent (for each volume of precedent miR sense solution we added two volumes of Hiperfect), which allows to the miR senses to penetrate in cells. Either the mimic335, the mimic324, the scramble was administered at the dose of 0.133 nmol per side intra hippocampus and intra ventral striatum and at the dose of 0.167 nmol per side intra cerebral ventricular (ICV). The drug administration was done through the canulates directly in the specific brain region 3 hours before the beginning of the training.

Data acquisition and analysis

All statistical analyses for behavioral data, Morris water maze task, provided by video tracking (Ethovision software), were performed using ANOVA repeated measures, followed by pairwise comparison (Tukey's post hoc test) when appropriated.

For the microarray data analysis, we filtered the data to exclude artefacts, saturated spots, and low signal spots. Assuming that most of the genes have unchanged expression, the Cy3/Cy5 ratios were normalized using Goulphar script (http://transcriptome.ens.fr/goulphar/index.php) running on R software using a Global Lowess Normalization. The hierarchical gene-clustering analyses were performed by TIGR MeV MultiExperiment ViewerVersion 4.0. The parameters used for the hierarchical clustering were the euclidean distance and the average linkage method.

Gene ontology analysis was performed using the MeV4 software. Selection of mRNA and miR was performed using t-test analysis and on the basis of different criterion and at different cutoff whereas to confirme microarray results by rt-PCR we used the Spearman correlation two tails.

For the LTP experiment, hippocampal slices were visualized with a Wild M3B (Heerbrugg, Switzerland).fEPSPs were recorded and filtered (1 kHz) with an Axopatch 200 A amplifier (Axon Instruments, CA) and digitised at 10 kHz with an A/D converter (Digidata 1322A, Axon Instruments). Data were stored on a computer using pClamp 9 software (Axon Instruments) and analyzed off-line with Clamp-fit 9 program (Axon Instruments). The values were reported as mean±

s.e.m with n, number of slices. A one-way analysis of variance (ANOVA) with pair wise comparison procedure (Holm-Sidak method) or Kruskal-Wallis One Way analysis of Variance on Rankswith pairwise comparison between treatment groups (significance level=0.05, SigmaPlot, 11.0 /a>.

III.3 Results

Microarray analysis

In this part of microarray analysis assessed first mRNAs expression after spatial learning and then miRs expression. The aim being to examine whether mRNAs expression changed after learning in two structures and after examine whether finer post-transcriptional regulators such as miRs can also change their expression after learning and see whether differences of miRs expression occurred between the hippocampus and the VS.

For the microarray analysis we used three experimental groups: naïve, trained and pseudo-trained as control group. We controlled (excepted for naïve group) that mice have learned (figure 1). [Figure R1A; ANOVA of session, $F(5,22) = 10.319$; $P = 0.0001$*** for trained group; Figure R1B, ANOVA of session, $F(5,23) = 12.529$; $P = 0.0001$*** for pseudo-trained group]

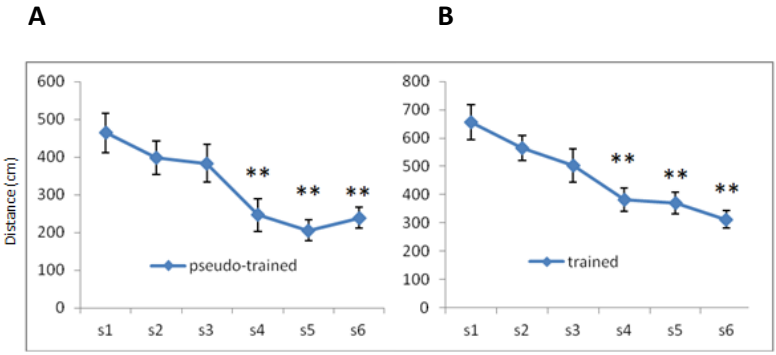


Figure 1: Pathlength during the training of mice used for microarray analysis.

mRNA expression:

First we focused our interest on global activation of miRs and mRNAs after the spatial or non-spatial training in the hippocampus and the VS. To did so we used, for miRs and mRNAs, a threshold of +/-0.41 (0.41 log2 = 25% of variation compared to the naïve mice) for each structure. Then we analyzed the percentage of miRs or mRNAs varied within total expressed.

Global mRNAs activation showed 25% and 22% of them varied in the hippocampus for trained and pseudo-trained groups respectively. While this analysis showed 5% and 30% of mRNA varied into the VS for trained and pseudo-trained groups respectively (figure R2). Global mRNAs activation didn't show any differences between both training versions in the hippocampus whereas the global activation in the VS decreased number of mRNAs varied in trained group.

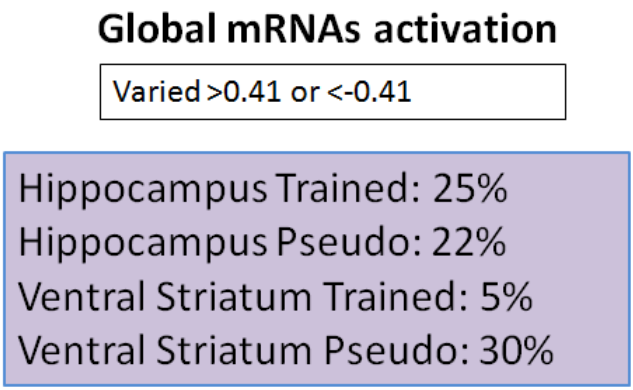


Figure R2: global mRNAs activation into the hippocampus and the ventral striatum for trained and pseudo-trained groups. The percentages show miRs varied more than +/-0.41 (25% of variation) respect to the totality of miRs expressed in microarray analysis.

In a second analysis, the microarray analysis showed mRNAs expressed in the hippocampus and the VS. We found in the hippocampus 13342 mRNAs expressed in the trained group (spatial version) and 9378 mRNAs expressed in the pseudo-trained group (control version). In the VS, we found 3352 mRNAs expressed in the trained group (spatial version) and 7783 mRNAs expressed in the pseudo-trained group (control version). We analyzed how many mRNAs were in common between the two training versions for each structure. We showed in the hippocampus 8921 mRNAs in common (green) between the two training versions and 4421 mRNAs trained specific (blue) and 457 mRNAs pseudo-trained specific (yellow) (figure R3A). In the VS 3344 mRNAs in common between the two training versions and 8 mRNAs trained specific and 4439 mRNAs pseudo-trained specific (figure R3B).

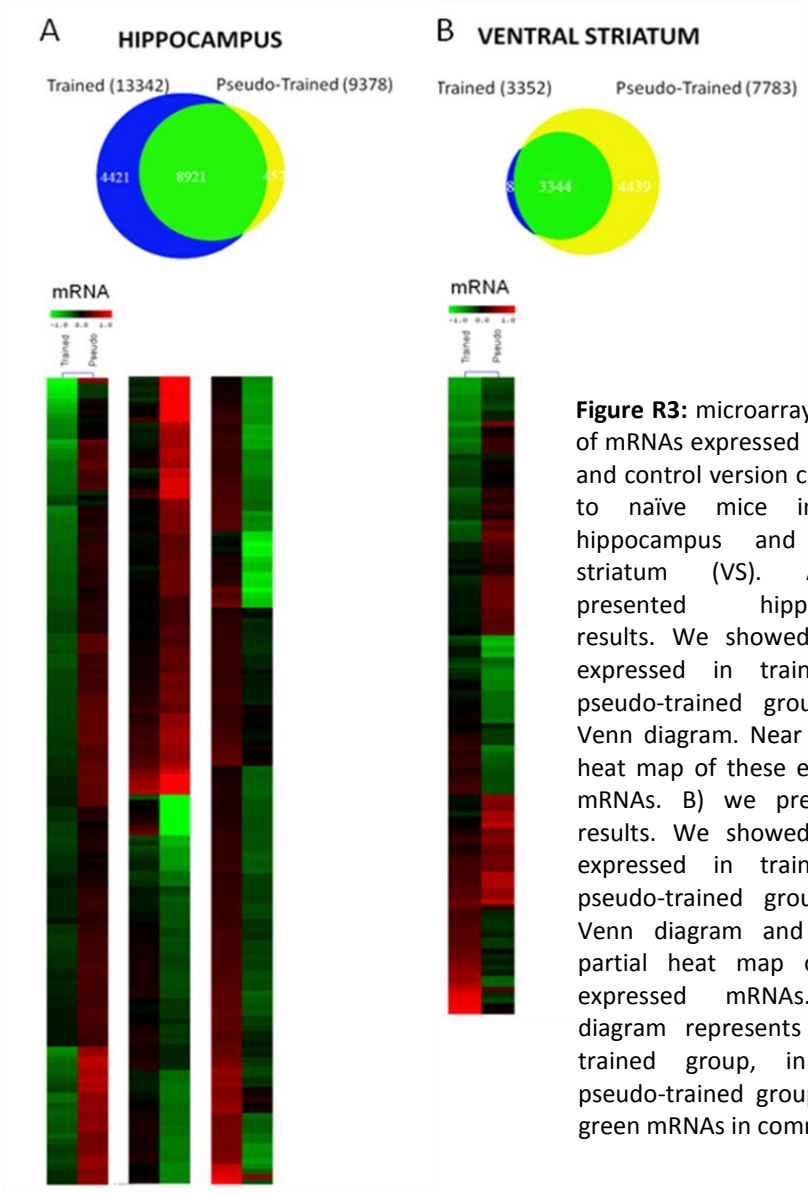


Figure R3: microarray analysis of mRNAs expressed in spatial and control version compared to naïve mice into the hippocampus and ventral striatum (VS). A) we presented hippocampus results. We showed mRNAs expressed in trained and pseudo-trained group using Venn diagram. Near a partial heat map of these expressed mRNAs. B) we present VS results. We showed mRNAs expressed in trained and pseudo-trained group using Venn diagram and near a partial heat map of these expressed mRNAs. Venn diagram represents in blue trained group, in yellow pseudo-trained group and in green mRNAs in common.

rtPCR was done to controlled microarray variation. We assessed 6 mRNAs in the hippocampus and the VS for the two training version that are 24 points (figure R4). The Spearman correlation for microarray versus rtPCR showed significant correlation between mRNAs variation in both technical analysis (microarray = -0.027 + 1.116* rtPCR; R^2 = 0.355; p-value=0.0289*).

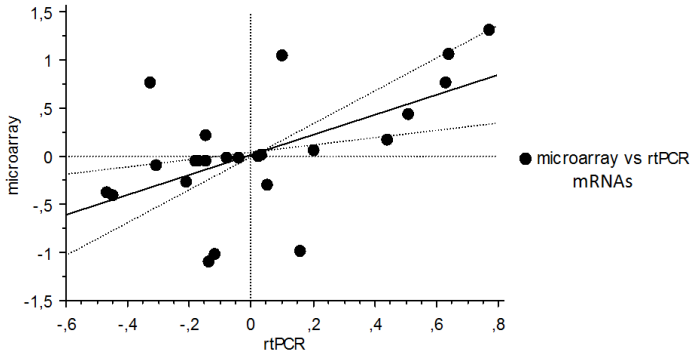


Figure R4: Spearman correlation for microarray versus rtPCR in mRNAs. The plot presents single microarray (y) and rtPCR (x) values by black point. The correlation shows significant correlation. Microarray = -0.027 + 1.116* rtPCR; R^2 = 0.355; p-value=0.0289*.

