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Sex-differences shape memory capacity declining during ageing: rescue effects of voluntary exercise

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Candidate Vittorio Loffredo

Tutor Prof. Andrea Mele

Supervisor Dr. Elvira De Leonibus

A mio padre Pasquale,
a mia madre Anna,
a mio fratello Luigi,
per la loro presenza
e l'incondizionato appoggio e sostegno.

A me stesso,
per l'indomabile voglia di pormi
degli obiettivi e nuove sfide da superare.

"I limiti, come le paure, spesso sono solo un'illusione."

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INTRODUCTION

MEMORY

Generalities

The term “memory” is generally referred as the ability to retain and recall facts and information acquired through senses or experience.

The first attempt in studying memory was made by Ebbinghaus, which in 1885 with his book *On Memory*, laid the groundwork for the experimental investigation of learning and memory (Rosenzwaig, 2007). Successively, Miller and Pilzecker in 1900, with their pivotal monograph, reported several experiments designed to identify aspects that govern memory formation and retrieval (Lechner et al, 1999). They introduced the *perseveration-consolidation hypothesis* claiming that “neural activity, initiated by a learning trial, continues and recurs for some time after the original stimulation has ceased, and, that this perseveration aids the consolidation of a stable memory trace” (Rosenzwaig, 2007). Time by time, experimental studies on memory became more and more so that now it is possible to widely speak about memory, focusing on the psychological, the neurobiological aspects and even the possibility to switch-on and off new memories (Tonegawa et al., 2015).

THE HIPPOCAMPUS

The hippocampal formation

The hippocampal formation (HF) is one of the most studied neuronal systems in the brain (Bird & Burgess, 2008) (Fig. 1). Placed in the medial temporal lobe (MTL), it includes many interconnected regions: the hippocampus proper (HP), dentate gyrus (DG), subiculum (SUB), pre-subiculum and para-subiculum, and entorhinal cortex (ENT) (Andersen et al., 2007). The HP has three subdivisions: the *cornu ammonis* 1, 2 and 3 (simply called CA1, CA2 and CA3; Andersen et al., 2007).

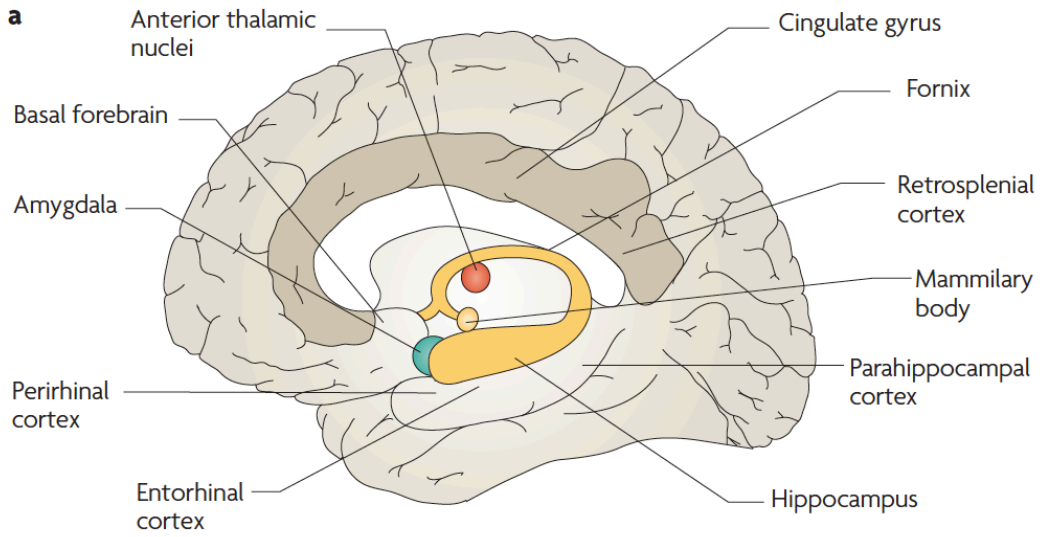

Figure 1. Representation of the human hippocampus and the main surrounding brain structures (Bird and Burgess, 2008).

Some authors consider part of the HF only the HP, the DG and the SUB, while the ENT (consisting of medial entorhinal – MEA – and lateral entorhinal – LEA – areas) is considered a part of the parahippoccampal region, together with the pre- and para-subiculum, the perirhinal (PRh) and the postrhinal cortices (POR) (van Strien et al., 2009).

In Fig. 2 is represented the HP organization in the rat brain (from van Strien et al., 2009).

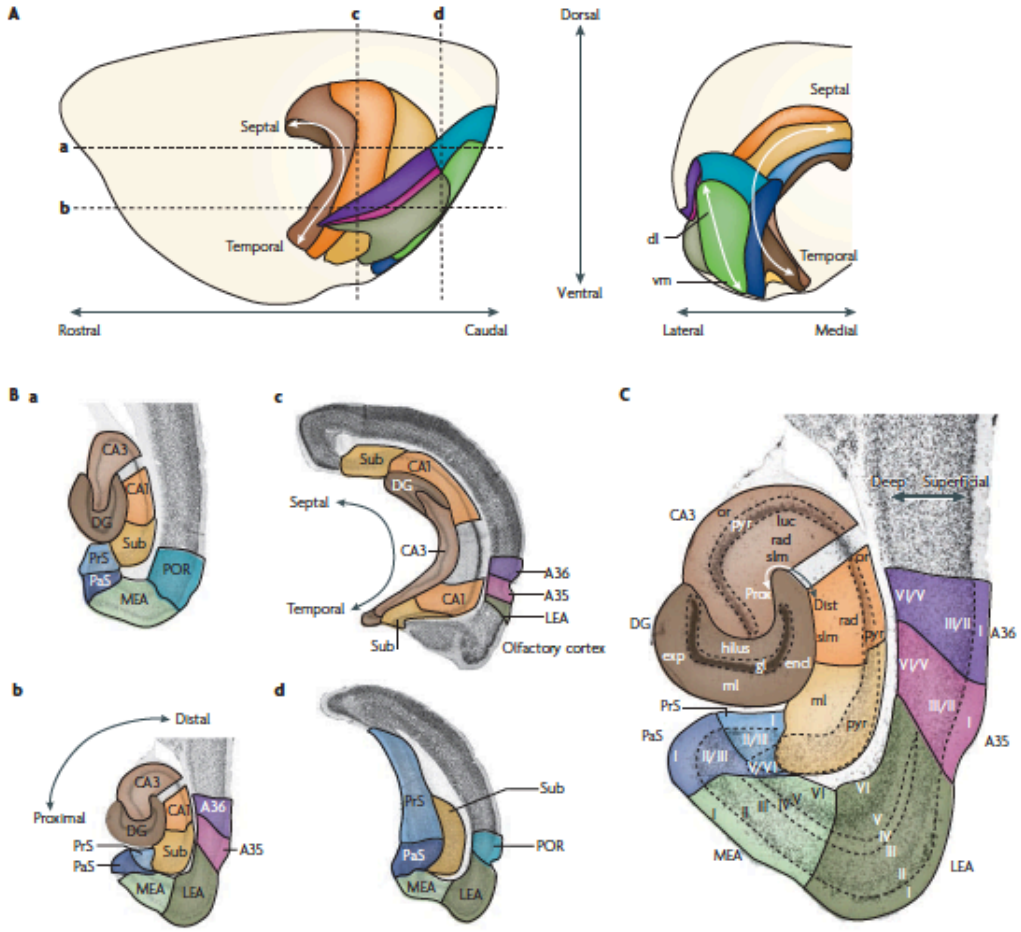

Figure 2. Representations of the hippocampal formation and the parahippocampal region in the rat brain (van Strien et al., 2009).

The cortex of the HF is structured in three layers. The first is a deep layer, called *hylus* in the DG and *stratum oriens* in the CA regions, where a mixture of afferent and efferent fibres and interneurons are included. Superficial to this deep layer, there is the *granule layer* in DG and *pyramidal* layer in the CA regions and the SUB constituted by the principal cells (glutamatergic pyramidal neurons) and the interneurons. The most superficial layer is the *molecular layer* (or *stratum*

moleculare) in DG and SUB, constituted by several sublayers in the CA regions (van Strien et al., 2009). In CA3, three sublayers of the stratum moleculare can be distinguished; the *stratum lucidum*, which receive inputs from the DG; the *stratum radiatum*, where there are the apical dendrites of the pyramidal cell layer; and most superficially the *stratum lacunosum-moleculare*, which comprises the apical tufts of the apical dendrites (van Strien et al., 2009). Similar sublayers can be founded in the CA2 and CA1, but not the *stratum lucidum* (van Strien et al., 2009).

In primates and rodents, the origins of information for the HP include virtually every (so several) neocortical association area. Each of these association area projects to the parahippocampal region, which consists of PRh, POR and ENT (Eichenbaum 2000). In this way parahippocampal regions represent a convergent site of cortical inputs and mediates the distribution of afferents to the HP as illustrated in Figure 3.

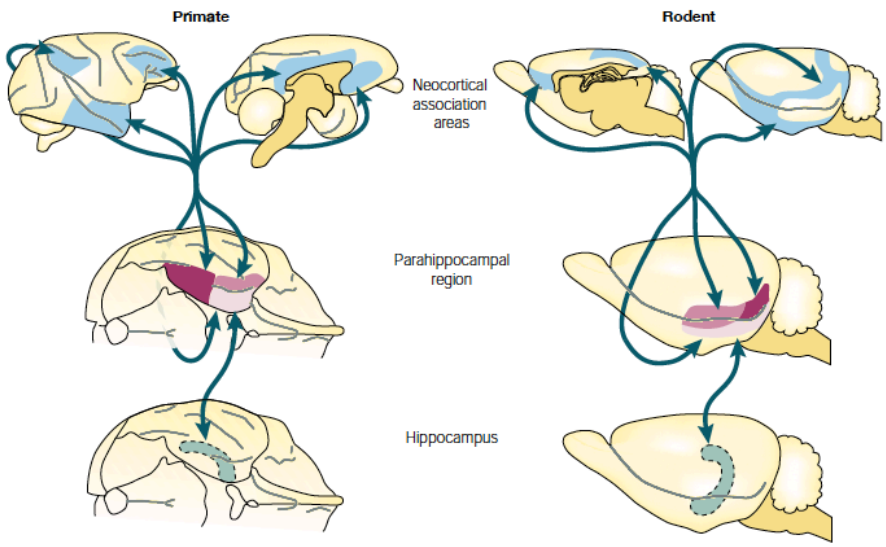

Figure 3. The hippocampus receives and sends back information to the parahippocampal regions, which in turn are connected with every neocortical association area.

The main input to the HP is represented by fibres from the *perforant pathway*, where the EC projections arrive to all subregions of the HF (van Strien et al., 2009).

Typically, the HP is known to have a polysynaptic inner pathway of connection. The first one is represented by the *mossy fibres*, which form unidirectional projections from the DG to the CA3; from here the *Schaffer collaterals*, originating in the CA3, project to the CA1. These connections constitute a well-organized circuit, with the distal part of the CA3 projecting to the proximal CA1, and the proximal CA3 projecting to the distal CA1 (Laurberg and Sorensen, 1981; Ishizuka et al., 1990). The last of these connections is represented by the projections from the CA1 to the SUB, constituting again, a similar circuit of connection as the previous one, with the proximal CA1 projecting to the distal SUB, and the distal CA1 projecting to the proximal SUB (Amaral et al., 1991; Naber et al., 2001).

Beyond the classical dorsal/ventral dichotomy in the hippocampus

A common view is to divide the hippocampus in a dorsal part, more involved in cognitive functions; and a ventral part, more involved in emotional responses. This dorsal-ventral dichotomy born from observations of the well-segregate inputs to the hippocampus (Strange et al., 2014).

However recent evidence suggests that is better to speak of a gradient-like organization (Strange et al., 2014). In rodents, a dorso-lateral to ventro-medial gradient of connection from the ENT targets the HP in its dorso-ventral axis (Fig. 3) (Witter et al. 2000). Recently it was also observed that there is a different pattern of gene expression between the dorsal and the ventral HP, likely associated to different neuronal function (Flore et al., 2016).

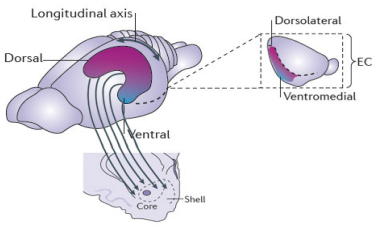

Figure 3. Representation of the topographical organization of EC-HP and HP-NAc connection (adapted from Strange

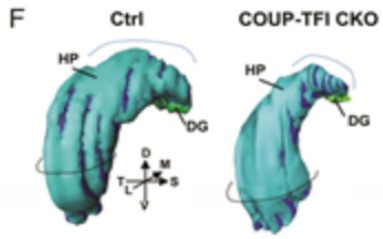

Figure 4. 3D reconstructions of the left hippocampal hemisphere in control and *COUP-TFI* CKO highlight the poor development of the dorsal mutant portion compared with the ventral one, which is more similar to controls. (Flore et al., 2016)

Indeed, it was demonstrated in male mice that genetic developmental removal of the

transcription factor COUP-TFI leads to a dramatic reduction in the volume of the dorsal HP, leaving almost intact the development of the ventral HP (Fig. 4).

GENERAL MEMORY CLASSIFICATION

The "what and how" of memory

Nowadays, the most common classification in literature distinguishes *declarative memory* from *procedural memory* (Fig. 5). This distinction originates from clinical observations showing that there were two types of memory that could be specifically affected in different clinical cases in humans (Squire, 2004; Tsien, 2006).