We then selected mRNAs specific varied for spatial training in the hippocampus and the VS. On the basis of log2 conversion we used a cutoff at +/-0.59 corresponding to 50% of variation compared to

naïve mice. In order to select specific mRNAs varied for spatial learning we excluded all mRNAs present also in the pseudo-trained group with a variation <0.41 (25% of variation). This analysis showed 218 mRNAs expressed in the hippocampus and 16 mRNAS in the VS. We found only one in common (figure R5A). We then assessed the number of down and up-regulated mRNAs. This analysis revealed 43 mRNAs up-regulated and 176 mRNAs down-regulated in the hippocampus, and 29 mRNAs up-regulated and 11 down-regulated in the VS (figure R5B).

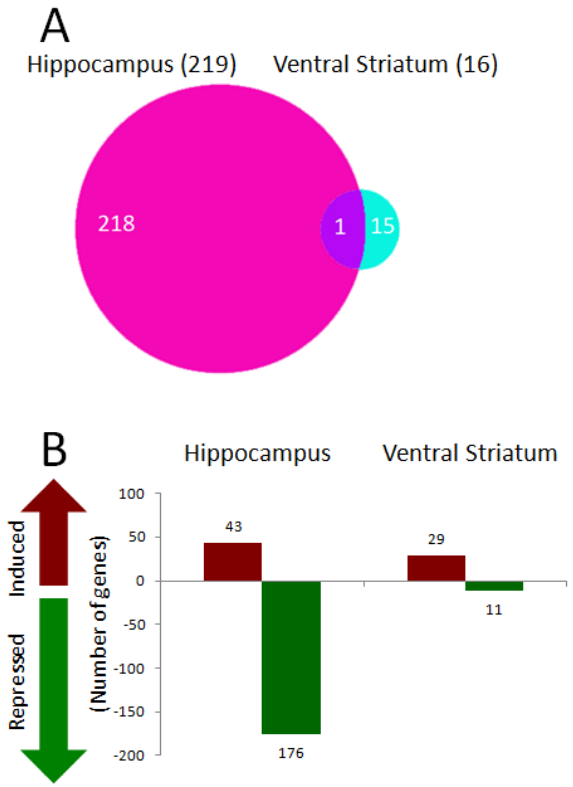


Figure R5: mRNAs specifically varied for spatial learning in the hippocampus and the ventral striatum (VS). A) we showed, in a Venn diagramme, the number of mRNAs varied after spatial learning in both structures. We exprimed in violet spatial mRNAs varied in the hippocampus, in light blue spatial mRNAs varied in the VS and in dark bleu spatial mRNAs in common. B) we showed also the number of mRNAs varied after spatial learning in both structures with separation between upregulated (red columns) and downregulated (green columns) miRNAs. mRNAs were selected by cutoff of

+/-0.59 (50% of variation) and mRNAs varied in trained group were at least +/-0.41 (25% of variation) away from mRNAs varied in pseudo-trained group.

miRs expression:

As exposed in the part *"mRNAs expression"*, first we focused our interest on global activation of miRs after the spatial or non-spatial version training in the hippocampus and the VS. To do so we assessed miRs varied more than +/-0.41 (0.41 log2 = 25% of variation compared to the naïve mice) for each structure and training version. Then we showed the percentage of miRs varied within total expressed.

Global miRs activation showed that 51% of them varied independently of the training version in the hippocampus. Whereas this analysis showed 50% and 19% of miRs varied into the VS for spatial training and pseudo-training groups respectively (figure R6). Global miRs activation didn't show any differences between both training versions in the hippocampus whereas the global activation in the VS decreased number of miRs varied in pseudo-trained group.

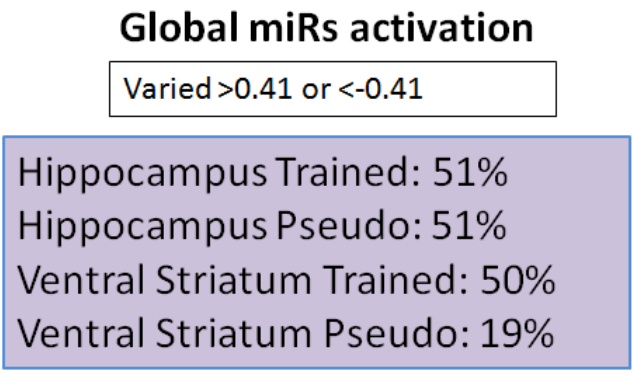


Figure R6: global miRs activation into the hippocampus and the ventral striatum for trained and pseudo-trained groups. The percentages show miRs varied more than +/-0.41 (25% of variation) respect to the totality of miRs expressed in microarray analysis.

In a second analysis, the microarray analysis showed microRNAs expressed in the hippocampus and VS. We found in the hippocampus 210 miRs expressed in the trained group (spatial version) and 164 miRs expressed in the pseudo-trained group (control version). In the VS, we found 170 miRs expressed in the trained group (spatial version) and 180 miRs expressed in the pseudo-trained group (control version). We analyzed how many miRs were in common between the two training versions for each structure. We revealed 163 miRs in common (green) between the two training procedures and 47 miRs trained specific (blue) and only 1 miR pseudo-trained specific (yellow) in the hippocampus (figure R7A). In the VS 161 miRs were found common between the two training procedures and 9 miRs trained specific and 19 miR pseudo-trained specific (figure R7B).

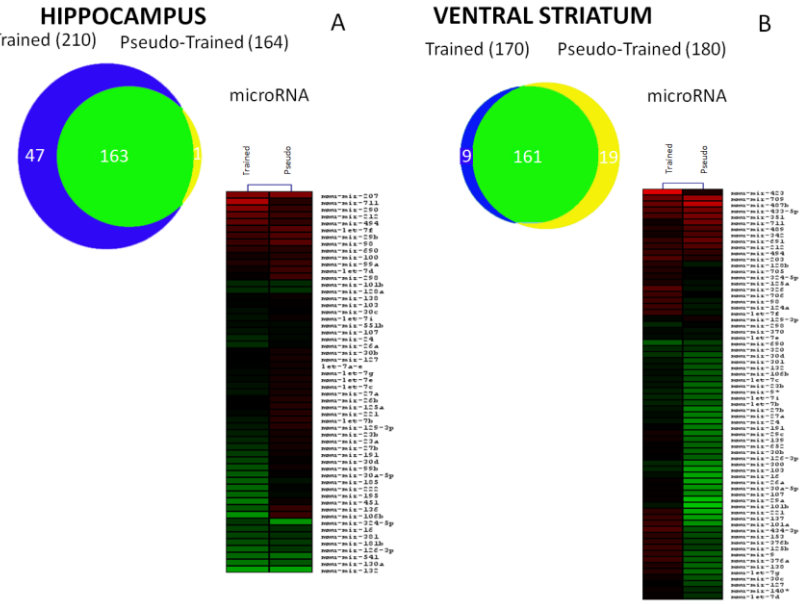


Figure R7: microarray analysis of microRNAs expressed in spatial and control version compared to naïve mice in the hippocampus and the ventral striatum (VS). A) we presented hippocampus results. We showed miRs expressed in trained and pseudo-trained group using Venn diagram. Near a partial heat map of these expressed miRs. B) we present VS results. We showed miRs expressed in trained and pseudo-trained group using Venn diagram and near a partial heat map of these expressed miRs. Venn diagram represents in blue trained group, in yellow pseudo-trained group and in green miRs in common.

rtPCR was done to controll microarray variation. We assessed 7 miRs in the hippocampus and the VS for the two training procedures (figure R8). The Spearman correlation for microarray versus rtPCR showed significant correlation between miRs variation in both technical analysis (microarray = -0.048 + 0.661* rtPCR; R^2 = 0.505; p-value=0.0003**).

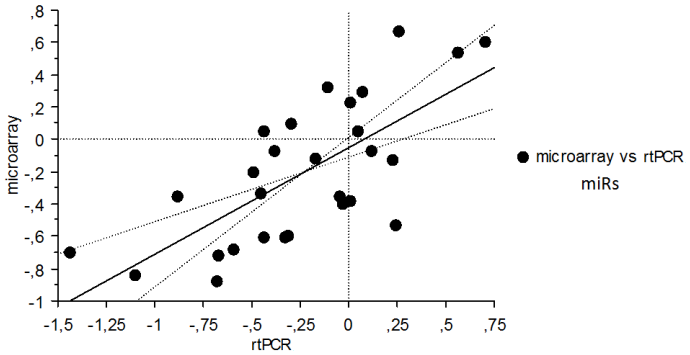


Figure R8: Spearman correlation for microarray versus rtPCR. The plot presents single microarray (y) and rtPCR (x) values by black point. The correlation shows significant correlation. Microarray = -0.048 + 0.661* rtPCR; R^2 = 0.505; p-value=0.0003**.

We then selected spatial specific miRs in the hippocampus and in the VS. On the base of log2 results we used a threshold of +/-0.59 corresponding to 50% of variation compared to naïve mice. In order to select specific miRs varied for spatial learning we excluded all miRs also present in the pseudo-trained group with difference <0.41 (<25% of variation). This analysis showed 13 miRs expressed in the

hippocampus and 8 miRS in the VS. We found only one in common (figure R9A). We also assessed the number of down and up-regulated miRs. This analysis revealed 5 miRs up-regulated and 8 miRs down-regulated in the hippocampus, and 3 miRs up-regulated and 5 down-regulated in the VS (figure R9B).

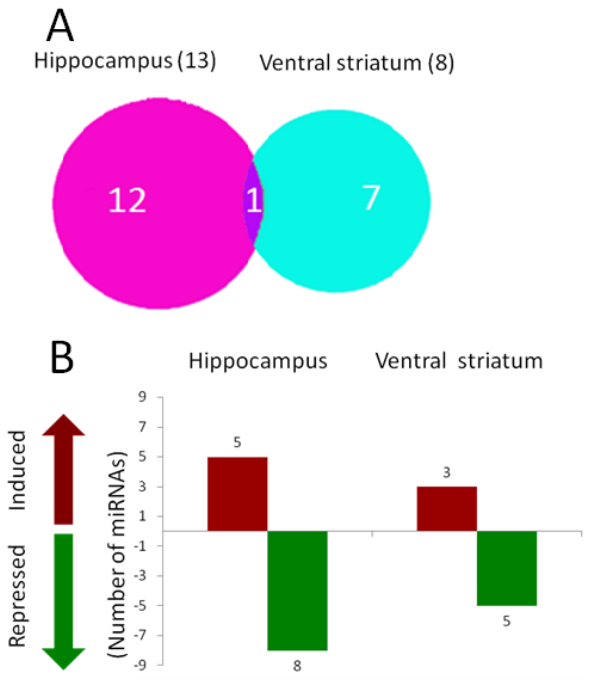


Figure R9: microRNAs specifically varied for spatial learning in the hippocampus and the VS. A) we showed, in a Venn diagramme, the number of miRs varied after spatial learning in both structures. We exprimed in violet spatial miRs varied in the hippocampus, in light blue spatial miRs

varied in the VS and in dark bleu spatial miRs in common. B) we showed also the number of miRs varied after spatial learning in both structures with separation between upregulated (red columns) and downregulated (green columns) miRs. miRs were selected by cutoff of +/-0.59 (50% of variation) and miRs varied in trained group were at least +/-0.41 (25% of variation) away from miRs varied in pseudo-trained group.