Declarative memory, also termed "explicit" memory, is represented by places, events, facts and people whose retrieval requires conscious or explicit recollection. This type of memory is mainly regulated by the medial temporal lobe system (MTL) and can be further subdivided in two classes: *episodic memory* and *semantic memory*. The first one refers to memory of episodic events that contains "what, when and where" information, while the second one refers to facts and knowledge that we acquire with single or repeated experience (Tsien, 2006).

Nondeclarative memory, also termed "implicit" memory, encloses motor learning skills and mental operations (Tsien, 2006) without conscious access, that are expressed and ameliorated through performance (Squire, 2004), such as skills, habits and associative learning (Kesner, 2007). A typical example is to learn how to ride a bike, a motor skill that we improve with exercise and can last lifelong.

This classification of memory is based, as mentioned above, on clinical evidence, showing that specific memory deficits are associated to the function of specific brain regions.

One of the most famous examples was the case of the patient H.M., who underwent the removal of a large portion of the hippocampus proper (HP) and related MTL cortices, due to a form of drug-resistant epilepsy. H.M. developed a *temporally-graded retrograde amnesia*, losing his memory of past events, with a particular severity for recent events, since he could still remember some events from his remote past. This deficit was specific for declarative memory. Indeed, H.M. could maintain mnemonic ability that we now include in the non-declarative type of memory.

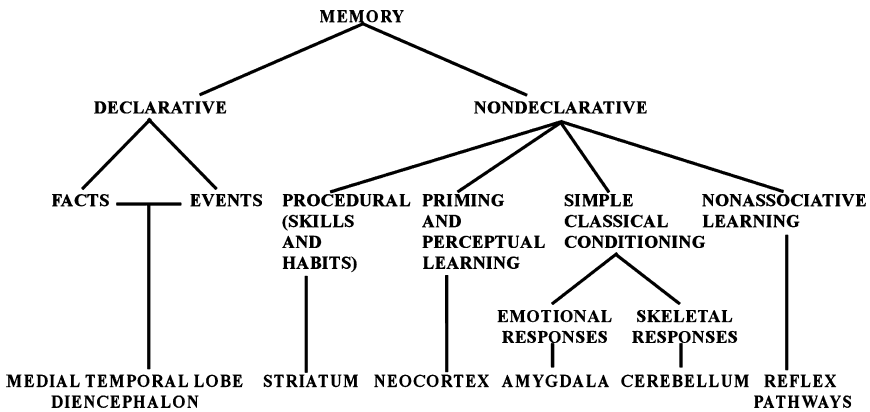

Figure 5. A taxonomy of mammalian long-term memory system (from Squire, 2004).

Short- and long-term memory

Beyond the division between declarative and non-declarative, memory can be classified based on its duration in *short-term memory* (STM), which lasts from seconds to hours, and *long-term memory* (LTM), that goes from days to years or lifelong.

The firsts who tried to include memory in a model of temporal division were Atkinson and Shiffrin in 1968 (Roncato, 1982), who claimed that there are three kinds of memory: *sensory memory*, STM and LTM. They posed that the first mnemonic information that we acquire are the sensorial ones, and these are retained in the so-called sensory memory, where information is processed in few seconds. Then, information is transferred to the STM storage. This is a limited storage (as described below) where information is temporarily stored for seconds to few

minutes. Finally, information can go or not to the LTM, which is a potentially unlimited storage where information can be stored for longer or endless periods (lifelong).

Accumulating evidence (McCarthy and Warrington, 1992; Atkinson and Shiffrin, 1968) demonstrated that STM and LTM can be selectively impaired in human neurological disorders and in animal models studies, thus suggesting that these two storage systems might also work in parallel. Izquierdo and colleagues in the 90s, showed that different treatments with specific molecular actions given into the HP, entorhinal or parietal cortex, immediately after one-trial avoidance training, were able to effectively block STM (1.5 h after learning) without affecting LTM (24 h after learning) formation. This evidence suggests that there are different neurobiological mechanisms for STM and LTM.

MEMORY CAPACITY

The term memory capacity (MC), or *memory span*, refers to the number of elements that can be actively held in memory for a short time interval. Typically, in this brief period, the amount of information retained is limited and this is a typical feature of short-term memory (STM).

The STM limited capacity was discussed already at the end of the 19th century when Joseph Jacobs observed that some subjects, after asking them to write sequences of numbers that he read out loud, remembered an average of seven or eight numbers (from McCarthy & Warrington, 1992). After this first observation, George Miller published in 1956 the article "*The Magical Number Seven, Plus or Minus Two: Some Limits on Our Capacity for Processing Information*".

In this pivotal article, Miller estimated that the number of elements that human mind can retain in STM is 7 ± 2. This indication remained valid for a long time, until an article was published (Cowan, 2001) that reassessed what Miller published indicating that the actual memory span for humans is 4 elements.

A common and simply method to study MC is to present to subjects a list of stimuli of different nature (letters, numbers, words, objects, etc.) and ask them to repeat the list after a short time period. It is important to underline that when talking about a limited number of elements that can be held in memory, the term "elements" refers to the groupings (or *chunks*) that the subject uses, which are the unitary representations of the stimuli. This process is inevitably influenced by the individuals' own knowledge, so by their long-term memory (LTM).

For example, if it is asked to remember the list of letters "H", "I", "P", "P", "O", "C", "A", "M", "P", "U", "S", the actual memory load will be of one because our brain associate this list of letters with the word "hippocampus", thus this is used by our memory as one element to remember. On the other hand, when the letters of a list are not easily associable in a single unit, the memory load will be higher than

before: for example, with the sequence "H", "N", "P", "K", "L", "T", "I", even if the number of letters is less than the previous example, the memory load is higher.
As mentioned before, the average MC of humans was estimated to be around 4 ± 2 elements (Cowan, 2001). Indeed, in a visual memory capacity study on humans, Luck and Vogel (1997) observed that the performance was optimal if the set of colored objects was composed from 1 to 3, while it declined systematically with a set of objects from 4 to 12 (Fig. 6) (Luck & Vogel, 1997).

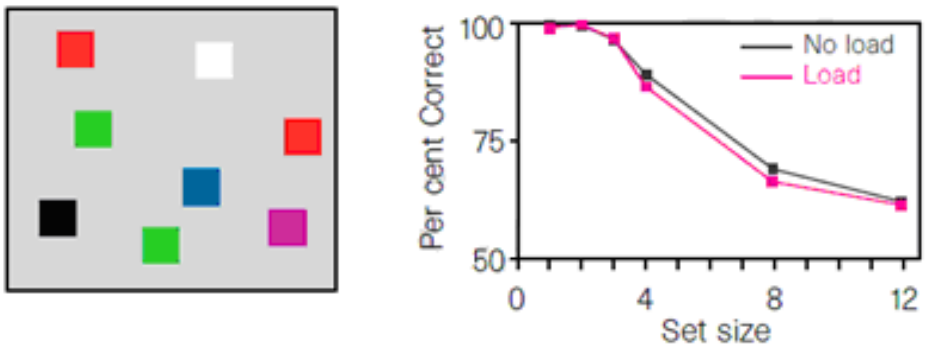

Figure 6. The results of the visual memory capacity experiment by Luck and Vogel (1997).

In this experiment, to exclude any bias such as the concomitant use of verbal and visual material, the subjects were asked to repeat out loud two elements that were presented; in this way, the performance of the subjects did not change (as indicated in Fig 15 by pink curve) indicating that the visual memory capacity in the task was not influenced by the verbal memory.
Using the described experimental protocol, Luria, Solokov and Klimkowski (1967) reported the case of two patients who showed a selective defect in their ability to repeat lists of numbers, words and syllables orally (Luria et al., 1967; McCarthy & Warrington, 1992). These patients were only able to repeat one element of a verbal list. However, when the same list was also visually presented (concomitant use of verbal and visual material), they were not impaired showing also no LTM deficits.

These findings support the idea that memory span or capacity can be impaired in conditions of normal working memory (ability to remember one or few elements) and that these deficits are often material-specific. Indeed, patients who show a reduced memory span for one type of stimulus (for example, auditory) do not necessarily have deficits with other types of material, such as visually presented stimuli (Luria et al., 1967; McCarthy & Warrington, 1992; Shallice & Warrington, 1970).

Methods for studying short-term memory and memory capacity

In humans, STM tasks start with the presentation of a stimulus to the subjects (such as a list of letters or numbers, a series of objects, etc.) for a few milliseconds, preceded by task-related instructions (such as to read the list of letters or to look at the set of objects). Once stimulus is presented, a retention interval of a few milliseconds follows, at the end of which the subject is asked to repeat the list presented before; a variant of this task might imply the presentation of a novel stimulus that must be recognized as different from previous ones. The subjects must, therefore, recollect/recognize the list of items or identify the new element. The *Digit Span Task* is probably the most famous behavioral task used in humans to evaluate memory span. It is also widely used for the identification of mild-cognitive impairment or memory deficits in Alzheimer's disease (Cherry, 2002; Kensinger, 2003). Although the original version of this task was verbally administered, recent versions are generally administered *via* computer. On each trial, participants are presented with a series of digits appearing sequentially on a computer screen (e.g., 3, 4, 1, 2, 7, 8). The task has two variants: *forward-span* and *backward-span*. In the forward-span variant, at the end of each list, participants attempt to recall the digits in the same order they appeared by typing them *via* keypress. In the backward-span variant, at the end of each list, participants attempt to recall the digits in the reverse order as they appeared. For both variants of the task, after each successfully completed trial, the number of digits presented increases by one for the next trial.

After a failed trial (if any digits are missing and/or if the exact order of digits is wrong), the number of digits presented remains the same for the next trial. The task ends after participants make errors for two consecutive trials for a given digit span. The dependent measure, digit span, is the maximum number of digits correctly recalled.

The study of memory capacity in animal models

We will refer here to STM without distinguishing it from working memory, as the two processes are difficult to distinguish in rodents. To study animals STM, different experimental procedures designed for both primates and rodents are used and inspired by those used for humans. In this study we aimed at exploring sex differences on MC using a mouse model; to this aim it is worth focusing on the main procedures used in rodents to study MC.

In general, most of the behavioral procedures used to study memory in rodents do not take into account the amount of information as a factor, but only the "duration" of the retention interval between the study/training and the testing phase. In the 1970s David Olton and Werner Honig, to study short-term visual memory, used the eight-arm radial maze (*Eight Arm Radial Maze* - Fig. 7), a task that was classically used to evaluate LTM in rodents (Dudchenko, 2004).

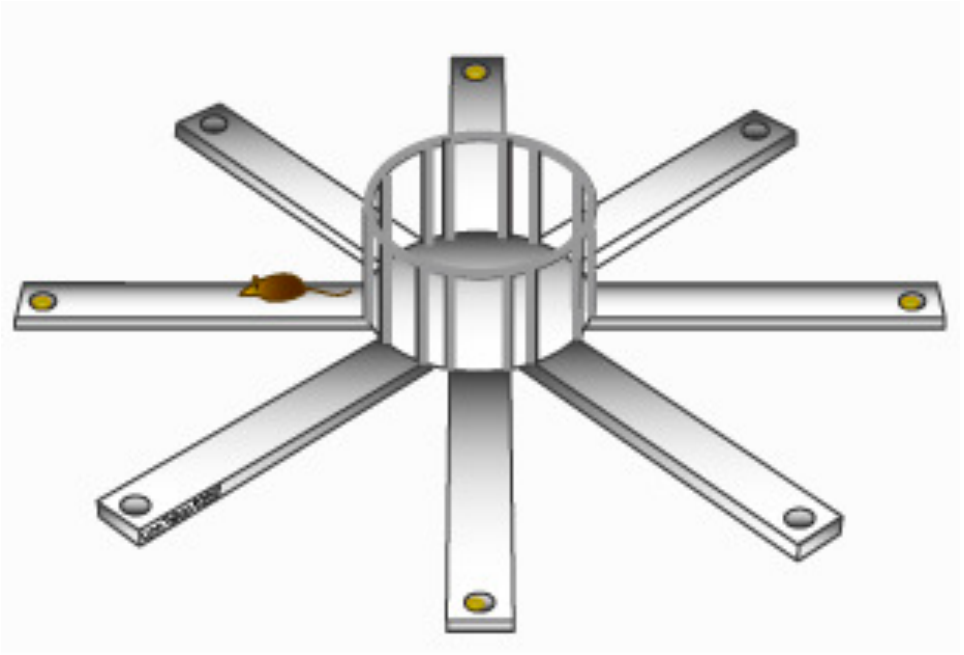

Figure 7. Representation of a rodent while performing an eight-arm radial maze.

For this task, the maze is made up of eight arms at the end of which a reinforcement (a food pellet) is placed. The animals are placed in the center of the apparatus and allowed to freely explore the maze. Food-deprived animals quickly learn to enter food-baited arms to retrieve the reward and to avoid re-entering into the same arms during the same trial. In this way, it was estimated that rats are able to make an average of 7 correct entries (entering baited arms) in a row before making an error (re-entering the same arm) (Dudchenko, 2004; Olton & Samuelson, 1976).

This task requires the ability to manage a set of information in memory during a single session. To be able to enter an arm containing food, animals must keep active the memory of the visited arms and the amount of information to remember increases as the animal visits each arm. Therefore, the eight-armed radial maze can be used to evaluate spatial memory capacity in rodents.

However, previous findings showed that animals can solve the spatial maze task by using a sequential exploration strategy (enter the arm on your right/left), which would not allow to evaluate MC. To study MC, in our laboratory we have modified the classical version of the eight-arm radial maze task, by manipulating the number of open/baited arms: 3, 6 and 9. We found that C57/BL6 male mice have a spatial

memory span or about 6 arms and do not use sequential strategies to solve it (Olivito et al, 2014).

Another type of task used to evaluate STM in rats is the *Delayed non-matching to sample task*, in which the animal has to remember a stimulus for a given interval of time when the stimulus is not present. After the retention interval, the familiar stimulus plus a new one is presented to the subject; the animal receives a reward when it recognizes the new stimulus (Dudchenko, 2004).

In the opposite version of the task, called *Delayed matched to sample*, the animal is rewarded when it selects the familiar stimulus. These two tasks can be performed using either objects or odors.

At this point, it is useful to describe also a modified version of the *Delayed non-matching to sample task,* used to evaluate memory span in primates, called *Delayed Recognition Span Task* (Beason-Held et al., 1999; Fig. 8). The task is similar to the *delayed non-matching to sample*, but in this case the number of stimuli is progressively increased. There are two versions of the task: the *spatial version* in which the stimuli consist of identical brown disks and the animal was presented with one baited disk placed in a predesignated position and it was required to displace the disk to obtain the reward. The addition of successive novel baited disks continued until the animal committed the first error, thus concluding the ‘‘recognition span.’’; and the *color* and *object version,* that differs from the spatial version, first, because

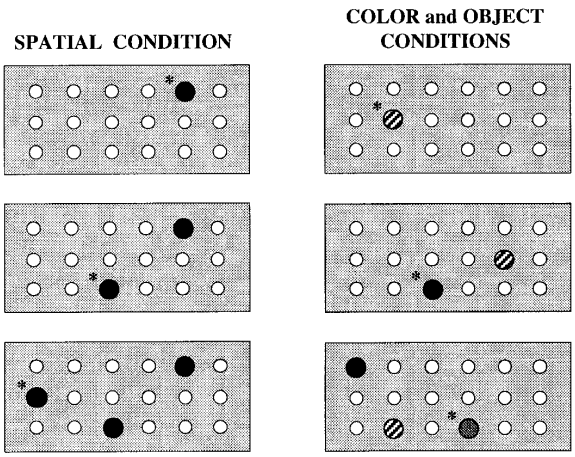

Figure 8. Sample of *Delayed Recognition Span Task* trial sequences. Each trial consists of a series of presentations. In the Spatial condition, a disk is added in a novel position for each presentation. The animal is required to displace the novel disk in each presentation. The span ends when the animal commits the first error. The Color and Object conditions require the animal to displace the novel stimulus in each presentation, but all previously presented stimuli are moved to new positions to preclude the use of spatial cues (shadings represent the different colored disks or objects). Asterisks denote the correct response in each of three sequential presentations. (Beason-Held et al, 1999).

stimuli consist of 15 different colored disks for the color condition and trial unique objects for the object condition and, second, previously presented stimuli are moved to new locations when each new stimulus is added to preclude the use of spatial cues. In this case, the memory span is determined by observing how many objects the subject can discriminate.

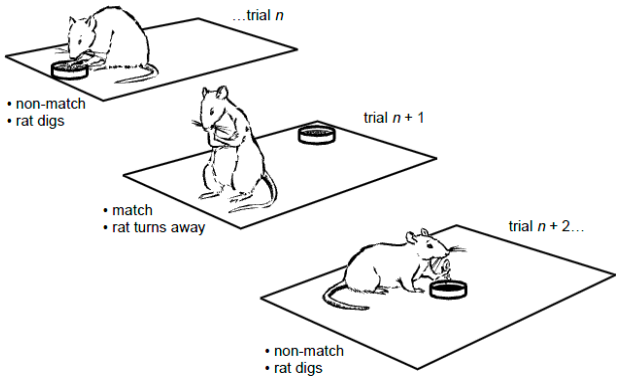

Figure 9. *Continuous non-matching to sample task.* Trial n represents a nonmatch trial where the odour differs from that presented on the previous trial, and the rat digs to find a buried reward. On the next trial (n + 1), the same odor is repeated, although in a different location. As no reward is available, animals learn not to dig on these match trials and to turn away from the cup. On the subsequent trial (n + 2), the odor again differs from that of the previous trial and the animal digs for a buried reward. Note that the position of the cup is independent of the match/non-match contingency (Wood et al, 1999)

The *Delayed non-matching to sample* task has been used on rats even with odors as stimuli (Fig. 9). The first to propose this version of the task were Tim Otto and Howard Eichenbaum (1992), who developed a procedure for studying odors, in which animals were rewarded when they chose an odor different from that presented in the previous session (Otto & Eichenbaum, 1992). Again, the performance of the animals in this task is time-dependent; however, compared to procedures using spatial or visual-tactile stimuli, this procedure is acquired by rats much more quickly (Otto & Eichenbaum, 1992). A modified version of this task has been used to evaluate odor span in rodents. In the *Odor Span Task* (Wood, Dudchenko, & Eichenbaum, 1999; Fig. 10) rats were trained to dig into plates filled with scented sand. Plates with different odors were presented one by one in sequential sessions. Animals learned that only novel odor were baited (Fig. 10). The task was modified so that animals could solve it using spatial information instead of odors.

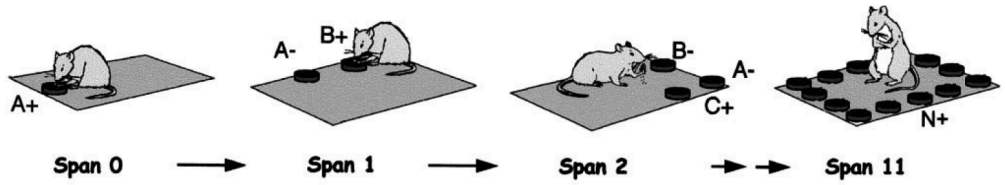

Figure 10. Schematic of the *odor span task*. Animals are first presented with a cup of sand scented with a specific odor (e.g., A). After digging in the sand and retrieving a buried reward, the animal is removed from the platform, and a second cup of sand, scented with a different odor (B) is added. The animal's task on being returned to the platform is to remember odor A (-) and dig at the different odor B (+). Additional cups of sand, scented with different odors, are presented in the same manner. (Dudchenko et al, 2000).

Animals are first presented with a scented plate with a specific odor (in the figure represented as A); after digging and finding the reinforcement the rat is moved, and a second scented plate with a new odor (B) is placed on the experimental apparatus. The number of scented plates is thus gradually increased. In the figure the signs "+" and "-" indicate respectively a new odor, in which the rat finds the reinforcement, and an already known odor, in which there is no reinforcement. For each rat the memory span was represented by the number of consecutive correct choice made before making an error. Using this task, the authors observed that animals maintained a good performance up to 24 different odors.

One of the difficulties in measuring MC in rodents comes from the necessity to avoid that animals use stimulus-associating strategies decreasing the effective load. In addition, the current methods for studying MC use procedures based on repeated exposure to a series of stimuli, rather than on the comparison of a single exposure session to the stimulus set and a test. Repeated exposure is necessary in these tasks in order to let the animals learn the task rule, such as learning the non-matching to sample rule.

To allow the animals to master the task requires many training sessions, which might recruit additional learning mechanisms interfering with the active processing of information. For this reason, in order to measure the number of elements that can be actively maintained in memory, behavioral procedures involving single-stimulus

exposure should be used with a subsequent test phase as it generally happens for procedures used in human studies.

Another big disadvantage of the procedures listed above is that it is necessary to motivate animals to seek reinforcement and, in general, the induction of motivation is done through food restriction procedures, which can introduce variables that potentially interfere with the animals' behavior. This aspect is particularly relevant in the context of this thesis as food deprivation might interact with hormonal factors (beyond the estrogens one that will be discussed in the next paragraphs) in an unpredictable manner. Indeed, starvation has been reported to activate the HPA axis with consequent rise of circulating glucocorticoid concentrations (Schwartz and Seeley, 1997).

In humans, learning is essentially motivated by curiosity, an autonomous motivational boost that spontaneously directs the cognitive activity to the acquisition of new knowledge and gives place to what is defined as *spontaneous* or *incidental learning*. However, this spontaneous learning ability driven by curiosity for novelty is also present in rodents. The *spontaneous object recognition task* is one of the most used behavioral procedures in rodents used to study spontaneous learning in rodents. Introduced for the first time by A. Ennaceur and J. Delacour (1988), this task showed for the first time that animals are motivated by curiosity to explore novel objects, and that, through this exploratory activity, they acquire information related to the objects. The task is generally based on a habituation/dishabituation paradigm conducted in three sessions: a habituation phase, a study phase and a test phase. The first session is used to familiarize the animals to the experimental context, which is usually an empty arena. In the study phase, two identical novel objects are presented and the animal is left to freely explore them for a certain amount of time. Finally, after a retention interval, which can be variable in order to evaluate the STM or LTM, a familiar object is presented along with a completely new one (Fig. 11).

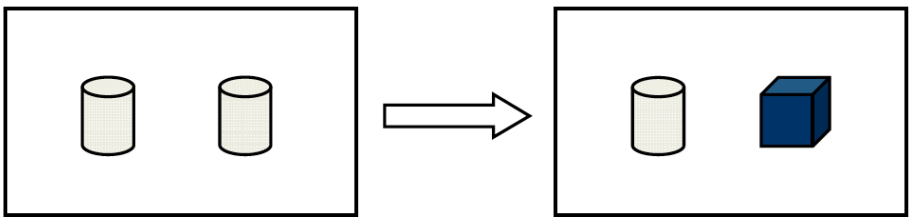

Figure 11. Schematic representation of study and test phase of *spontaneous object recognition task*.

It is generally observed that the new object is explored significantly more than the familiar one (Antunes and Biala, 2012; Ennaceur and Delacour, 1988). Moreover, presenting two familiar objects instead of a new one in the test phase, there is a significant reduction in object exploration compared to the study phase (Antunes and Biala, 2012; Poucet, 1989). The reduction in exploration after repeated exposure to the object indicates that the animal has lost interest on it, and therefore implies that it recognized the characteristics of familiarity (Ennaceur and Delacour, 1988; Ennaceur, 2010; Poucet, 1989). At the same time, the re-newed interest for the novel object presented during the test phase, indicates that the lack of exploration of the familiar objects is not due to a general animal fatigue or to a loss of interest for the stimuli in general, but it is due to the specific characteristics of the new object, which is new in shape, color, size (Antunes and Biala, 2012) attracting the animal interest. This task, for its high face, construct and predictive validity, is one of the most widely used to study the neurobiological basis of object memory in rodents (Antunes and Biala, 2012; Ennaceur, 2010).

Ennaceur and Delacour, indeed, showed that, as typically observed also in other memory tasks (as the previous ones described above), performance is retention interval-dependent (Ennaceur and Delacour, 1988). Finally, another big advantage of this task is that, unlike others, it is not necessary to repeat the training but, as a single exposure to the objects, is sufficient for spontaneous learning. The classical version of the spontaneous object recognition task has a very low memory load, 1 or maximum 2 different objects to remember.

In our lab we have modified the procedure in the *Different/Identical Object Recognition Task* (DOT/IOT; Fig. 12) to use for the study of object memory span in rodents (Sannino et al., 2012).

To manipulate the amount of information to be learn in the DOT, animals are exposed to 3, 4, 6 and up to 9 different objects during the study phase. The control task is represented by the IOT, where we have exposed the animals to 3, 6 or 9 identical objects, which control for variables related to objects configuration in the arena and/or motivational factors without increasing the memory load. Using this task, it has been shown that male mice have an object memory span of 6 different objects (Sannino et al, 2012; Olivito et al, 2014).

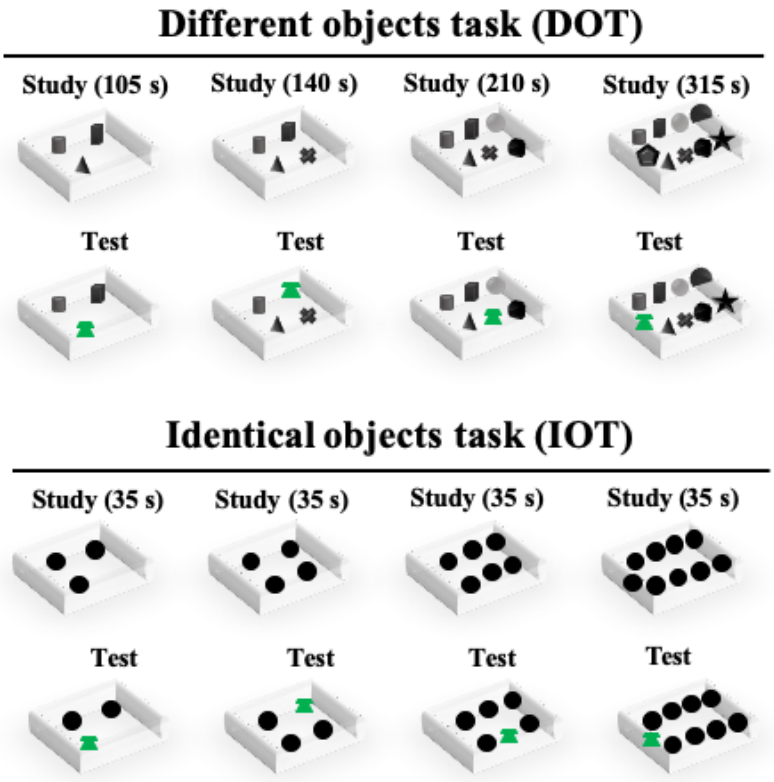

Figure 12: Schematic representation of the *Different/Identical Object Recognition Task* (DOT/IOT; adapted from Sannino et al., 2012).