The single analysis of the seven microRNAs, examined in Spearman correlation (figure R8), was done to verify the own variation of each miR before starting the in vivo manipulation.

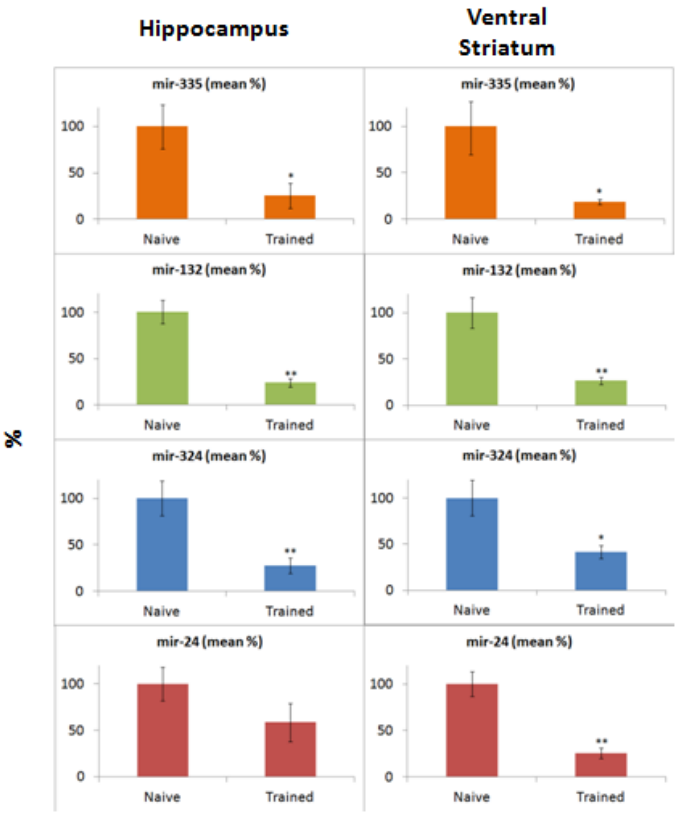


Figure R10: rt-PCR of selected miRs varied in a new mice pool. For each real time PCR group was used 8 pools of two mice. We analized by an unpaired t-test to assess the variation between naïve and trained group. In the hippocampus trained groups were significantly different from naïve groups (p=0.0179*; p=0.0001**; p=0.0035**, for mir-335-5p, mir-132, mir-324-5p respectively) but not mir-24. In the VS trained every groups were significantly different from naïve groups (p=0.0186*; p=0.0007**;

p=0.0129*; p=0.0001**, for mir-335-5p, mir-132, mir-324-5p and mir-24 respectively)

These results confirmed all data from microarray analysis excepted for mir-324-5p. selectively varied in the hippocampus and not in the VS during microarray analysis.

In vivo manipulation of selected microRNAs

First we tested miR manipulation in the whole brain to see whether a single miR was able to induce changes in vitro and in vivo In the two following experiments we studied the mir-335-5p which was down-regulated during spatial learning and was alone to vary in both structures. In order to induce a deficit we injected a mimic of mir-335-5p (mimic335) to overexpress it in the whole brain using an intracerebro-ventricular (ICV) injection. As a control we used a scramble mimic that is the same molecule with random sequence of nucleic acids.

Intracerebro-ventricular overexpression of mmu-mir-335-5P in mice

In vitro assessment: an LTP experiment
(An experiment in collaboration with Laura Maggi from Sapienza-University of Rome, Dept. of Physiology and Pharmacology).

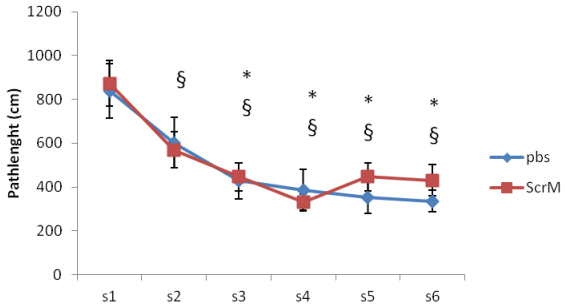
Long term potentiation (LTP) was assessed in mice injected with mir-335-5p mimic (mimic335) or scramble mimic (ScrM) 24 hours before. During this experiment we tested LTP in the hippocampus. In control

mice, injected with ScrM, the mean fEPSP slope potentiation, measured 46-50 min after LTP induction, was 1.42 ± 0.01 (10 slices/4 mice). In treated mice, which received mimic335, LTP induction produced fEPSP slope reduction of 1.25 ± 0.01 (12 slices/4 mice, p<0,05* vs scramble mice) (appendix 2). The mir-335-5p overexpression in mice didn't change early LTP whereas late LTP wasn't constant as well as scramble mice. The mir-335-5p overexpression showed deficit in hippocampal cells to maintain electrical activity in late LTP.

In vivo assessment : a spatial memory task

To extend the LTP result, we verified the effects of mimic335, after ICV injections, on spatial memory performance in the Morris water maze task. Mice were trained in a water maze using a single-day massed procedure (Ferretti et al., 2011), (PBS, n=11; ScrM, n=11; mimic335, n=11). During training we analyzed the pathlenght (mean of the distance in centimeters to go to the platform) and the velocity (cm/s) of mice. We showed for each group a significant decrease of distance along the six sessions to find the platform demonstrating that mice have learned (figure R11). The repeated measures ANOVA analysis showed for control groups, PBS and ScrM, a significant session effect ($F_{5,20}$ = 13.157, p < 0.001), no treatment effect ($F_{5,20}$ = 0.141, p = 0.7112) and no interaction between the two factors ($F_{5,20}$ = 0.351, p = 0.8806) and for ScrM vs mimic335, a significant session effect ($F_{5,20}$ = 14.899, p < 0.001), no treatment effect ($F_{5,20}$ = 0.087, p = 0.7709) and no interaction between the two factors ($F_{5,20}$ = 0.910, p = 0.4776).

A



B

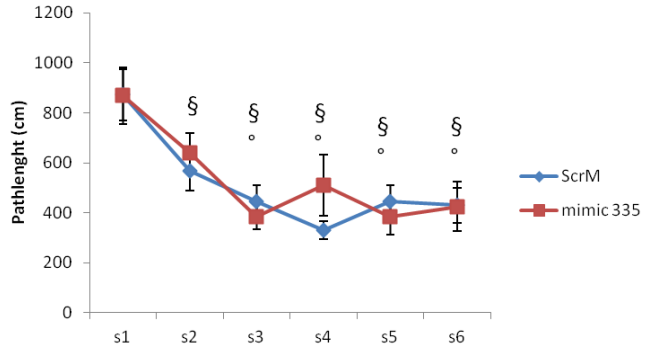
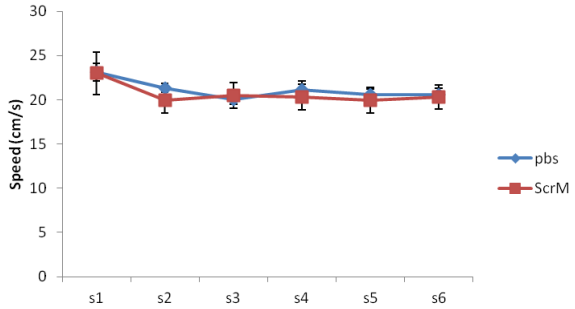


Figure R11: distance to rally the platform decreases during the six sessions training. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The curves represent the mean distance (in cm ± SEM) during each session.

Moreover we controlled locomotors activity along the six session and we didn't find any differences. The repeated measures ANOVA

analysis showed for control groups, PBS and ScrM, no treatment effect ($F_{5,20}$ = 0.095, p = 0.7609) and no interaction between the two factors ($F_{5,20}$ = 0.269, p = 0.9290) and for ScrM vs mimic335, no treatment effect ($F_{5,20}$ = 0.449, p = 0.5104) and no interaction between the two factors ($F_{5,20}$ = 0.297, p = 0.9138).

A



B

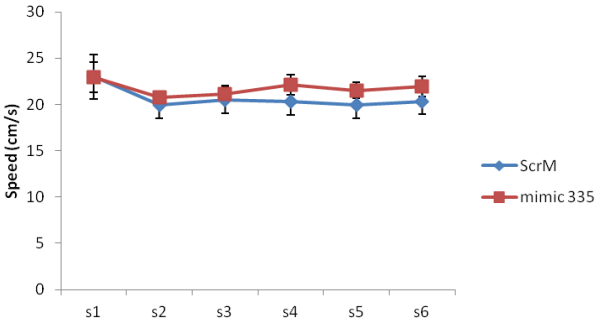
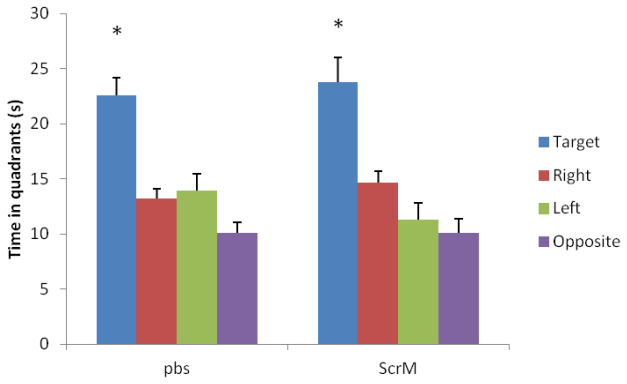


Figure R12: unchanged velocity along the six sessions training. A) compared the two control groups. B) compared the test of mimic335 mice versus

scramble mice. The curves represent the mean velocity (in cm/sec ± SEM) during each session.

On the probe test, PBS and scramble mimic showed similar performance spending more time in the correct quadrant than in the other three [figure R13A; ANOVA of treatment, $F(3,20) = 0.422$; $P = 0.5232$; quadrant preference, $F(3,20) = 22.455$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.607$; $P = 0.6129$]. On the contrary, mice injected with mimic335 spent equivalent time in the four quadrants during the probe trial, thus proving to be unable to correctly locate the platform [figure R13B; ANOVA of treatment, $F(3,20) = 0.238$; $P = 0.6309$; quadrant preference, $F(3,20) = 7.206$, $P = 0.0003$**; treatment × quadrant preference, $F(3,20) = 6.279$; $P = 0.0009$**].

A



B

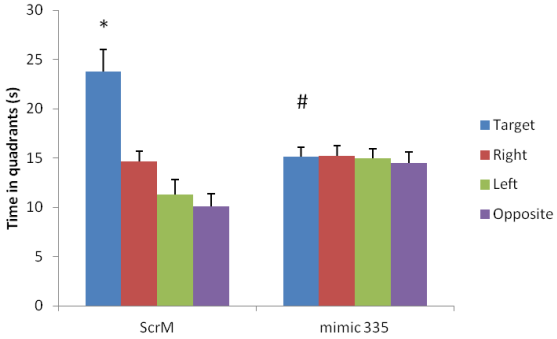
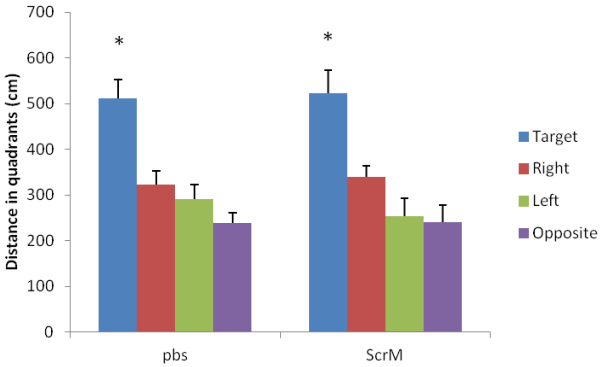


Figure R13 : mimic 335-5p injected in ICV 3 h before training impairs long-term memory in Water maze task. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the mean time (in sec ± SEM) spent in the four quadrants. *$P \leq$ 0.05, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 335 group.

We also showed the distance in the correct quadrant during the probe test. PBS and scramble mimic showed similar performance spending longer distance in the correct quadrant than in the other three [figure R14A; ANOVA of treatment, $F(3,20) = 0.011$; $P = 0.9179$; quadrant preference, $F(3,20) = 23.380$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.219$; $P = 0.8829$]. On the contrary, mice injected with mimic335 spent equivalent distance in the four quadrants during the probe trial, thus proving to be unable to correctly locate the platform [figure R14B; ANOVA of treatment, $F(3,20) = 0.525$; $P = 0.4771$; quadrant preference, $F(3,20) = 9.115$, $P =$

0.0001***; treatment × quadrant preference, $F(3,20) = 6.090$; $P = 0.0011$*].

A



B

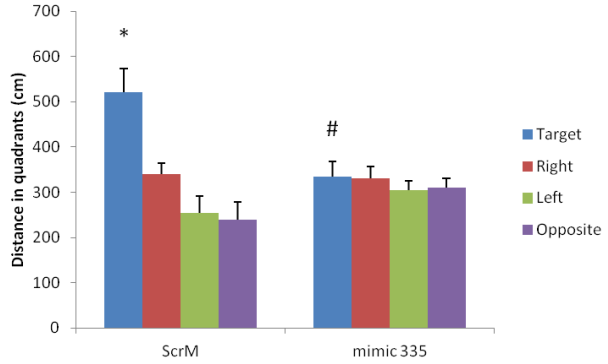
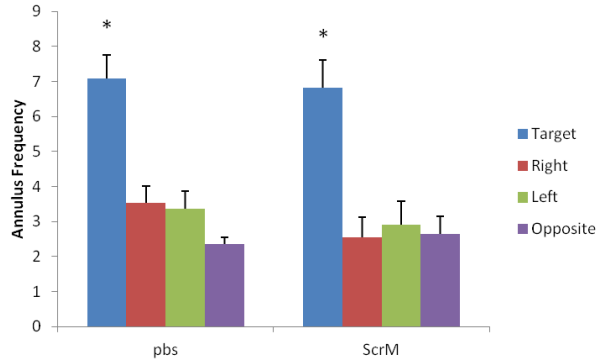


Figure R14 : mimic 335-5p injected in ICV 3 h before training impairs long-term memory in Water maze task. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the mean distance (in cm ± SEM) moved in the four quadrants. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 335 group.

We also showed the crossing frequency of annulus during the probe test. PBS and scramble mimic showed similar performance crossing more time the correct annulus than in the other three [figure R15A; ANOVA of treatment, $F(3,20) = 0.733$; $P = 0.4020$; quadrant preference, $F(3,20) = 27.293$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.447$; $P = 0.7200$]. On the contrary, mice injected with mimic335 crossed the four annuli during the probe trial with a similar frequency, thus proving to be unable to correctly locate the platform [figure R15B; ANOVA of treatment, $F(3,20) = 2.196$; $P = 0.1540$; quadrant preference, $F(3,20) = 12.106$, $P = 0.0001$***; treatment × quadrant preference, $F(3,20) = 6.193$; $P = 0.0010$**].

A



B

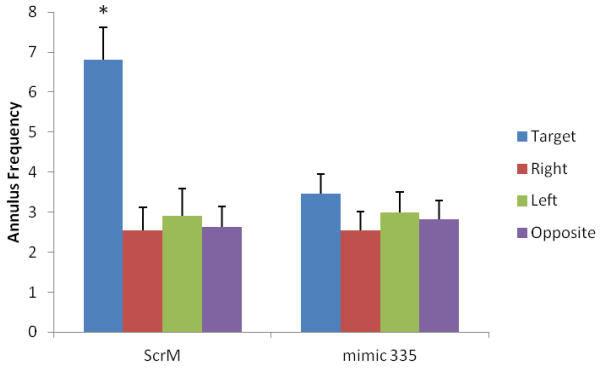
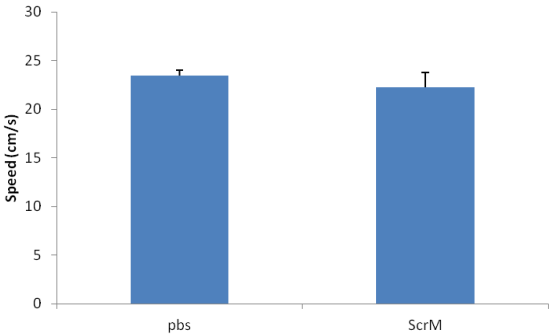


Figure R15 : mimic 335-5p injected in ICV 3 h before training impairs long-term memory in Water maze task. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the mean of crossing annulus (in number of crossing ± SEM) for the four annuli. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within

groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 335 group.

During the probe test we controlled effects on the locomotors activity using the mean velocity (cm/s) of the test. We didn't found any differences between the different groups (PBS vs ScrM, p=0.46 and ScrM vs mimic335, p=0.90).

A



B

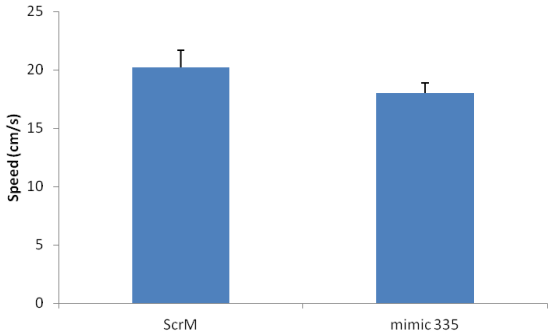


Figure R16: velocity isn't affected during the test. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the velocity mean mice (in cm/sec ± SEM).

Overexpression of mmu-mir-324-5p in mice hippocampus

The following experiment examined whether a miR varied selectvely in the hippocampus or the VS can also affect memory, manipulating in vivo this varied miR directly into the specific structure.

Thus in this second part we injected in the hippocampus or the VS mimic of the mir-324-5p down-regulated specifically in the hippocampus, one hour after spatial training in the water maze task. The injections are performed as described in the materials and the figure R18 shows injection positions. All the injections are localized in the dorsal hippocampus.

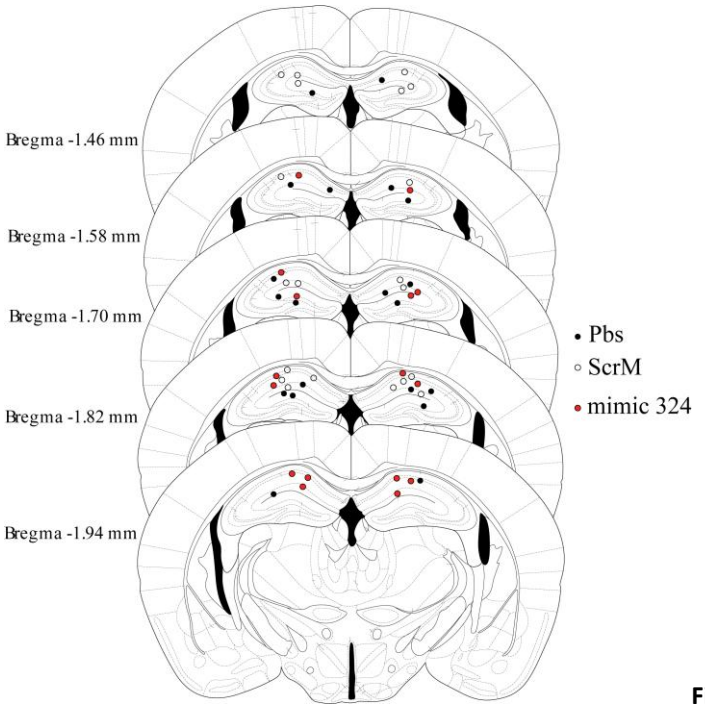
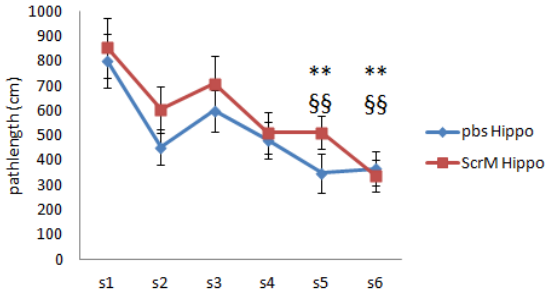


Figure R18: Schematic representation of cannula placements in hippocampus. Each symbol represents the site of injection for one animal (*) vehicle; (○) scramble 0.136 nmol/side; (●) mimic324 0.136 nmol/side.

Mice were trained in the same procedure of water maze task, (PBS, n=12; ScrM, n=10; mimic324, n=11). During the training we analyzed the pathlenght (mean of the distance in centimeters to go to the platform) and the velocity (cm/s) of mice. We showed for each group a significant decrease of distance along the six sessions to find the

platform demonstrating that mice have learned (figure R19). The repeated measures ANOVA analysis showed for control groups, PBS and ScrM, a significant session effect ($F_{5,20}$ = 8.963, p < 0.0001***), no treatment effect ($F_{5,20}$ = 1.408, p = 0.2493) and no interaction between the two factors ($F_{5,20}$ = 0.431, p = 0.8260) and for ScrM vs mimic324, a significant session effect ($F_{5,20}$ = 6.203, p < 0.0001***), no treatment effect ($F_{5,20}$ = 1.207, p = 0.2857) and no interaction between the two factors ($F_{5,20}$ = 0.636, p = 0.6730).

A



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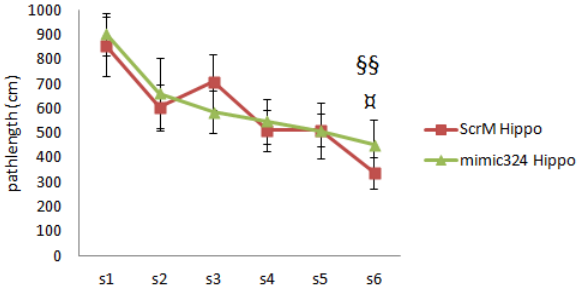
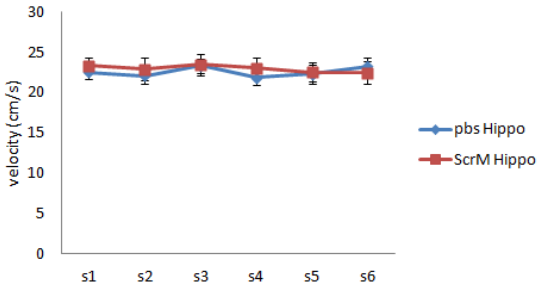


Figure R19: distance to rally the platform decreases during the six sessions training. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The curves represent the mean distance (in cm ± SEM) during each session.