Role of the hippocampus in memory capacity

The hippocampus (HP) and more in general the medial temporal lobe (MTL), is involved in LTM. Its role in STM has been largely debated in the literature. The general accepted view is that the HP is involved in STM or working memory for spatial information, but not for item memory, such as memory for objects and odors where short-term information processing is classically associated with the frontal lobe, in particular the prefrontal cortex (PFC), a brain structure involved in planning and behavior control (Zigmond et al., 2001).

However, recent evidence has shown that STM deficits may also be related to MTL lesions, but only in particular conditions. Findings in humans, show that patients with MLT lesions have reduced olfactory and visual memory span as compared to control subjects (Levy et al., 2003, 2004).

An interesting experiment conducted in 2008 by the group of Larry Squire and colleagues shows that patients with MTL lesion show the same performance as healthy controls in the repetition of a list of 3 words after a retention interval of 14 s or in the discrimination of the position of 3 objects; however, they are not able to solve the task when they have to remember the list for more than 14 s and when they have to discriminate the position of 6 objects (Shrager et al., 2008; Fig. 13), meaning that MTL lesioned patients show a delay-dependent impairment in MC.

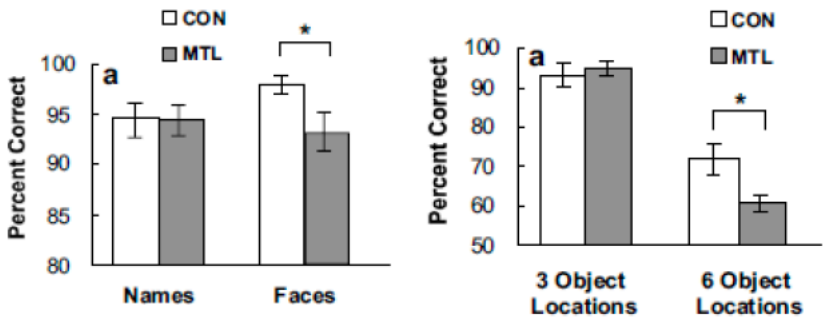

Figure. 13 Results of the experiment performed by Squire and colleagues in 2008 to evaluate the role of the HP in STM. **a (left).** Controls (CON) and patients with medial temporal lobe damage (MTL) tried to remember three names for 14 s or a single face for 14 s. **a (right).** Controls (CON) and

patients with medial temporal lobe damage (MTL) tried to remember three object locations or six object locations for 8 s (16 trials per condition). (Shrager et al., 2008).

Another study indicates that the performance of patients with MTL injury may depend on the type of material presented, and not just on the retention interval (McCarthy & Warrington, 1992). However, in a similar set of studies Lewy et al. (2003, 2004) showed that patients with MTL lesion show reduced memory span for designs and pictures, confirming that, in condition of high memory load, the hippocampus is recruited to solve STM task independently of the type of information to be processed.

Human lesion studies do not allow to specifically dissect the role of the hippocampus. Animal models studies about the hippocampal function in memory capacity produced conflicting results.

A study conducted by Beason-Held and colleagues (1999) showed that hippocampal lesioned primates are not able to solve the *Delayed Recognition Span Task* (described in the previous paragraph). The memory span was measured on three different visual, spatial, color and object modes and, in all cases, the authors found reduced memory span in animals with hippocampal lesion as compared to their relative intact controls. On the contrary, a study performed by Murray and Mishkin (1998) showed that primates with hippocampal and amygdala lesions are able to recognize up to 40 objects in a memory span task, while they are not able to perform the same task in a spatial version, in which they must recognize the position of a stimulus instead of a new stimulus. The main difference between the two studies is that while in the first study animals were trained in the task only after the lesion, in the second study animals were trained before surgery. Therefore, these contradictory results might be due to the fact that the hippocampus has a role in the acquisition, but not in the maintenance, of the task rule. Alternatively, the pre-lesion training might have made most of the components of the task familiar, therefore lowering the memory load as compared to post-lesion condition.

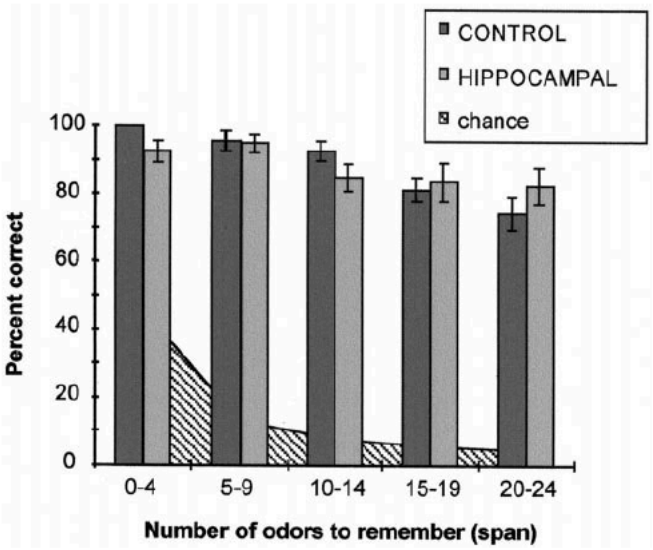

Figure 14. Hippocampal lesioned and control rats' performance in the *Odor Span Task.* Percentage of correct responses for the control and hippocampal groups in the 25-odor span probe session. Spans (number of odors to be remembered) are shown in blocks of five. (Dudchenko et al 2000)

In rodents there are few experimental works in the literature that investigate the role of the HP in MC. Indeed, Dudchenko and colleagues in 2000, showed that rats with hippocampal lesions are able to solve the *Odor Span Task* (described in the previous paragraph) also in the presence of 24 odors (Fig. 14). In the same study the authors found an effect of the lesion only at long retention intervals and in the spatial version of the task. Therefore, they concluded that the hippocampus is not involved in olfactory MC at very short retention intervals (such as 20 s), while it is necessary for the same task for longer retention interval or in its spatial version.

In both Murray and Mishkin's study on primates and in that of Dudchenko and colleagues on rats, the stimuli (objects or odors) of the behavioral procedure for the study of the *memory span*, are presented one by one in consecutive sessions, spaced by a standard time. Therefore, the stimuli presented during the initial sessions are present in the arena until the last session, which is sometimes delayed from the first by several minutes (for example, in Murray and Mishkin's experiment was about 40 minutes).

As described in the previous paragraphs, with this kind of behavioral procedures it is likely that the repeated exposure to the same stimulus may bring into different mechanisms from those typical of STM capacity: the fact that some stimuli are

presented several times and for a longer time than others make them “familiar”, and their recognition may depend on other brain structures, such as the perirhinal cortex. A recent study performed in our laboratory with the *Different/Identical Object Recognition Task* procedure, showed that selective lesions of the dorsal HP in mice cause a deficit in the new object discrimination ability only in condition of high memory load (Fig. 15), reducing the object memory span from 6 to 4 objects (Sannino et al., 2012).

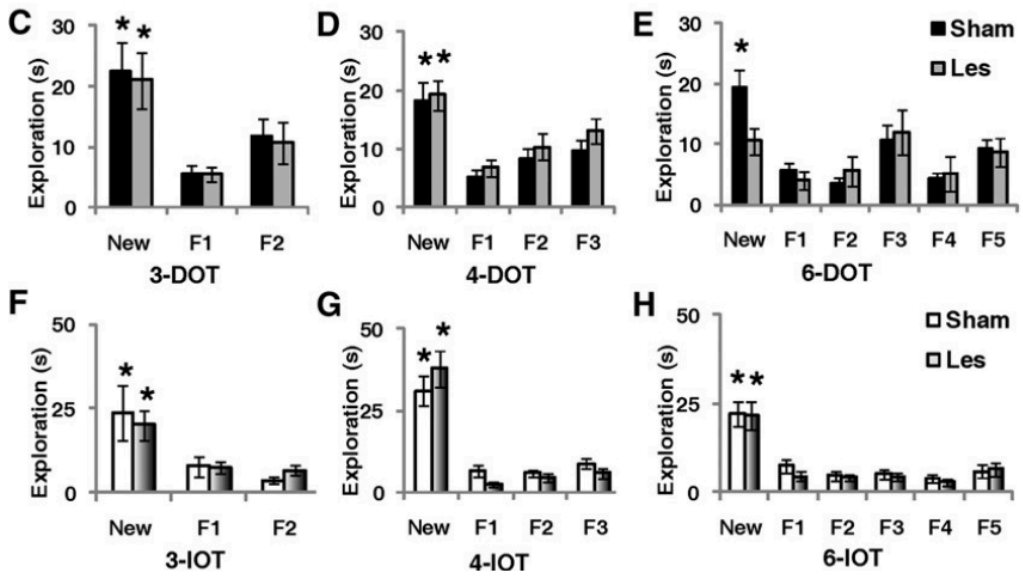

Figure 15. Effect of the hippocampal lesion on the discrimination ability with an increasing number of different (3, 4, 6) or identical (control condition) objects (Sannino et al., 2012). Animal with hippocampal damage (Les) are not able to solve the task with 6 different objects (high memory load condition) with a retention interval of 1 min compared to sham (control) group (**E)**. Since the task is solved correctly by both groups with lower memory load conditions (**C**, **D**, **F**, **G**, **H)**, this suggested that the HP is recruited in object STM in high memory load conditions.

Remarkably, with the introduction of DOT/IOT, it has been observed that the HP, generally recruited in LTM, it is also necessary for object STM in condition of high load.

Memory formation

According to Josselyn et al., 2015 (Fig. 16), the present view about how memory is formed, states that memory is a process that involves strong connections between collections of neurons (neuronal ensemble) that are active during an experience.

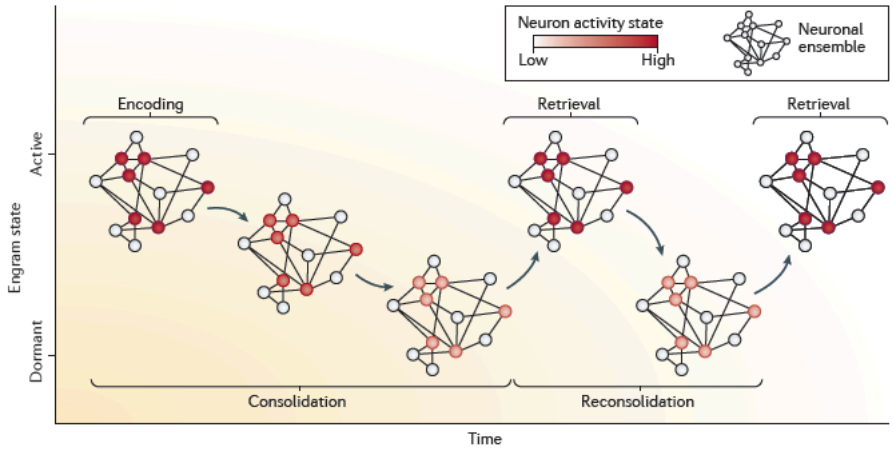

Figure 16. A model of memory formation showing the different phases of memory formation (Josselyn et al, 2015).

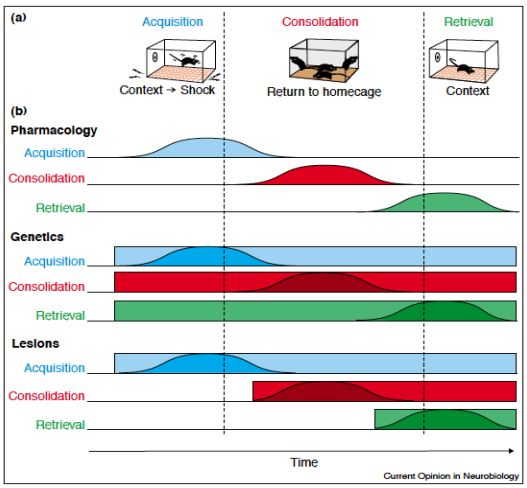

Figure 17. Illustration of the temporal selectivity allowed by different experimental approaches for studying different phase of memory in animal models (from Abel and Lattal, 2001).

According to this view, memory formation can be divided in three main temporal stages: *acquisition* (learning), *consolidation* and *retrieval*. The use of drugs, genetic manipulation and/or lesion approaches helped defining the brain circuits and the molecular processes underlying these three memory stages (Abel & Lattal, 2001). As shown in Fig. 17, the use of drugs is one of the most selective way to specifically interfere with a single phase because they can modulate a relatively short time window. One of the characteristics

of the acquisition/memorization phase is that the newly learned information is sensitive to disruption. This well-accepted concept was explored experimentally by Duncan in 1945, who showed that administration of electroshock to rats immediately after training in a maze was able to induce amnesia compared to controls (Duncan, 1945).

Subsequently, McGaugh and colleagues further confirmed the labile-state of new learned information by showing that electroshock treatments produce retrograde amnesia in rats exclusively when administered immediately after training in a one-trial inhibitory learning task (passive avoidance). The same treatment administered 2 h after training did not have major effects. This is one of the very first experimental evidence suggesting that memories are initially labile (new learned information) and sensitive to proactive interference and gradually they converted in a more stable state, through a process termed memory consolidation.

Memory consolidation

The phase and the processes through which memory become stable is commonly called memory consolidation.

As Dudai (2004) wrote "in the domain of memory research and theory, consolidation or memory consolidation, refers to the progressive post-acquisition stabilization of long-term memory, as well as to the memory phase(s) during which such presumed stabilization takes place".

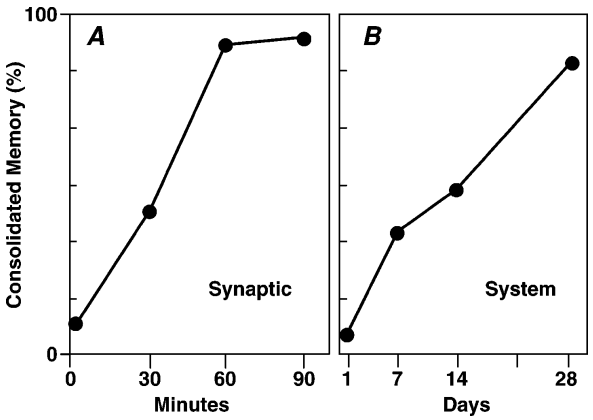

Figure 18. Types of consolidation (from Dudai 2004), in which consolidation is measured based on the time-dependent resistance to protein synthesis inhibitors. In A) data show different sensitivity of memory to protein inhibition. In B) data show time course of system consolidation, determined by measuring the sensitivity of memory to hippocampal damage and indicate that memory become hippocampal-independent in about one month.

Since in 1900, Muller and Pilzecker observed that if interfering stimuli were presented just after training, they can impair memory retrieval (a phenomenon they termed "retroactive inhibition") and that there is a post-training interval during which information are consolidated in memory (Dudai, 2004). Based on this first evidence many other studies confirmed that memory consolidation requires the activation of several biological processes that take place both during wakefulness and during sleep at multiple levels in the brain (nuclear, synaptic, cellular, and system level) and over a temporal window ranging from seconds to months to years.

Nowadays, memory consolidation refers to two related processes occurring at cellular/synaptic level and at brain system level (Dudai 1996; Dudai and Morris, 2000; Dudai, 2004; Dudai et al., 2015; Fig.18).

Synaptic consolidation

Synaptic consolidation refers to a process accomplished within the first minutes to hours after learning, in which information acquired during learning are stored in LTM.

The current idea is that LTM stabilization requires gene expression and *de novo* protein synthesis that lead to the formation of new dendritic spines. Mechanisms such as the activation of intracellular signaling cascades, post-translational modifications, modulation of gene expression and new protein synthesis have been identified as the main crucial processes through which memory are stabilized in the brain (Lamprecht and Ledoux 2004; Dudai, 2004; Kandel, 2012).

What is now thought about synaptic consolidation is that it depends on activity and connections of neuronal circuits while the subject is generating an internal representation of the stimuli. This idea arose from the studies of Hebb (1949), suggesting that when two neurons are repeatedly active at the same time something happens between them, such that when they are stimulated again later in time and the activity of one leads to activity in the other (reported by Lamprecht and LeDoux, 2004), a process referred as "Hebbian plasticity".

In other words, when two connected cells are simultaneously activated, the strength of their connection increases, providing the basis for memory persistence; this mechanism is thought to be mediated by intracellular signaling cascades activated by the learning experience (Bredt and Nicoll, 2003).

The activation of intracellular signaling cascades are thought to be pivotal for consolidation of short- to long-term memory. It was widely reported and reviewed (by Lamprecht and LeDoux, 2004,) that NMDA (N-methyl-D-aspartate) receptors and also voltage-gated calcium channels (VGCC), mediate the calcium influx at the post-synaptic level, which in turn trigger Hebbian plasticity events.

Calcium influx into post-synaptic cells rapidly increases (from seconds to minutes) synaptic efficiency by AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor phosphorylation (through activation of protein kinases, such as calcium/calmodulin-dependent protein kinases – CAMKs – and protein kinase A and C – PKA, PKC) and, after several minutes , gene transcription is activated for protein synthesis modulating spine morphology and density. In particular the activation of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREBs) and its gene expression modulation has been observed to be crucial for synaptic consolidation (reviewed in Kandel et al., 2012).

System consolidation

With the term *system consolidation,* it is indicated a process that, differently from synaptic consolidation, takes weeks, months or even years to be accomplished, and involves the reorganization of brain circuits that encode memory, possibly based on a transfer of information from one region to another, in particular from the HP to the cortex (Dudai 2004; Dudai et al., 2015). In few words, it is a kind of "macroscopic" consolidation if compared to the synaptic one. Indeed, synaptic consolidation has thought to be a sort of subroutine in system consolidation because the recurrent processes of synaptic consolidation are responsible for the reorganization of brain systems at a higher level (Dudai, 2015).

System consolidation was originally considered to explain clinical evidence showing decreased sensitivity of declarative memory to hippocampal damage over time (Dudai, 2015). Patients with hippocampal damage suffer temporally graded retrograde amnesia, but spared remote memory, while patients with neocortical damage show flat extensive retrograde amnesia (Squire et al., 2004). These findings were replicated in animal lesion and brain activation studies (Squire, 1986; Kim and Fanselow, 1992; Nadel and Moscovitch 1997; Clark et al., 2002; Zigmond et al., 2001; Winocur et al., 2007), suggesting that the hippocacampal formation (HF) has a transitory role in LTM storage, occurring before they are finally transferred to neocortex.

Starting from this evidence, several theories were elaborated about system consolidation.

The first was the *Standard Consolidation Theory* (SCT) (Squire, 1986, 2004), in which it was assumed that the HP is only temporarily required for long-term storage, and that information are then transferred and reorganized in the neocortex in a long-lasting and hippocampal-independent manner (Dudai, 2012).

Since it has been shown through functional neuroimaging studies that the HF is still activated in healthy individuals who are required to remember remote autobiographical memory (Dudai, 2012), Nadel and Moscovitch (1997) proposed the *multiple-trace theory* (MTT), suggesting that episodic information are immediately encoded by the HP and distributed in hippocampal-neocortical circuit to build a coherent representation.

In 2010, there was an update of the MTT: the *trace-transformation theory* (TTT) proposed by Winocur and collegues in 2010 (Winocur et al., 2010, Winocur and Moscovich, 2011). It posits that resulting semantic memories coexist and interact with those circuits in which the episodic information of the original event are retained, and that are still HF-dependent (Winocur et al., 2007).

The last model of system consolidation is the *schema assimilation model* (SAM) that has been proposed by Tse and collegues (2007). According to the SAM, system

consolidation is not always a gradual and slow process and can occur relatively quickly if previous established information are available and new information can be integrated into a “schema” previously created, and those information are still maintained after hippocampal lesion (Tse et al., 2007).

SEX-DIFFERENCES IN MEMORY

Several data have been published on memory and MC, but the majority of these studies only considered male subjects (Beason-Held et al., 1999; Levy et al., 2004; Sannino et al., 2012).

Although gender differences on memory are reported (Pauls at al., 2013; Zilles et al., 2012), there is not an exhaustive comprehension of the mechanisms underlying these differences and, further, there are few information about MC in females for different memory load conditions.

Remarkably, the study of sex differences becomes necessary since women during ageing are more prone to develop dementia compared to men (Alzheimer's Association, 2010).

Sex differences in memory tasks: human studies

It is known that women perform better than men in social cognition tasks, while men are better in motor and spatial skills (reviewed in Andreano and Cahill, 2009). Sex-related connectivity across brain development have been correlated to these behavioural sexual dimorphisms. Indeed, brain networks related to social motivation, attention and memory tasks have been found highly connected in females, while males showed higher connectivity between motor, executive functions and sensory systems and increased segregation of brain structures possibly linked to functional specialization (Tunc et al., 2016).

The hippocampus (HP) and more in general the hippocampal formation mediates learning and memory, with spatial memory as one of the main specializations of this cerebral domain. Many studies elucidated the role of the HP as the key brain structure for spatial navigation and place mapping (Maguire et al., 1998; Burgess, 2014; Lisman et al., 2017; Eichenbaum, 2017).

In spatial navigation there are two main kinds of strategies adopted for learning: the allocentric and the egocentric one, that refers respectively to navigation guided by

external landmarks or by self-motion information. Extensive literature has demonstrated that these two kind of strategies are controlled by different neuronal circuitries, with the hippocampal formation that support allocentric navigation and the frontostriatal system that supports egocentric navigation (Hartley et al., 2003; Iaria et al., 2003; Bohbot et al., 2004; Maguire et al., 1998; reviewed in Chersi and Burgess, 2015).

There are substantial experimental evidence reporting men outperforming women in spatial navigation tasks (Miller and Santoni, 1986; Lawton, 1994; Schmitz, 1997; Dabbs et al., 1998; Astur et al., 2004; Sneider et al., 2015; Korthauer et al., 2017). However, a careful analysis of the results suggests that women and men use different spatial strategy to solve navigational tasks. Women tend to use more landmark information, while men use Euclidean information, which support egocentric and allocentric strategy, respectively.

This difference can be already observed in young children, by the age of 4 years old (Levine et al., 1999). The preference for egocentric strategies in women does not preclude learning of other strategies, since both sexes are still able to perform well when asked to accomplish the task in a non-preferred strategy (Spriggs et al., 2018). All together, these findings suggest that sex-regulated differences exist in cognitive strategies used to solve complex spatial tasks, with women favoring non-hippocampal dependent strategies.

Another field in which men have been found to outperform women are visuo-spatial abilities. Mental rotation tasks are commonly used in human studies to evaluate visuo-spatial abilities and literature confirms higher performance of men compared to women in this concern (reviewed in Andreano and Cahill, 2009). Mental rotation tasks mainly involve the parietal lobe recruitment (Harris et al., 2000), suggesting another functional sex difference. Interestingly, in a mirror-tracing task, although the task is visuo-spatial one, females were found faster than male subjects, with larger involvement of lateral prefrontal cortex and caudate and no association of performance with the HP (Kennedy et al., 2005).

On the contrary, women have better performance in verbal memory and in perceptual-motor skill learning (reviewed in Andreano and Cahill, 2009; but see also Jäncke, 2018; Mehl et al., 2007). These tasks are used to measure executive control functions that require the frontostriatal system engagement (Kennedy et al., 2005; Schwartz and Baldo, 2001; Leisman et al., 2016). These findings suggest that in women there is a lower recruitment of the parietal-hippocampal circuit, which favors men performance in tasks recruiting this neuronal pathway, but women tend to be more performative in tasks requiring the frontostriatal system activation.

Concerning sex-differences in MC few information have been reported.

In 2004, Miller and Bichsel reported that male and female subjects showed no differences in verbal (reading span task) and visual (paper-folding task) MC tasks. However, testing again subjects, after a preliminary basic or applied mathematical performance, men outperform women in both task after the applied mathematical performance, while no gender-differences were found in both tasks after the basic mathematical performance.

Again, in 2005, Geiger and colleagues observed that, testing male and female subjects in verbal and spatial MC tasks, males showed both a larger verbal and spatial MC. Furthermore, analyzing the recall and question data, they found that males comprehended the material presented better than females.

In 2013, Pauls et al., testing male and female subjects of different ages in episodic and visual MC tasks, showed that women tended to outperform men on tasks that required remembering verbally-encoded items, whereas men showed higher level performances on subtests measuring visuo-spatial processing and data remained stable also considering the course of age.

Finally, Lynn in 2008 reviewed the gender differences in the *digit span task*, one of the most used tasks to diagnose some kind of dementia such as the AD. In his review, he reported that children and adolescent female subjects outperform male subjects. However, in adulthood, the data were reverted with males outperforming female subjects.

Sex differences in memory tasks: animal studies

Rodent models' studies support human studies results, showing male subjects outperforming females in spatial processing (reviewed in D'Hooge and Deyn, 2001; McCarthy and Konkle, 2005; Duarte-Guterman et al., 2015), while the opposite is true for conditioning tasks and activity levels (reviewed in Dalla and Shors, 2009; Rosenfeld, 2017).

Opposite results or equal performance between males and females in spatial navigation also exist, again suggesting that the performance may be dependent on the strategy employed (reviewed in McCarthy, 2016). As observed in human studies, also rodent models report that males and females use opposite navigational strategies when the environmental cues allow for that, with male more prone to use an allocentric reference and female an egocentric one, as well reviewed in McCarthy and Konkle, 2005.

As reported above, women successfully solve spatial tasks by using different cognitive strategies; this suggests that female subjects naturally engage different brain circuitries compared to male ones. This is supported by findings in rodents showing that female outperform males in classical and operant conditioning task paradigms (Dawson, 1975; Stenbergen et al., 1990; reviewed in Dalla and Shors, 2009). For example, the percentage of conditioned response in an eye-blink conditioning paradigm has been shown to be higher in female than male rats (Dalla et al., 2009). Moreover, in one- or two-avoidance tasks or lever presses tasks, where subjects have to learn the rule to avoid a shock, females are faster than males (Dalla et al., 2009; Heinsbroek et al., 1983).

Concerning sex-differences on MC in animal models, the majority of studies reported in literature, as intuitable from what reported above, focus their attention on spatial memory tasks.

Jonasson in 2005 reviewed studies investigating the performance of male and female rodents (rats and mice) in spatial memory tasks (Morris water maze and radial maze),

including different rat and mouse strains. He reported that, independently from the strain, male rats outperform females in spatial tasks while, in mice, females outperform males in the water maze but not in the radial maze. He concluded that the data collected in rats were in line with human studies concerning spatial cognition and learning (Voyer et al., 1995; Saucier et al., 2002).

Similar findings were reported in primates where male subjects, in the spatial version of the *Delayed Recognition Span Test*, showed greater scores than female subjects (Lacreuse et al., 2005).