Moreover we controlled locomotors activity along the six session and we didn't find any differences (figure R20). The repeated measures ANOVA analysis showed for control groups, PBS and ScrM, no treatment effect ($F_{5,20}$ = 0.081, p = 0.7787) and no interaction between the two factors ($F_{5,20}$ = 0.781, p = 0.5654) and for ScrM vs mimic324, no treatment effect ($F_{5,20}$ = 0.001, p = 0.9787) and no interaction between the two factors ($F_{5,20}$ = 0.393, p = 0.8522).

A



B

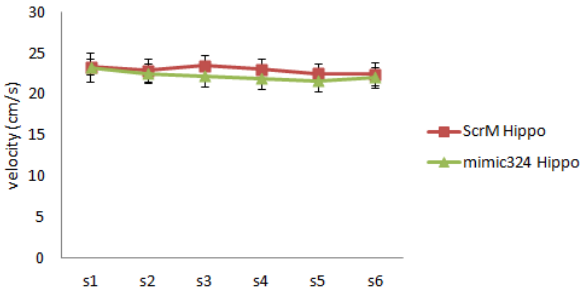
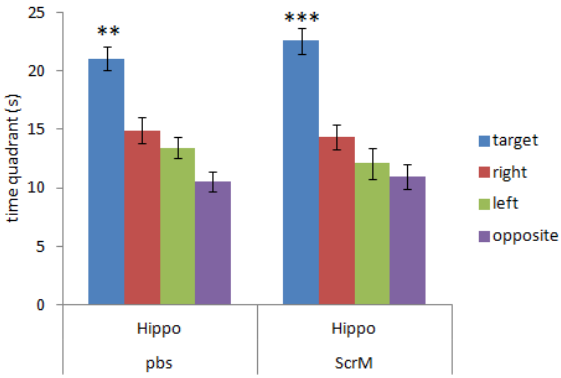


Figure R20: unchanged velocity along the six sessions training. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The curves represent the mean velocity (in cm/sec ± SEM) during each session.

On the probe test, PBS and scramble mimic showed similar performance spending more time in the correct quadrant than in the other three [figure R21A; ANOVA of treatment, $F(3,20) = 0.339$; $P = 0.5671$; quadrant preference, $F(3,20) = 31.968$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.514$; $P = 0.6740$]. On the contrary, mice injected with mimic324 spent equivalent time in the four quadrants during the probe trial, thus proving to be unable to correctly locate the platform [figure R21B; ANOVA of treatment, $F(3,20) = 0.279$; $P = 0.6032$; quadrant preference, $F(3,20) = 18.360$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 4.254$; $P = 0.0088$**].

A



B

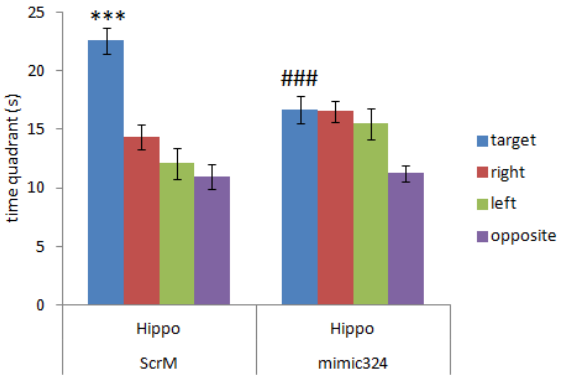
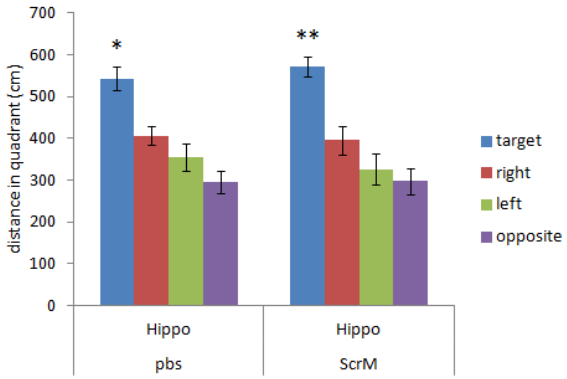


Figure R21: mimic 324-5p injected in the hippocampus 3 h before training impairs long-term memory in water maze task. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The

histograms represent the mean time (in sec ± SEM) spent in the four quadrants. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

We also showed the distance in the correct quadrant during the probe test. PBS and scramble mimic showed similar performance spending longer distance in the correct quadrant than in the other three [figure R22A; ANOVA of treatment, $F(3,20) = 0.010$; $P = 0.9215$; quadrant preference, $F(3,20) = 33.750$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.372$; $P = 0.7738$]. On the contrary, mice injected with mimic324 spent equivalent distance in the four quadrants during the probe trial, thus proving to be unable to correctly locate the platform [figure R22B; ANOVA of treatment, $F(3,20) = 0.142$; $P = 0.7103$; quadrant preference, $F(3,20) = 18.651$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 4.318$; $P = 0.0082$**].

A



B

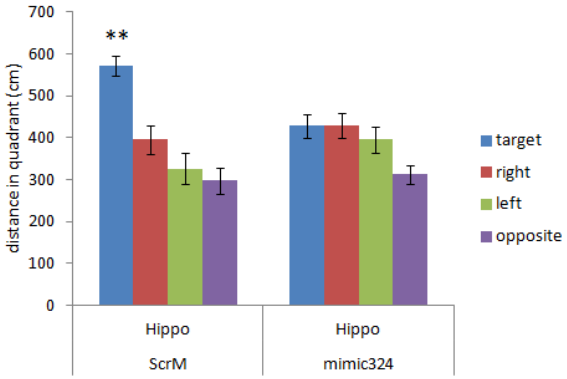
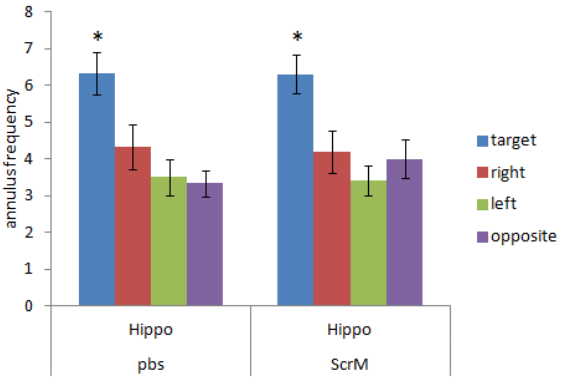


Figure R22: mimic 324-5p injected in the hippocampus 3 h before training impairs long-term memory in water maze task. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The

histograms represent the mean distance (in cm ± SEM) moved in the four quadrants. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

We also showed the crossing frequency of annulus during the probe test. PBS and scramble mimic showed similar performance crossing more time the correct annulus than in the other three [figure R23A; ANOVA of treatment, $F(3,20) = 0.039$; $P = 0.8452$; quadrant preference, $F(3,20) = 37.303$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.389$; $P = 0.7616$]. On the contrary, mice injected with mimic324 acrossed equivalent time the four annuli during the probe trial, thus proving to be unable to correctly locate the platform [figure R23B; ANOVA of treatment, $F(3,20) = 3.314$; $P = 0.0845$; quadrant preference, $F(3,20) = 9.572$, $P = 0.0001$***; treatment × quadrant preference, $F(3,20) = 3.688$; $P = 0.0170$*].

A



B

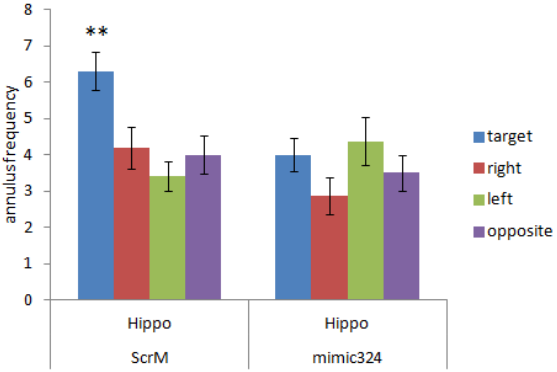
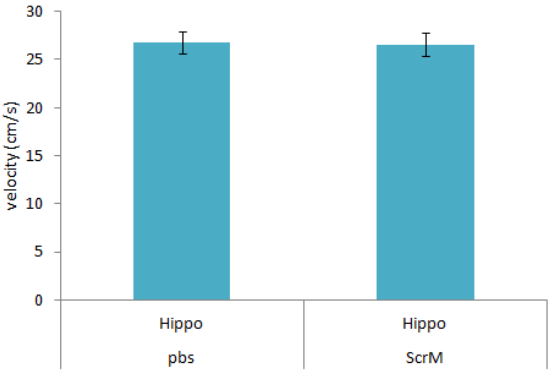


Figure R23: mimic 324-5p injected in the hippocampus 3 h before training impairs long-term memory in Water maze task. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the mean of crossing annulus (in number of crossing ± SEM) for the four annuli. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

During the probe test we controlled effects on the locomotors activity using the mean velocity (cm/s) of the test. We didn't found any differences between the different groups (PBS vs ScrM, p=0.91 and ScrM vs mimic324, p=0.70).

A



B

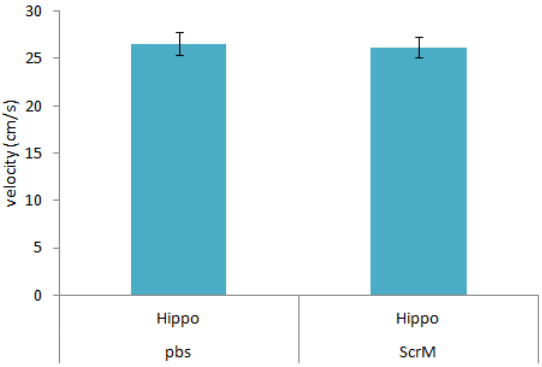


Figure R24: velocity isn't affected during the test. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The histograms represent the velocity mean mice (in cm/sec ± SEM).

Overexpression of mmu-mir-324-5p in mice VS

In this experiment we injected in the VS mimic of the mir-324-5p down-regulated specifically in the hippocampus but not in the VS, one hour after spatial training in the water maze task. The injections are performed as described in the materials and the figure R25 shows injection positions. All the injections are localized in the nucleus accumbens (core and shell).