Other sex-related differences in rodents were reported by Ghi and colleagues in 1999, when they tested male and female rats in the classical version of the spontaneous object recognition task in which, in condition of low memory load (animals are exposed only to two objects), they found that female subjects are able to discriminate the new object up to 90 min delay compared to male subjects performance that was good up to 60 min evidencing a better performance in this task in females compared to males.

AGEING

Ageing: a general view

Ageing is a normal and complex biological process characterized by a steady decline in various physiological functions that result in both physical and cognitive deficits (Deary et al., 2009).

Beyond being a natural process, several factors can impact upon the rate and magnitude of ageing which varies greatly between individuals (Deary et al., 2009), such as changes in diet and lifestyle, inflammation, genetics and the presence of confounding illness.

López-Otín et al (2013) proposed nine possible factors contributing to the ageing process and these include features such as genomic instability, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication and epigenetics. Indeed, as individuals age, their genes become increasingly susceptible to damage from endogenous and exogenous sources. Errors in DNA replication, point mutations, reactive oxygen species (ROS) generation and alterations in DNA methylation all contribute to accelerated ageing (Moskalev et al., 2013).

The brain ageing

Ageing is probably the biggest risk factor for the development of neurodegenerative diseases including Alzheimer's disease (AD) and other disorders such as Parkinson's disease.

During normal ageing, various neuroanatomical regions are affected with structural changes that are not identical in all brain regions. For example, in early adulthood, grey matter volume (for instance the prefrontal cortex and the hippocampus), decreases in size (Harada et al., 2013). In fact, the whole brain volume, as estimated in longitudinal studies, decreases in size by 0.2 – 0.5% every year (Fjell and Walhovd, 2010). That being said, white matter changes during ageing are more

prominent than that of grey matter (Harada et al., 2013). While there is a steady increase in structural grey matter alterations from childhood to adulthood, the white matter has significant alterations in adulthood where it reaches a plateau condition and resumes in old age (Madden et al., 2008). The largest volumetric changes recorded include the putamen, the nucleus accumbens, the thalamus and the frontal and temporal cortices (Fjell and Walhovd, 2010).

It is clear, therefore, that structural changes occur in various brain regions across age. The HP, however, is an area of particular interest in ageing and cognition as it governs spatial learning, memory and MC; processes that are intimately linked to cognitive decline during ageing (VanGuilder and Freeman, 2011; Morra et al., 2001; Gilchrist et al., 2008). Hippocampal atrophy is one of the defining anatomical alterations that occur during ageing, and, it is likely due to a reduction in synaptic density and neuronal size (Harada et al., 2013). The HP, as described in detail previously, is made up of several structures namely the *cornu ammonis* areas (CA1-4) which together form what is known as the "hippocampus proper" and along with the dentate gyrus and the subiculum are collectively known as the "hippocampal formation". During normal ageing, neuronal loss has been observed in the dentate gyrus and the subiculum but not in the hippocampus proper (Jagust, 2013).

Several studies suggested that the HP may be the hallmark in the discrimination of "healthy" and "pathological" ageing. Indeed, studies have shown that neuronal loss in the dentate gyrus and subiculum of the hippocampus are due to age-related changes only, while neuronal loss in CA1 and the entorhinal cortex are implicated in neurodegenerative diseases and particularly in Alzheimer's disease (Jagust, 2013). In contrast to this view, others suggested that older adults display decreased volumes of the dentate gyrus and the CA3 region of the hippocampus (Shing et al., 2011) while others have found decreased volume of CA1 in memory impaired patients (Yassa et al., 2010).

Specifically, decreased synaptic density has been observed in the dentate gyrus and the CA3 region of the hippocampus but not in the CA1 region, as evidenced in aged

rats exhibiting a loss of synapses in these regions (Hara and Morrison, 2014). Similarly, in a study performed on aged female monkeys, there were a reduction in the number of presynaptic buttons and thus, fewer connections made in multiple synapses of the dentate gyrus.

Interestingly, the loss of excitatory synapses has been correlated with cognitive disability in aged monkeys, even if the amount of both excitatory and inhibitory synaptic loss during ageing is similar (Petralia et al., 2014).

Moreover, it is known that synaptic release of neurotransmitters modulates memory, executive function, hormone release and motor function and it is, therefore, essential for normal functioning of the central nervous system (Azpurua and Eaton, 2015). During ageing, both excitatory and inhibitory neurotransmitters play a role in the destabilization of the synapses which significantly contributes to cognitive decline. Indeed, changes in the activity of excitatory glutamatergic synapses during ageing, which are heavily implicated in learning and memory, are evidenced by several anatomical and chemical mechanisms.

Key hippocampal proteins are decreased in aged mice such as the glutamate receptors AMPA and NMDA, the presynaptic protein SNAP25 and postsynaptic protein PSD95 (Adlard et al., 2010; Canas et al., 2009). In addition, several markers of synaptic plasticity such as CaMKII (calmodulin-dependent kinase II) and synaptophysin have also been found decreased in aged mice (Adlard et al., 2014; VanGuilder and Freeman, 2011).

On the other hand, inhibitory neurons control overall excitability of neural circuits in order to maintain the homeostatic balance in the brain (Rozycka and Liguz-Lecznar, 2017). During ageing, this control is deficient and therefore contributes to cognitive decline in old age. Indeed, in the HP of aged rats, Stanley et al. (2012) observed a decrease in inhibitory input and reduced GABA release in the entorhinal cortex. Furthermore, these synaptic alterations further contribute to cognitive decline by avoiding the induction of long-term potentiation (LTP) and encouraging long-term depression (LTD; which opposes LTP; Barnes et al., 2000).

Finally, the dysregulation of neuronal synapses observed during ageing are also influenced by age-related epigenetic changes in gene expression.

Ageing individuals undergo changes in histone methylation, acetylation and DNA demethylation which have been reported to modulate the observed age-related changes in synapse loss and structural changes (Azpurua and Eaton, 2015).

However, these changes in the ageing brain may only be partly attributed to neuronal loss, rather, it seems that reductions in spine density and decreased neuron sizes and number of synapses, as showed by Fjell and Walhovd, in 2010, more likely contribute to the volumetric alterations observed during normal ageing.

Indeed, during ageing, neurons radically change their morphology through the decrease in synaptic connections and the number and length of dendritic spines and this has been demonstrated in humans, rats and non-human primates (Morisson and Baxter, 2012).

The effects of ageing on cognition

During ageing, several cognitive domains decline such as reasoning, memory and processing speed (Ballesteros et al., 2009). It is important to note that the rate and degree of cognitive decline varies widely across individuals and there are various reasons to explain this.

Remarkably, individuals may have inherent differences in plasticity based on different life experiences or even due to differing levels of physical activity throughout their lives (Hedden and Gabrieli, 2004). Based on their genetic background and epigenetic influences throughout the lifespan, certain individuals could be more susceptible to cognitive decline.

The effects of ageing on memory are remarkable also in terms of capacity. MC is reduced with ageing (Balota et al., 2000).

Salthouse, Mitchell, Skovronek, and Babcock (1989), indeed, demonstrated that older adults' performance in some working memory tasks (verbal reasoning and spatial visualization) was lower as compared to young subjects.

A meta-analysis study on the effects of ageing on memory span reported that older adults were outperformed by young subjects in the forward- and in the backward-digit span task (Gregoire and Van der Linden, 1997; Myerson et al., 2003; Park et al., 2001).

Studies in rodents confirm the existence of age-dependent cognitive decline.

In the T-maze delayed alternation task, used to evaluate working memory in rats, it has been found that aged rats (24 months old) are impaired compared to young animals (3 months old). In the odor recognition task, 12 and 18 months old rats were found also impaired compared to younger (3 and 6 months old) rats.

Similar deficits were observed in the social recognition task (Prediger et al., 2005).

Notably, in rodents, cognitive decline with ageing were also found using the spontaneous object recognition task (Blalock et al., 2003); specifically, in the hippocampal-dependent version of the task (described by Squire et al., 2000), the *Object-Place Recognition Task*, aged mice resulted impaired when tested with young control mice (Wimmer et al., 2012).

Exercise training and its effect on memory

Exercise training (ET) has been largely observed to induce beneficial effects on animal cognition and physiology (Cotman and Berchtold, 2002).

In 2006, Colcombe and colleagues reported a significant increase in brain volume, both at the level of gray and white matter and primary in the temporal and prefrontal lobe, in older volunteers, after 6 months of randomized controlled trial in which they were submitted to aerobic training as compared to coetaneous controls that underwent stretching and toning trials. This effect was not valid for younger participants, indicating a specific effect on ageing brain health.

The result of the study was very relevant since it suggested a possible easy strategy to prevent brain volume decrease that is naturally associated to ageing (Mouiha and Duchesne, 2011).

Meta-analyses of randomized controlled trials showed how aerobic exercise training, which stands for 20-30 min of aerobic activity, shows good improvement on cognition, specifically on attention, processing speed, memory and executive functions (Smith et al., 2010).

Recent evidence reported in literature showed that aerobic exercise resulted in improvement of mood states, increase in hippocampal volume and prevention of hippocampal volume decrease with ageing (Erickson, 2011; Frodl et al., 2019).

After the discovery of the beneficial effects of ET on brain neurogenesis and plasticity during ageing, many studies attempted to use this strategy to rescue mild cognitive impairment (MCI, an intermediate condition between health ageing and dementia often predictive of future AD development), dementia and AD related symptoms in humans and animal models.

The role of sex was specifically investigated as influencing factor of ET in patients diagnosed with MCI, dementia and AD. In a randomized controlled clinical trial, the effect of high intensity aerobic exercise was compared to that of stretching as a control, in women and men separately (Baker et al., 2010). Data showed more beneficial effects of ET in women compared to men, with improved performance in executive function tests and some positive physiological effects such as reduced insulin, cortisol and Brain Derived Neurotrophic Factor (BDNF), while a non-significant trend to decreased plasma levels of Aβ-42 was found across genders compared to stretching controls (Baker et al., 2010).

However, already in 2003 it was reported the greatest effects of aerobic training on cognition was in women (Colcombe and Kramer, 2003).

Similar effects have been observed on a randomised placebo-controlled trial on MCI patients, showing that 1 year (2 time per week for 1 hour) of moderate intensity walking was sufficient to improve memory in men and improve both memory and attention in women (van Uffelen et al., 2008), confirming again a higher responsivity of women to the beneficial effects of exercise training.

The biological mechanisms through which ET improves cognition and exerts a neuroprotective effect have been largely investigated in animal models. One of the first evidence found was the increase of neurogenesis in the dentate gyrus (DG) of mice, with associated improved hippocampal-dependent cognition and structural plasticity observed as better performance in spatial memory (Morris water maze), potentiated LTP and increased number of dividing cells on the DG (van Praag et al., 1999a; 1999b; Kempermann et al., 1997).

Neurogenesis and potentiated LTP are among the first mechanisms discovered (van Praag et al., 1999) and largely confirmed (Berchtold et al., 2010) to be improved by ET, but they are not the only ones. Increase in brain expression of neurotrophins such as BDNF (Berchtold et al., 2010, reviewed in Liu and Nusslock, 2018), influence on synaptic plasticity genes (Stranahan et al., 2010) and proteins such as synapsin I and synaptophysin (Vaynman et al., 2004, 2006), increase in CREB expression and energy metabolism related proteins (Gomez-Pinilla et al., 2008) have all been observed to be induced by ET.

From a behavioral point of view, some sex-related studies on rodents are reported in literature.

Among them, it has been observed that aged male (18 months old) or female (24 months old) rats (Kim et al., 2010) or mice (Harburger et al., 2007), when tested in the Morris Water Maze after 4-5 weeks of voluntary ET, had an improved performance compared to sedentary (no ET) controls. Meta-analysis by Bahra (2007), showed that, after comparing data of male and female subjects of these studies, the improved cognitive performance did not differ between sexes.

Also in other tasks, such as the spontaneous object recognition task and the object-place recognition task, 20 months old female rats showed an improved cognitive performance compared to their sedentary controls (Siette et al., 2013).

Similar results were also found when male and female aged rodents were tested for conditioned avoidance memory. However, in these non-spatial memory tasks,

voluntary ET had greater cognitive benefits in male compared to female subjects (Adlard et al., 2011).

However, meta-analysis by Bahra (2007) showed that the real sex-related differences in rodents were displayed in some of pathways involved in ET.

Indeed, in the majority of the studies it has been showed that ET rodents had increased BDNF levels compared to their sedentary controls, with greater effects in female (Kim et al., 2010) compared to male subjects (O'Callaghan et al., 2009; Costa et al., 2012).

Even in some AD models it has been found beneficial effects after voluntary AD. 3xTg-AD male and female mice after treadmill exercise, indeed, showed an improved cognitive performance in the Morris Water Maze compared to sedentary controls, with greater results in female than in male subjects (Gimenez-Llort et al., 2010).

Sex-regulated anatomical differences in the hippocampus: human studies

The HP has been observed to consistently differ between male and female in the anatomy, cellular make-up and physiology (reviewed in Madeira and Liebermann, 1995; Cahill, 2006). From *in vivo* imaging studies, normalizing on brain total size, it was observed that HP has a larger volume in women than men (Filipek et al., 1994; Goldstein et al., 2001; Pletzer et al., 2010; Perlaki et al., 2014), a difference that has also been attributed to the posterior HP, found larger in young women than men (Persson et al., 2014). The HP, together with the amygdala (another area of the medial temporal lobe involved in emotional processing and memory), are two brain structures in which androgen and estrogen receptors are abundant (Clark et al., 1988; Morse et al., 1986).