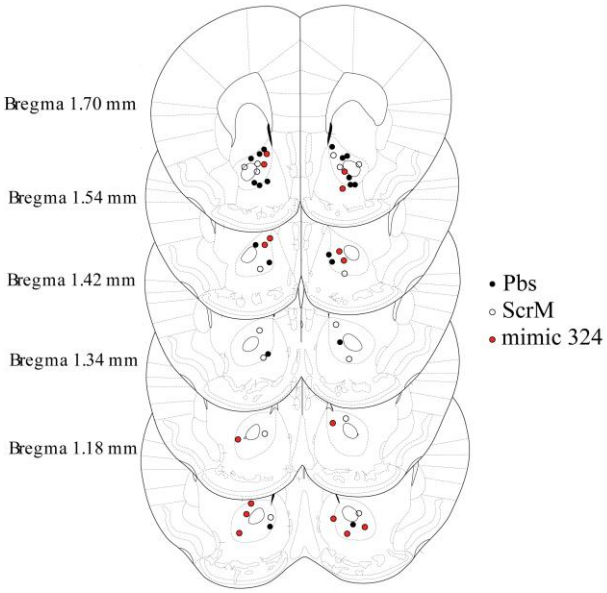
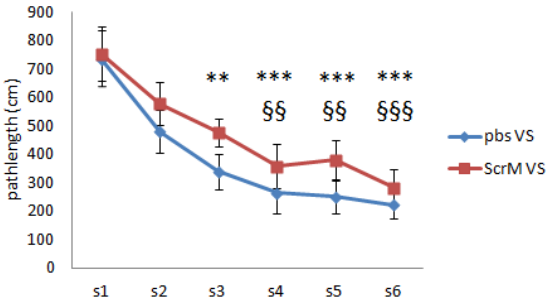


Figure R25: Schematic representation of cannula placements in VS. Each symbol represents the site of injection for one animal (*) vehicle; (○) scramble 0.136 nmol/side; (●) mimic324 0.136 nmol/side.

Mice were trained in the same procedure of water maze task, (PBS, n=11; ScrM, n=11; mimic324, n=9). During the training we analyzed the pathlenght (mean of the distance in centimeters to go to the platform) and the velocity (cm/s) of mice. We showed for each group a significant decrease of distance along the six sessions to find the platform demonstrating that mice have learned (figure R26). The repeated measures ANOVA analysis showed for control groups, PBS and ScrM, a significant session effect ($F_{5,20}$ = 17.245, p < 0.0001***), no treatment effect ($F_{5,20}$ = 2.017, p = 0.1710) and no interaction between the two factors ($F_{5,20}$ = 0.258, p = 0.9348) and for ScrM vs mimic324, a significant session effect ($F_{5,20}$ = 11.135, p < 0.0001***), no treatment effect ($F_{5,20}$ = 0.111, p = 0.7425) and no interaction between the two factors ($F_{5,20}$ = 0.538, p = 0.7468).

A



B

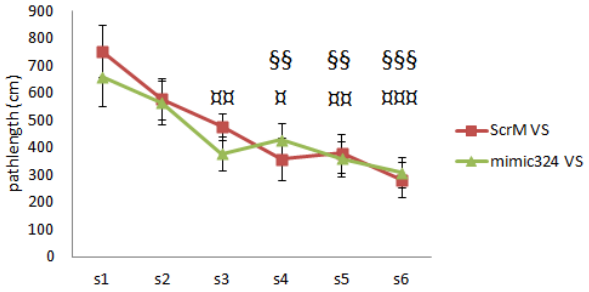
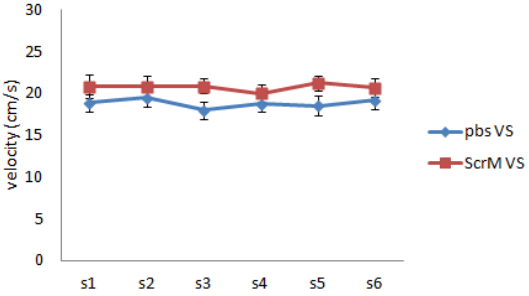


Figure R26: to rally the platform decreases during the six sessions training. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The curves represent the mean distance (in cm ± SEM) during each session.

Moreover we controlled locomotors activity along the six session and we didn't find any differences (figure R27). The repeated measures ANOVA analysis showed for control groups, PBS and ScrM, no treatment effect ($F_{5,20}$ = 1.935, p = 0.1795) and no interaction between the two factors ($F_{5,20}$ = 0.782, p = 0.5651) and for ScrM vs mimic324, no treatment effect ($F_{5,20}$ = 0.030, p = 0.8635) and no interaction between the two factors ($F_{5,20}$ = 0.702, p = 0.6232).

A



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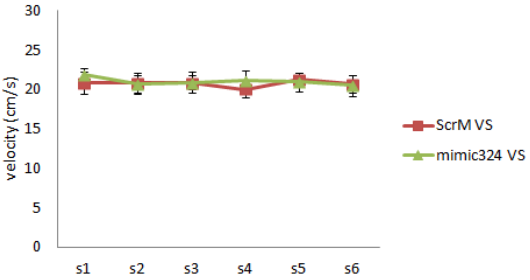
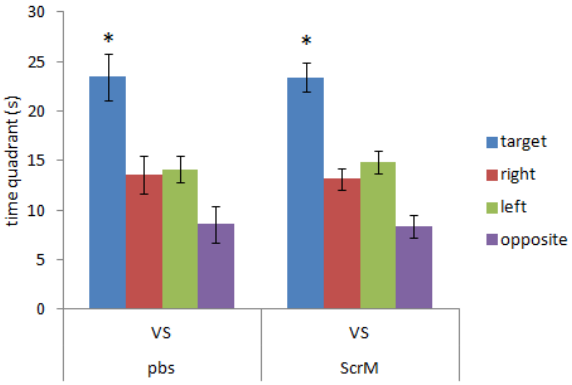


Figure R27: unchanged velocity along the six sessions training. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The curves represent the mean velocity (in cm/sec ± SEM) during each session.

On the probe test, PBS and scramble mimic showed similar performance spending more time in the correct quadrant than in the other three [figure R28A; ANOVA of treatment, $F(3,20) = 0.106$; P =

0.7477; quadrant preference, $F(3,20) = 22.737$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.036$; $P = 0.9899$]. Mice mimic324 spent equivalent time to the ScrM group in the target quadrant during the probe trial. Mice injected in the VS with mimic324 can correctly locate the platform [figure R28B; ANOVA of treatment, $F(3,20) = 0.279$; $P = 0.6032$; quadrant preference, $F(3,20) = 18.360$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 4.254$; $P = 0.0088$**].

A



B

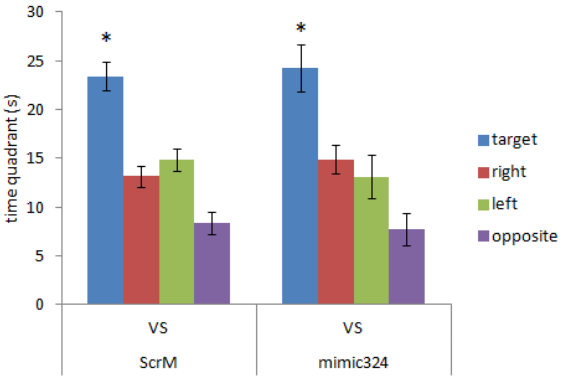
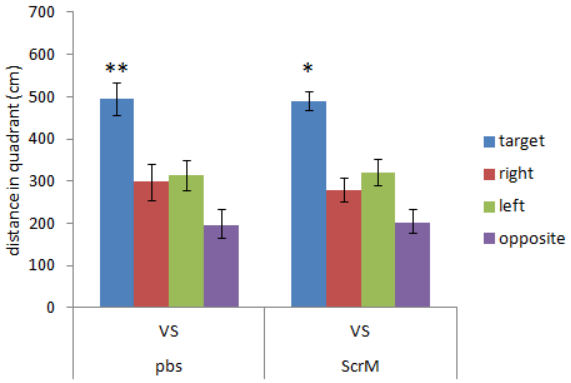


Figure R28: mimic 324-5p injected in the VS 3 h before training didn't affect long-term memory in water maze task. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The histograms represent the mean time (in sec ± SEM) spent in the four quadrants. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

We also showed the distance in the correct quadrant during the probe test. PBS and scramble mimic showed similar performance spending longer distance in the correct quadrant than in the other three [figure R29A; ANOVA of treatment, $F(3,20) = 0.008$; $P = 0.9304$; quadrant preference, $F(3,20) = 27.882$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.065$; $P = 0.9782$]. Mice mimic324 spent equivalent distance to the ScrM group in the target quadrant during the probe trial. Mice injected in the VS with mimic324 can

correctly locate the platform. [figure R29B; ANOVA of treatment, $F(3,20) = 3.214$; $P = 0.0898$; quadrant preference, $F(3,20) = 26.772$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 1.366$; $P = 0.2629$].

A



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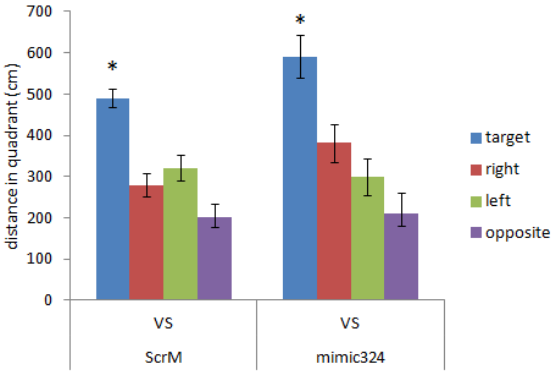
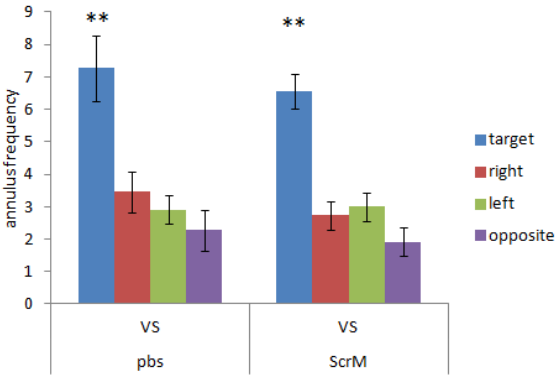


Figure R29: mimic 324-5p injected in the VS 3 h before training didn't affect long-term memory in water maze task. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The histograms represent the mean distance (in cm ± SEM) moved in the four quadrants. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

We also showed the crossing frequency of annulus during the probe test. PBS and scramble mimic showed similar performance crossing more time the correct annulus than in the other three [figure R30A; ANOVA of treatment, $F(3,20) = 0.1.376$; $P = 0.2546$; quadrant preference, $F(3,20) = 23.517$, $P < 0.0001$***; treatment × quadrant preference, $F(3,20) = 0.193$; $P = 0.9009$]. Mice mimic324 acrossed equivalent time to the ScrM group the target annulus during the probe trial. Mice injected in the VS with mimic324 can correctly locate the platform. [figure R30B; ANOVA of treatment, $F(3,20) =$

4.523; P = 0.0475; quadrant preference, $F(3,20)$ = 29.196, P = 0.0001***; treatment × quadrant preference, $F(3,20)$ = 0.884; P = 0.4551].