The origin of sex-related differences in brain formation starts in the prenatal and early post-natal period, and they are completely manifested during puberty (Paus et al., 2017; Koolschijn et al., 2014; Lin et al., 2013), two fundamental stages when sexual hormones exert their effect on brain shaping. In accordance, women with

gonadal aplasia have smaller HP volumes correlated with visuo-spatial deficits (Murphy et al., 1993).

Previous studies suggested that the larger volumes of the HP of female subjects, which is a condition extensively demonstrated also in adulthood (Filipek et al., 1994; Goldstein et al., 2001; Perlaki et al., 2014), is related to the lower circulating testosterone levels in females during pubertal age compared to males (Neufang et al., 2009).

Age and sex are both factors that can influence the anatomy and connectivity of brain specific structures. In a recent study of MRI conducted on healthy people ranging from 18 to 69 years old, age-by-sex interactions on hippocampal formation have been investigated (Khurt et al, 2017). A significant effect of age and sex independently was found, with age-associated volume loss starting from 50 years old and females showing larger volumes than males for all the subregions considered, except for the entorhinal cortex. However, no significant effect of age-by-sex interaction was found, indicating an equal amount of atrophy on both sexes with ageing. However, there are also studies reporting no differences for age (Mouiha and Duchesne, 2011) or sex (Tan et al., 2016). A possible reason for these conflicting findings could be the different sampling between studies (for example see Perlaki et al., 2014, Tan et al., 2016), but also the influence of circulating hormones during testing. Interestingly, it has been observed that hippocampal volume and grey matter volume in general, can change as a function of the menstrual cycle in women (Protopopescu et al., 2008; Hagemann et al., 2011).

Sex-dependent differences in brain connectivity that result in differential activity and excitability levels between men and women are also reported. Indeed, young females show higher interhemispheric connection compared to young males, who, on the other hand, show more intrahemispheric connection (Ingalhalikar et al., 2014; Szalkai et al., 2015; Tyan et al., 2017; but see also Joel et al., 2015). It is possible that the increased interregional connectivity in females is linked with their higher HP volume (Tyan et al., 2017).

Overall, data from human imaging studies report bigger volume of the HP and higher interhemispheric connectivity in women compared to men.

Sex-regulated anatomical differences in the hippocampus: animal studies

Animal studies are important in defining sex-regulated anatomical differences as they allow to evaluate also the cellular density and to extrapolate differences in hippocampal subfields (Zhao et al., 2010; Van Praag et al., 2002; reviewed in Balu and Lucki, 2009 and Urban and Guillemot, 2014).

As mentioned previously, the volume of the HP is higher for females than for males in humans. However, it is reported a lower cellular density in females than males in rodents. A possible explanation for this discrepancy is that most of the analyses conducted in rodents are performed *ex vivo* without volumetric measures with functional imaging techniques.

Few studies have investigated sexual dimorphism in the mouse brain using 3D MRI and partially resemble data from humans. In Spring et al. (2007) the total brain volume of mice was found higher in males than females; concerning the HP, when removing the global size difference across sexes, the posterior part was found larger in males than females and the anterior was found larger in females than males.

Studies based on the density of cells have observed that in the granular layer of the DG, male rodents have higher density as compared to females (reviewed in Madeira and Lieberman, 1995). Furthermore, it has been shown that the HP of female rats has smaller density of the pyramidal cells of the CA1 compared to male subjects, but differently from the DG, this difference is consistent across development and adult life (Madeira et al., 1992; reviewed in Madeira and Lieberman, 1995). Concerning the CA3, more controversial results have been reported, with both no differences between sexes (Madeira et al., 1992) or lower volume in females (Isgor and Sengelaub, 1998).

Sex-differences in hippocampal synaptic plasticity

Another main sex-related difference is the one observed in long-term potentiation (LTP) expression.

LTP is a form of Hebbian plasticity mechanism thought to be at the base of memory formation and consolidation (Bliss and Lomo, 1973; Nabavi et al., 2014). It has been observed how females do not express, or at least express a lower LTP compared to males. High-frequency stimulation (HFS) of the perforant pathway in the HP evoked a significant lower excitatory post-synaptic potential (EPSP) slope in female rats compared to males at the age of 35 and 60 days but not at 20 days (Maren et al., 1994), an effect possibly dependent on the developmental differentiation in the structure, which is already established at this age (Wimer et al., 1988; Qui et al., 2018; reviewed in Madeira and Lieberman, 1995). Moreover, HFS induced a robust and 30 min stable hippocampal LTP in males and a rapidly decaying short-term potentiation in females (Maren et al., 1994). In accordance, the magnitude of the NMDA receptors activation, key receptors for LTP induction and maintenance in the HP (reviewed in Luscher and Malenka, 2012), was found correlated to the LTP features in male and females. The smaller and fast-decaying LTP in the HP of female subjects was also paralleled by a LTM deficit (24 h) compared to male subjects in the contextual fear condition (Maren et al., 1994), a behavioral paradigm well known to be dependent on hippocampal functionality. It is of note that although females in the cited study acquired the conditioning response, its magnitude was higher in males than females and this effect was specific for hippocampal-dependent fear conditioning (Maren et al., 1994).

ESTROGENS

A focus on the estrogens

Estrogens are steroid hormones synthesized in the gonads, of which three forms circulate in the female body: estrone (E1), estradiol (E2) and estriol (E3). The 17β-estradiol (E2) is the principal circulating estrogen synthetized by the enzyme aromatase from testosterone. Its circulating levels substantially fluctuate during the estrous cycle in mice. Even if the estradiol is mainly synthetised in the gonads, a part of it, acting in the brain, is synthetized directly by neurons that express the aromatase with different distribution in the brain (reviewed in Rosselli, 2007).

Estrogens exert a direct neuromodulator effect on several mechanisms, from synaptic plasticity to cognition. The amount of circulating estrogen in females varies depending on the phase of the estrous cycle. A typical estrous cycle in mice lasts 4-5 days and it is divided in four consecutive stages: proestrus, estrus, metestrus and diestrus (Fig. 19). The highest peak of E2 serum levels occurs prior ovulation during the proestrus phase (56 pg/ml in CD1 mice), while the lowest peak occurs in the estrus/early metestrus (35 pg/ml in CD1 mice) (Walmer et al., 1992; Fata et al., 2001; Becker et al., 2005), while an opposite trend is instead followed by progesterone. These fluctuations have an effect on plasticity both physiologically and in response to external stimuli (stressors, learning, etc.). The effect of estrous cycle and estrogens on structural synaptic plasticity (Fig. 19) has been reported on different plasticity mechanisms in the HP (reviewed in Sheppard et al., 2019).

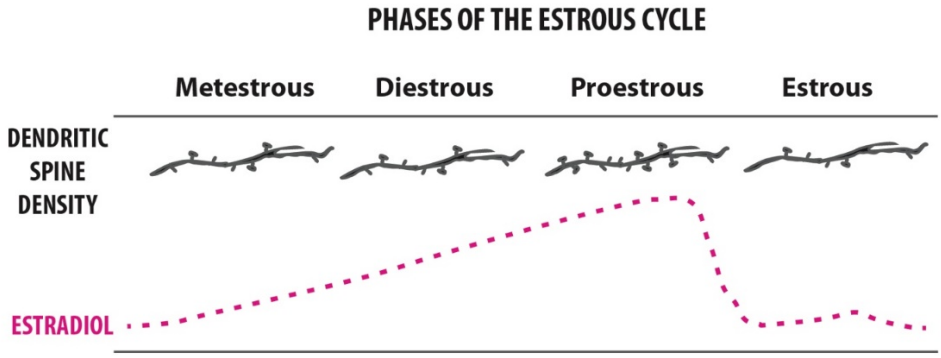

Figure 19. The level of circulating estrogens produced by ovaries varies across the phase of the rodent estrous cycle, with the highest peak in the proestrous and the lowest in the estrous (Torromino and De Leonibus, *Progress in Neurobiology,* under revision).

In women the menstrual cycle has been observed to influence the volume of the HP, with increase of the gray matter volume in the postmenstrual phase compared to the premenstrual phase (Protopopescu et al., 2008; Pletzer et al., 2010; Lisofsky et al., 2015).

Estrogens receptors and signalling cascades

Sex-hormones effects are exerted after their interaction with specific receptors expressed in the brain and leading to the activation of biological cascades involved in apoptosis, synaptogenesis, gene expression regulation and epigenetic control (Le May et al., 2006; Kooptiwut et al., 2018; Vegeto et al., 2003, 2006; Mann et al., 2007; Lone et al., 2004; Zhao et al., 2010; Yamamoto, 1985, Green et al., 1986).

Estrogen can bind two kinds of intracellular receptors (ERs), ER-α and ER-β, which are members of ligand-dependent transcriptional factors superfamily, functionally distinct and with different distribution.

Estrogens have been shown to activate different signaling pathways in the cells. E2 binding indeed increases calcium, nitric oxide (NO) and cAMP, activates the mitogen-activated protein kinase (MAPK) cascade, including the extracellular

signal-activated kinase (ERK1/2); activates phospholipase C, the protein kinases A, B and C (PKA, PKB and PKC) and the phosphatidylinositol-3-OH kinase (PI3K), the insulin-like growth factor-1 receptor (IGFR-1) and G-protein coupled receptors (GPCRs) (Singh, 1999, 2000; Kuroki et al., 2000, reviewed in Ho et al., 2002). All these cascades (Fig. 20) are activated independently of gene transcription, but they can in turn mediate gene transcription regulation, a fact that can make difficult to measure the "real" effect of the estrogens after the activation of these cascades. For instance, ER-α binds to the regulatory subunit (p85α) of PI3K and brings to the activation of the protein kinase B (PKB/AKT) in a way that is independent of gene transcription (Simoncini et al., 2000). It is known that ER-α is more transcriptionally active than ER-β (Bean et al., 2014), indeed the latter that also need higher E2 concentration to reach the full activity (Cowley et al., 1997; Pettersson et al., 1997; Watanabe et al., 1997 from Petterson et al., 2000). Despite to the different homology between the receptors, the reason of different transcriptional activities seems to rely on the structure of ER-α and ER-β. Indeed, the affinity of the two receptors for E2 is different, in a way that E2 preferentially binds ER-α (Bean et al., 2014).

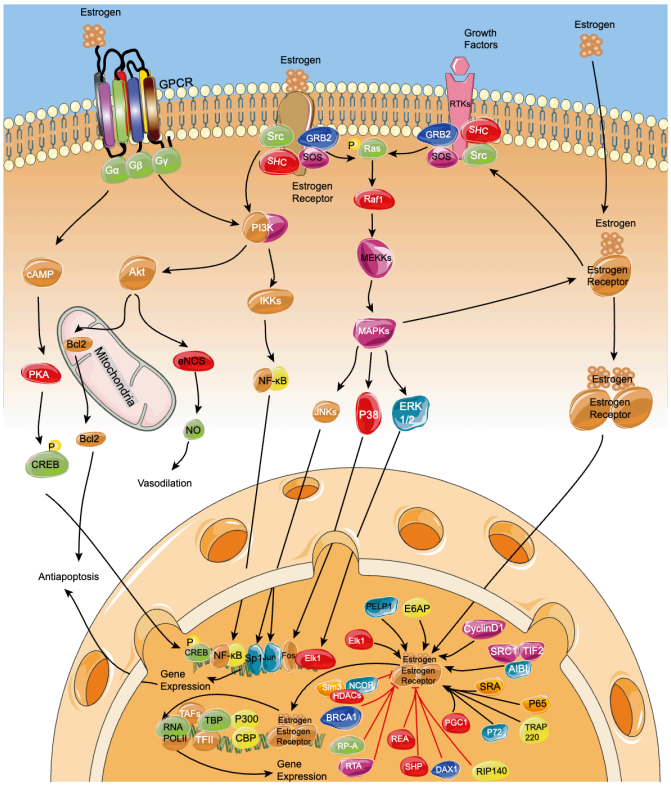

Figure 20. Schematic representation of estrogens signalling pathways

After E2 stimulation there is an increased level of membrane localization of the ER-α, that, in normal condition, is mainly expressed at the level of the nucleus (Simoncini et al., 2000). In condition of low concentration of E2, the two receptors subunits can form heterodimers, a state in which ER-β lowers the transcriptional power of ER-α (Petterson et al., 2000), finally bringing to reduced transcriptional activity suggesting that ER-β can modulate ER-α activity. Thus, in the presence of heterodimers the transcriptional activity is different than in the presence of homodimers (Petterson et al., 2000). ER-βKO mice display increased levels of transcriptional activity (Foster, 2012 from Bean et al., 2014). This is also true for the HP of ovariectomized ER-βKO mice in absence of E2 replacement, a mechanism possibly dependent on local E2 transcription in the brain (Han et al., 2013 from Bean et al., 2014); however since this effect is reduced by treatment with E2, it seems clear the occurrence of feedback mechanisms on ERs-regulated transcription in response to E2.

As mentioned above, estrogens are also present and produced in the brain and they have been proposed as neuromodulator molecules (Balthazar and Ball, 2006). This is quite predictable when looking at the signalling cascade that are activated by ERs, which have all been correlated to learning and memory functions.

BRAIN MAPPING

Neuroimaging (or brain scanning) techniques include the use of various tools to directly or indirectly display the structure and function of the brain (Fulham, 2004). It can be divided in two broad categories: the *structural* imaging, which investigates the structure of the brain and it is useful to diagnose tumor or brain injury (Henson and Gonzalez, 2012); and the *functional* imaging, which is used to diagnose metabolic diseases (such as Alzheimer's disease), and also for neurological and cognitive-psychology research (Steele and Lawrie, 2010).