A



B

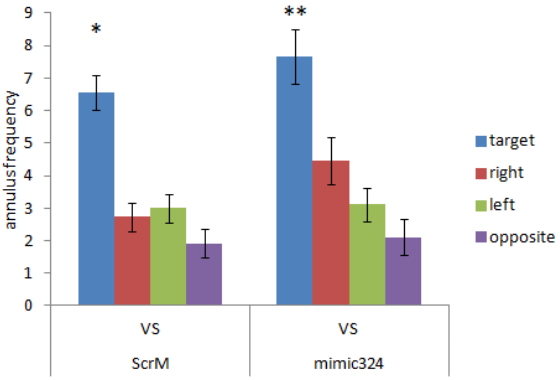
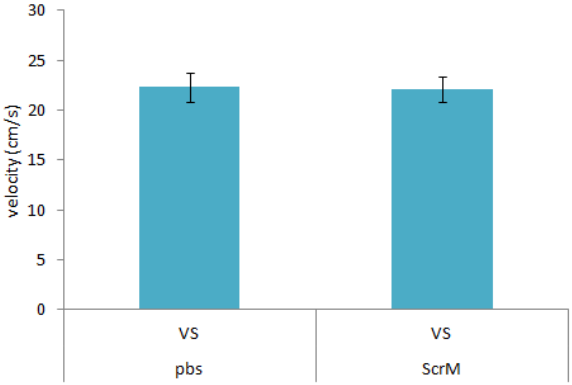


Figure R30: mimic 324-5p injected in the VS 3 h before training didn't affect long-term memory in Water maze task. A) compared the two control groups. B) compared the test of mimic335 mice versus scramble mice. The histograms represent the mean of crossing annulus (in number of crossing ± SEM) for the four annuli. *$P \leq 0.05$, correct vs. opposite, right, left quadrants, within groups. #$P \leq 0.05$, correct quadrant, scramble mimic (ScrM) vs. mimic 324 group.

During the probe test we controlled effects on the locomotors activity using the mean velocity (cm/s) of the test. We didn't found any differences between the different groups (PBS vs ScrM, p=0.92 and ScrM vs mimic324, p=0.17).

A



B

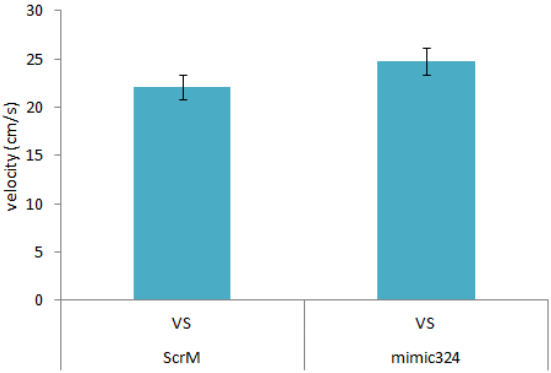


Figure R31: velocity isn't affected during the test. A) compared the two control groups. B) compared the test of mimic324 mice versus scramble mice. The histograms represent the velocity mean mice (in cm/sec ± SEM).

IV. Discussion

Microarray analysis:

In my study I examined molecular expression of miRs amnd mRNA after spatial learning in two key structures, the hippocampus and the VS to verify whether the molecular mechanisms were structure specific.

At the beginning, we decided to perform a large scale screening of mRNAs and miRs expression in mice after spatial training in Morris water maze. According to the literature we decided to examine this variation one hour after the training. This time point is relevant by its correspondence with the gene transcription phase and when new protein synthesis has been described (Krug et al., 1984; Kaczmarek, 1995; Alberini et al., 1994; Bailey et al. 1996; Guzowski and Mc Gaugh., 1997; Guzowski et al., 2001; Davis and Squire, 1984; Bourtchouladze et al., 1998; Bourtchouladze et al., 1998; Bailey et al., 1999; Bozon et al., 2002; Vazdarjanova et al., 2004; Artinian et al., 2008; kelley et al., 1997, 2004; Ferretti et al., 2011).

Changes in messenger RNAs expression was assessed by microarray analysis. First analysis showed a global activity in the hippocampus and the VS. The global activity revelaed percent of mRNAs varied compared to the naïve group. These results showed similar activation of mRNAs, in the hippocampus, for the two procedures: spatial (trained) and non spatial (pseudo-trained). While, in the VS we

revealed a minor mRNA activation after spatial procedure. These findings were the representation of all mRNAs expressed in both structures and we hadn't for basal mRNA expression in naïve mice alone, so we couldn't say if the down- or up-expression of mRNAs was representative of the involvement of the structure.

Then we showed single mRNA expression and we found a higher numerous of spatial mRNAs in the hippocampus (4421) than the VS (8) (figure R3). However using a cutoff of at least 50% increase compared to the naïve group and a variation of 25% compared to the non-spatial group, in order to select significatively varied mRNAs, the number of varied mRNA could be sensibly reduced. In the hippocampus 218 mRNAs were varied, which the bigger part was down-regulated and in the VS 16 mRNAs were varied, which had an equivalent distribution between down- and up-regulated (figure R5). Only one mRNA was found in common. Real time PCR confirmed the mRNAs variation in microarray analysis. The spatial procedure triggered bigger mRNA variation in the hippocampus than the VS and the pattern expression was different in both structures. This result was in accord with the suggested involvement of the two structures in spatial memory (Ferretti et al., 2010), but in disagreed with the working hypothesys that similar molecular mechamisms were involved in the two structures.

Different hypotheses suggest that finer regulations occur during consolidation processes able to control and regulate transcription and post-transcription. Recently microRNAs have been demonstrated involved in post-transcriptional regulation and suggested to have a role in plasticity *in vitro* and *in vivo* (Vo et al., 2005; Gao et al., 2010; Kawasaki et al., 2004; Williams, 2008; Konopka et al., 2010; Siegel et al., 2009; Fukazawa et al., 2003; Hansen et al., 2010). Moreover

bigger part of those should be localized at the somatodendritic level (Kye et al., 2007), suggesting a synaptic local role that would support the synaptic tagging model (Frey and Morris, 1998). Thus in second time we proposed to explore microRNAs expression and variation, which is supposed to have an active role in learning and memory.

The microarray analysis of miRs expression showed first the global activation of miRs in the hippocampus and the VS (figure R6). As mRNAs global activity the hippocampus showed high variation of expression in both spatial and non-spatial training. However in the VS we found again differences of miR variation between the two procedures showing an activity dependent of miRs for the treatment of spatial or non-spatial information. These results could suggest, paradoxically, that the VS but not the hippocampus was able to distinguish spatial and non-spatial information. Intrerestingly we found the same general conclusions during mRNAs analysis. On the basis of the findings it could be suggested that the hippocampus plays an equivalent role in spatial and non-spatial information whereas the VS shows different mRNAs and miRs pattern expressions depending of the type of information. To conclude on global miRs activity, these analyses support the idea that the hippocampus might be less selective in the kind of information treated while the VS could be selective for specific information or might use different molecular processes under different information.

As for mRNAs expression we showed single miR expression without cutoff and we also found a higher numerous of spatial miRs in the hippocampus (47) than the VS (9) (figure R7). Analysis with a cutoff, the same used during mRNAs analysis, revealed 13 miRs in the hippocampus and 8 miRs in the VS specifically varied during spatial

learning (figure 9). miRs microarray variation was confirmed by rt-PCR (figure R8). Also in this case we found only one miR in common. In this miRs analysis we found again two different patterns of miR variation after spatial learning between the two structures. Interestingly the analysis showed for the two structures a higher number of miRs down-regulated after spatial learning. Different analyses, not showed here, presented always a major down-regulation of miRs. According to their hypothetical role to block translation (Schratt et al., 2009), our data suggested thus an increase of translation so enabling new protein synthesis important during memory stabilization. These findings suggested an involvement of the both structures in fine post-transcriptional regulation after learning but supposed also different molecular mechanisms between the two structures.

To conclude mRNAs and miRs, after spatial learning, varied in both structures the hippocampus and the VS but the expression pattern was different for each structure. The microarray analysis shows that there were variation of mRNAs and miRs suggesting an involvement of these two structures in spatial memory and moreover suggesting that the molecular mechanisms should be different for the same kind of information. That isn't still shown at this molecular level.

In vivo manipulation of selected microRNAs

To demonstrate the role of miRs in the memory we decided to manipulate miR expression directly into the brain. To do so, we manipulated selected miR varied on the microarray analysis during spatial learning. The first step to verificate miR implication in memory

was to teste miR manipulation in the whole brain to see whether a single miR was able to induce changes in vitro and in vivo.

Microarray analysis has revealed only one miR in common between the hippocampus and the VS. The expression of this miR, the mmu-mir-335-5p, down-regulated, was controlled only in these two structures and we supposed its possible expression and variation in others brain structures. Thus we induced an overexpression in the whole brain injecting mimic molecule of the mir-335-5p (mimic335) intracerebro-ventricular (ICV). Mimic molecules was never been used in vivo and exists only for cellular cultures yet. The mimic molecule is double strends RNA that is able to pernetrate in the cell when it is associated to lipofectamine (hitperfect®) molecule. When the mimic is inside the cell, it is processed and maturated in the miRs pathway and finishes associated to the Risc complex thus ready to act as well as the endogenous miRs. We did experiments on the vehicle (hitperfect® as lipofectamine), not available here, showing not significative but important degradation in the memory maintain. Cellular studies demonstrated that the hitperfect alone unties cells themselves (Mannironi, 2010). Consequently we assessed whether lipofectamine associated with scramble mimic affect memory and we demonstrated that mice infused with lipofectamine (hitperfect) + scramble (ScrM) had a memory performance similar to PBS (saline) group. Thus during the whole study used ScrM (the same molecule with random sequence of nucleic acids) mice as control group always compared to PBS group. Moreover mimic molecule used in cellular studies is highly diluted. For ICV and focal infusion we concentrated as much as possible and we obtained 1nmol/µl before precipitation of the RNA at the bottom of the solution. Along different

experiments the volume of injection was as much as possible to inject and make impossible the dose response.

In an experiment in collaboration with Laura Maggi from Sapienza-university of Rome, *dept. of physiology and pharmacology*, we explore the effect of mir-335-5p overexpression in the whole brain during long term potentiation (LTP). We showed that hippocampal cells had deficit during late LTP revealing deficit to maintain a stabilized electrical activity but not during early. This result indicated that the mir-335-5p had a role in molecular mechanisms sustaining late LTP, LTP as a model of plasticity. The deficit in late LTP, associated to molecular mechanisms involved in long term memory, demonstrates that miRs are involved in molecular mechanisms and can modify neuronal electrical activity property.

To extend the in vivo effect we overexpressed in a new mice goup the mir-335-5p and assessed their memory capacity in water maze task again. The results didn't show any deficit during the training. And the locomotor activity was similar to the control mice. 24 hours later during the probe, test we showed a deficit in the mimic335 group in all parametters, pathlength, time and crossing of annulus (figure R13, R14, R15). First an experiment with a higher volume of mimic335 (1µl/side) was done but showed also deficit in control mice (not show here). We didn't found any deficit in the velocityin all groups. The overexpression of the mir-335-5p in the whole brain impaired long term memory but not the acquisition. As it has been supposed, miRs might regulate traduction needed during long term stabilization, by LTP experiment and behavior experiment. We demonstrated that at least one microRNA, the mir-335-5p, is

necessary to block long term memory and has a key role in molecular mechanisms of memory stabilization.

The last experiments of this study address the question of possible region specific modulation of memory by miRs. To do so, we now selected miRs varied only in one of the two structures studied. The hypothesis being, whether a miR is varied specifically in the hippocampus after spatial learning but not in the VS, consequently preventing this miR variation during spatial learning might have an effect in the hippocampus but not in the VS, vice-versa.

Thus we selected miR varied in the hippocampus but not in the VS after spatial learning. The most interessant miR by its value in microarray analysis was the mmu-mir-324-5p, a down-regulated miR, which is abundant in the hippocampus. As previously described for mir-335-5p, we assessed an overexpression of the mir-324-5p either in the hippocampus (dorsal) or in the VS (nucleus accumbens) and we explored injected mice in water maze. Mice injected received a bitter volume (0.4µl) than ICV (0.5µl) injection to do not affect mice capacities. The results didn't show any deficit during the training. Locomotor activity was similar to the control mice, in the hippocampus and in the VS. 24 hours later during the probe test we showed a deficit in the mimic324 hippocampus group in all parametters, pathlength, time and crossing of annulus (figure R21, R22, R23). On the contrary, we didn't find deficit in mimic324 mice injected in the VS (figure 28). We didn't find any deficit in the velocity neither in the hippocampus nor in the VS. These results showed a specific deficit in long term memory when the overexpression of the mir-324-5p was localized in the hippocampus but didn't affect

performance when it was injected in the VS as hypothezied previously. This experiment demonstrated again that miRs manipulation *in vivo* affects long term memory. This last experiment demonstrated that among miRs there are miRs involved memory molecular processes probably in common between structures in the spatial circuitry and there are others miRs specific for a one structure. This specificity could suggest molecular differences in the elaboration of spatial information essential to maintain long term spatial information.

V. General conclusion

In this dense literature of learning and memory, each day more specific, we add new evidence for the science. The molecular results of our microarray analysis suggest that the hippocampus is able to treat spatial information as well as non-spatial information. Although the hippocampus is described as a structure spatial-dependent (Morris, 1984), studies suggest that different kinds of information transit in the hippocampus to join its final destination responsible of most associative learning context dependent, such as posttraumatic memory, or noise, savour, odor association (Brewin, 2001; Shors et al., 2000). Thus mRNA and miR activity in the hippocampus might be due to the transit of the both information. However we showed two different patterns of mRNAs and miRs expression between the two procedures, suggesting different analysis of the information under either procedure. We observed that the bigger part of mRNAs and miRs expression in the non-spatial learning was included in those expressed for the spatial learning. And the spatial learning showed a higher number of mRNAs and miRs suggesting that the hippocampus used the same molecular base to treat both information, but the spatial information required more molecular expression. Based on the same line of reasoning, we can hypothesize that the VS is able to differenciate the two types of information by its different global activity under each procedure. However we can't say if an increase or a decrease of global activity observed indicates the implication or not of the structures. As our results are opposed between mRNAs and miRs in the VS under the two procedures, we supposed that increase

as well as decrease of global activity indicates an implication of the structure but without naïve control mice we cannot conclude.

The specific spatial learning mRNAs and miRs revealed different patterns between the two structures. The mRNAs analysis showed higher varied number in the hippocampus than the VS whereas miRs analysis showed the same number of varied miRs in both structures. Important we demonstrated that mRNAs and miRs varied after spatial learning were different between the two structures. We found only one in common in each category. The higher number of mRNAs varied in the hippocampus suggests a more important involvement in spatial learning of the hippocampus than the VS. When the same level of miRs varied in the hippocampus and the VS suggests the same involvement of both structures. Numerous molecular regulation and structural interaction could be the cause of different pattern expressions. Moreover we know that mRNA expression can be modified by intracellular communication and it is more probable that miR expression is modulated at the intercellular level. Thus the differences saw in the level of mRNAs varied could be due to structural interaction, and the equal level of miR varied provides the evidence that the two structures are implicated in spatial information treatment. But the different patterns show that different information need different molecular mechanisms.

In order to verify the major hypothesis that the microRNA could involved in spatial memory, we manipulated miR expression in vivo. The long term potentiation (LTP) showed deficits in late LTP demonstrating impairment in the maintaining of electrical activity cells. This model of plasticity was confirmed by the impairment in

water maze task where showed deficits in long term memory. These results confirmed my hypothesis of the role of microRNAs in spatial long term memory thus giving the first evidence in vivo. This study reinforces the hypothetic mechanisms of miRs regulation during learning (Schratt et al., 2009) and validates that microRNAs are necessary in spatial learning, here the mir-335-5p and the mir-324-5p. Before just once the mir-335-5p was study. It was involved in the climbing fibers in the cerebellum (Barmack et al., 2010) and anything about mir-324-5p.

Our results demonstrated moreover that miR can be necessary in the hippocampus but not in the VS. This mir-324-5p chose in specific varied miRs from the hippocampus was tested as well as others miRs: the mir-24 down-regulated in the VS and the mir-136 up-regulated in the VS. These preliminaries studies, not shown here, didn't reveal any deficit. We demonstrated, by the long term memory deficit manipulating the mir-324-5p, that structures from the same circuitry have different molecular mechanisms. And our results showed that not all miRs varied after spatial learning, revealed by microarray analysis, can exacerbate or inhibat long or short term memory. In fact an important observation that can be done, is the lack of effect on short term memory. The structures were taken one hour after learning and that has been described as the transition phase between short and long term memory. Thus it is possible to suggest that genes varied are not involved in short term memory. Alternatively we haven't found miRs involved in short term memory suggesting that miRs are specifically implicated in post-transcriptional regulation needed in new protein synthesis for memory stabilization.

Interestingly most varied miRs, a part from mir-542-3p, were somatodentritic. Thus supporting the hypothesis that varied miRs

after learning are mainly localize at the post-synapses level. This evidence reinforces the synaptic tag hypothesis.

The large scale screaning approach could seem too long and too expensive but to allow quick knowledges in this domain the microarray analysis could help for the beginning and save us to wait for singly miR work as Izquierdo for neuroreceptor's studies. The following approach possible should be studies on putative target of miRs and explore protein variation due to miRs manipulation. We tried to analyzied two validated proteins (retinoblastoma 1 (RB1) and JAG-1 (Jagged-1) not show here), the results are not yet conclusive. For this exploration it will be recommended to use cellular model thus without cellular interaction and circuitry artifact. However sever pathologies, such as Alagille syndrome or William's syndrome, are caused in part by problems of JAG-1 expression. Interestingly these two pathologies have several symptoms in common, however only William's syndrome shows abnormalities in the cerebellum, where the mir-335 was showed in climbing fibers and this syndrome shows also deficits in visuo-spatial capacity. But these pathologies are the result of 26 gene deletion in the chromosome 7. Thus we cannot conclude but I hope add a puzzle piece.

Finally, all data of this thesis shows clear evidence that molecular mechanisms sustaining memory consolidation and the maintaining of long term memory cannot be hypothezied as a linear mechanisms but as process with different level, such as structural level, cellular level and molecular level, and with different regulation,such as feedback regulation, transcription regulation and post-transcriptional regulation,…etc and probably also epigenetic regulation and transposonic regulation experience-dependent. Thus it is possible to do hypotheses but our results have a high probability to reflect

another combination that gives the same results. Moreover our findings show the importance to continue microRNAs exploration to understand its molecular mechanism and maybe create a new tool in memory diseases.

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Appendix

LTP

Hippocampal slices preparation

The experiments were performed in agreement with international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609 EEC). Hippocampal slices were routinely obtained from CD1 mice. Briefly, the animals were decapitated after being anesthetized with halothane. Whole brains were rapidly removed from the skull and immersed for 10 min in ice-cold artificial cerebrospinal fluid (ACSF) solution containing (in mM): NaCl 125, KCl 4.4, CaCl2 2.5, MgSO4 1.5, NaHPO4 1, NaHCO3 26 and glucose 10. The ACSF was continuously oxygenated with 95% O2, 5% CO2 to maintain the proper pH (7.4). Transverse 350 µm thick slices were cut at 4 °C with a vibratome (DSK, Japan) and the appropriate slices were placed in a chamber containing oxygenated ACSF. After their preparation, slices were allowed to recover for 2 h. Individual slices were then transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc.) with a total fluid dead space of approximately 3 ml. Slices were maintained at room temperature (22–25 °C), and constantly superfused at the rate of 1.5 ml/min.

Electrophysiological recordings

At the beginning of each recording, a concentric bipolar stimulating electrode (SNE-100X 50 mm long Elektronik-Harvard Apparatus

GmbH) was positioned in the stratum radiatum for stimulation of Schaffer collateral pathway projections to CA1. An ACSF-filled glass micropipette (0.5–1 MΩ) was positioned 200–600 µm from the stimulating electrode for recording orthodromically-evoked fEPSPs. Stimuli consisted of 100 µs constant current square pulses, applied at 0.05 Hz. The intensity of the stimulus was adjusted in each experiment to evoke ~50% of the maximal field potential amplitude without appreciable population spike contamination. Evoked responses were monitored online and stable baseline responses were recorded for at least 10min before applying LTP stimulation. Only the slices that showed stable fEPSP amplitudes were included in the experiments. To analyze the time course of fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n=3) and then normalized to the baseline values (100%) 1 min preceding the LTP induction. LTP was induced by applying 4 trains (100 Hz, each 1 s duration, test strength), spaced 3 s apart.

Results:

Long term potentiation (LTP) was assessed in mice injected with mir-335-5p mimic (mimic335) or scramble mimic (ScrM) 24 hours before. During this experiment we tested LTP in the hippocampus. In control mice, which were injected with ScrM, the mean fEPSP slope potentiation, measured 46-50 min after LTP induction, was 1.42 ± 0.01 (10 slices/4 mice). In treated mice, which received mimic335,

LTP induction produced fEPSP slope reduction of 1.25 ± 0.01 (12 slices/4 mice, p<0,05* vs scramble mice) (figure R10). The mir-335-5p overexpression in mice didn't change early LTP whereas late LTP wasn't constant as well as scramble mice. The mir-335-5p overexpression showed deficit in hippocampal cells to maintain electrical activity in late LTP.

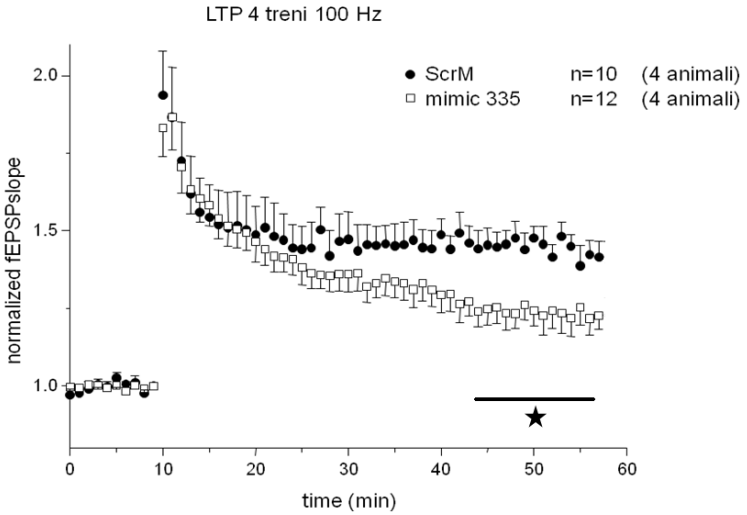

Figure R10: Altered electrophysiological properties in the hippocampus of mir-335-5p overexpressed in the whole brain mice. Altered LTP in area CA1 after tetanic stimulation in treated mice (mimic335) compared to inactive treatment in control mice (ScrM), (n=12 slices/4 mice infused with mimic 335-5p, ○; *n* = 10 slices/4 mice infused with scramble mimic, ●). (mimic 335-5p mice, p<0,05* vs scramble mice).