The most common types of brain scan are the electroencephalography (EEG), the Positron Emission Tomography (PET), the Magnetic and Functional Magnetic Resonance Imaging (MRI and fMRI respectively) (Fulham, 2004).

These techniques are largely used in humans, but they can be also applied to animal studies (Howell, 2008; Jonckers et al., 2015; Wakeman, 2015; Bajic et al., 2017; Morita et al., 2016; Martucci, 2018).

However, in animal studies, similar and cheaper results than classical neuroimaging methods can be achieved by evaluating, through chemical methods, the expression of immediate early genes (IEG) in brain areas of interest after a behavioral procedure. Indeed, the IEG such as c-fos, have long been known as molecular marker of neural activity (Chung, 2015).

c-fos signaling pathway

Several external stimuli can induce c-fos expression by increasing neuronal activity (Morgan and Curran, 1991; Herdegen and Leah, 1998).

These stimuli, first, determine, as shown in Figure 21, the calcium intracellular influx through NMDA receptors and L-type voltage-sensitive calcium channels (Chaudhuri et al., 2000). The increased intracellular calcium can activate multiple kinase

pathways including MAPK, CaMKIV, CaMKII and PKA. Concerning c-fos, its expression is mainly mediated by MAPK (Chaudhuri et al., 2000). The MAPK activates two transcription factors binding to the c-fos promoter in the nucleus (Lyons & West, 2011). First, CREB (cyclin AMP-response element-binding protein) is phosphorylated and it binds the calcium response element (or cyclic AMP response element) in the c-fos promoter; second, phosphorylated Elk-1 in association with serum response factor (SRF) binds to the serum response element in the c-fos promoter (Cruz et al., 2014).

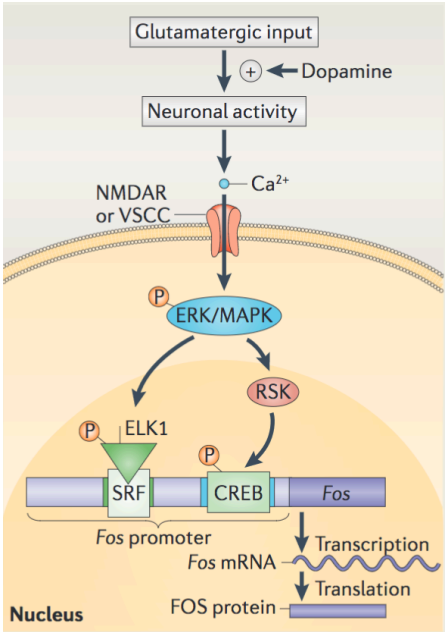

Figure 21. Schematic representation of c-fos signaling pathway (Cruz et al, 2014)

Fos protein translated in the cytoplasm moves back into nucleus where together with Jun (another IEG) make up the AP-1 complex that acts as a transcription factor for "late" genes.

As mentioned before, c-fos signaling pathway can be induced by several external stimuli. For this reason, c-fos has been largely used as neuronal marker of activation in behavioral studies performed in rodents through chemical methods such as immunohistochemical (IHC) analysis (Bullit, 1990; Guzowski et al., 2001; De Leonibus et al., 2002; Maviel et al., 2004; Cruz et al., 2014).

Indeed, c-fos was successfully used in rats to evaluate the hippocampal encoding of spatial and non-spatial information after spatial and cued water tasks (Guzowski et al., 2001). In this work rats were sacrificed 30 min post training and c-fos expression was evaluated through fluorescent *in situ* hybridization and RNA assays, finding that c-fos RNA levels were rapidly increased in the pyramidal cell layers and in the granule cell layers of the dentate gyrus of the dorsal HP after training both in spatial and non-spatial tasks.

c-fos was also well exploited in mice to test the impact of post-training silencing (with DREADDs) of different network nodes on fear memory consolidation (Vetere et al, 2017). Mice were sacrificed 90 min after the fear conditioning training and c-fos expression was evaluated through IHC to assess the chemogenetic inhibition of neurons infected.

BRAIN MANIPULATION

Tools to manipulate neural circuits: a general overview

At the beginning of the last century, understanding how neural circuits work or communicate each other was challenging. With the recent evolution of genetics (and technologies, in general) several tools in order to manipulate brain circuits were introduced beyond the classical usage of brain lesions or drugs. Indeed, brain lesions have several limitations such as their non reversible effect (Park and Carmel, 2016). Nowadays, brain circuits can be specifically manipulated with genetic tools covering various time windows from milliseconds to weeks.

Brain regions can be manipulated considering their genetic identity, connectivity, or a combination thereof, with the use of transgenic animals or approaches such as the optogenetics or chemogenetics.

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs): the chemogenetic approach

The Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) were developed by Bryan Roth and colleagues (Armbruster et al., 2007).

DREADDs are modified muscarinic G-protein coupled receptors (GPCRs) typically introduced into cells by viral vectors and they provide a lock-and-key approach to selectively modulate cellular activity by chemical means.

This technology has been used to control the activity of a wide range of cell types. The selective targeting of DREADDs to a cell population can be achieved by using a cell type-specific promoter to drive DREADDs expression, and the expression of this promoter can be further controlled using a recombinase-based (Cre) system (Whissell et al., 2016).

DREADDs possess a low affinity for endogenous ligands and little constitutive activity but may be activated by synthetic compounds. Most DREADDs are

selectively responsive to a drug, the clozapine N-oxide (CNO; Armbruster et al., 2007). This drug is considered inert, even if, since it is a metabolite of the atypical antipsychotic clozapine, recently it was discussed about its possible binding also with other types of receptors such as the serotoninergic ones, exerting aspecific effects (Manvich et al., 2017).

The effect of CNO on a DREADD depends upon the signaling cascade activated by the G-protein to which that DREADD is coupled (Fig. 22). DREADDs are coupled to an inhibitory (Gi) or excitatory (Gq, Gs) signaling cascade.

CNO activation of the modified human M4 muscarinic DREADD, which is coupled to Gi signaling, silences neuronal activity (hM4Di receptor; Armbruster et al., 2007). Conversely, CNO activation of the modified human M3 muscarinic DREADD receptor, which is coupled to Gq signaling, elicits burst firing in neurons (hM3Dq receptor; Alexander et al., 2009). Recently, a novel DREADDs has been developed that incorporates an engineered kappa-opioid receptor coupled to an inhibitory Gi-signaling cascade (Fig. 25). This kappa-opioid receptor DREADD (termed KORD) is insensitive to endogenous ligands, including the dynorphins, which are the natural ligands for kappa-opioid receptors. The KORD is exclusively activated by the inert ligand, salvinorin B (SALB), which is a semi-synthetic analog of the natural psychotropic agent salvinorin A.

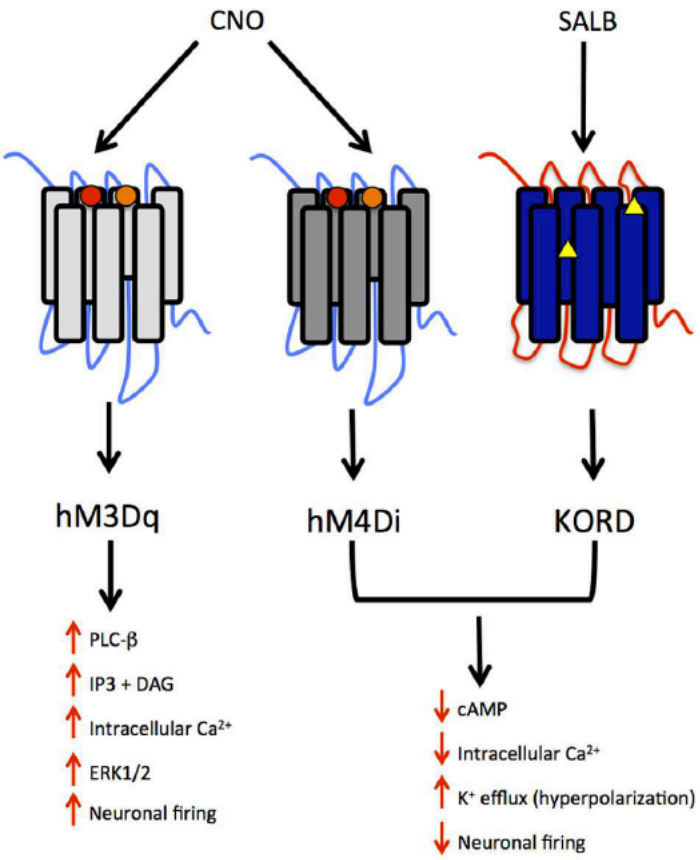

Figure 22. Modified human muscarinic (hM) and Kappa-opioid receptor (KOR) designer receptors exclusively activated by designer drugs (DREADD) subtypes and pathways activated (from Whissell et al, 2016).

The temporal properties of DREADDs activation depends on the pharmacokinetic properties of CNO. Following peripheral administration (intra-peritoneal injection), plasma levels of the CNO peak within 30 min in the brain (Roth et al., 2016) and sharply decline over the subsequent 2 h (Guettier et al., 2009). Even if plasma levels of CNO decline quickly, behavioral effects of the drug may be evident for up to 6 h (Alexander et al., 2009). Thus, DREADDs technology is preferred in studies where the activity of neurons must be manipulated over a longer time period (hours to days). CNO is commonly administered via injection, but the drug can also be mixed into food chow or drinking water (Urban et al., 2016).

Nowadays, DREADDs application in modulating behavior are several; they were successfully used in fields such as associative learning, mood and depression, memory and reward-guided behaviors (Whissel et al. 2016; Fig. 23).

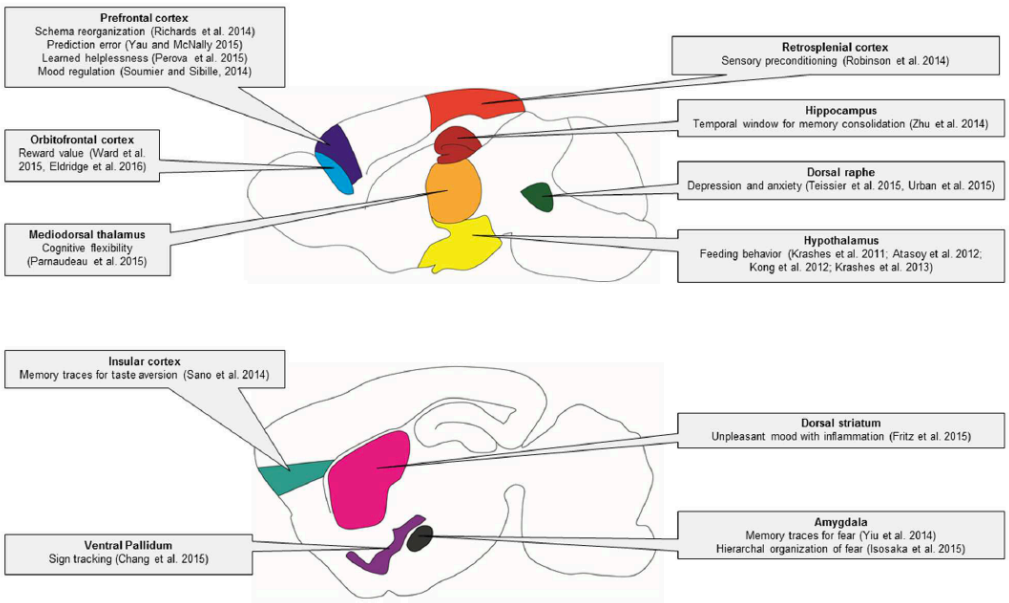

Figure 23. Behavioral circuits manipulated with DREADDs in rodents studies (from Whissell et al, 2016).

DREADDs and memory

Since their introduction, DREADDs have been extensively used to study the physiological factors governing the encoding, consolidation and updating of memory (Whissel et al., 2016).

Using DREADDs and other techniques, Yiu et al. in 2014 addressed whether the excitability of neurons during encoding affected their probability of being incorporated into a memory trace. In testing this possibility, it was selectively expressed hM3Dq receptors in lateral amygdala neurons and mice were injected with CNO pre-training in a fear conditioning task. If excitability was a factor in determining the composition of the memory trace, CNO effect during training should increase the probability that hM3Dq neurons are incorporated into the trace. Indeed, the authors observed that CNO injection before training greatly increased the

probability that hM3Dq neurons were active during retrieval of the fear memory, suggesting that these neurons represented the fear memory trace.

Recently, the timeline for contextual fear memory consolidation was investigated using DREADDs techniques (Zhu et al., 2014; Varela et al., 2016). In this study, hM4Di receptors were selectively expressed in CaMKIIa neurons in the HP. Mice underwent training in the fear conditioning task and were subsequently injected with CNO at either 0 – 4 h or 6 – 10 h after training. Strikingly, mice injected 0 – 4 h after training, but not 6 – 10 h, demonstrated impaired contextual fear conditioning. Surprisingly, the authors found also that the inhibition of CaMKIIa neurons in the ventral hippocampus (vHPC) impaired the consolidation of contextual fear memory without inhibiting neurons in the dorsal HP, suggesting that the 'window' for memory consolidation of contextual fear memory in the vHPC is within 6 h of training.

Yau and McNally (2015) recently demonstrated that chemogenetic excitation of neurons in the medial prefrontal cortex (mPFC) can renew prediction error and promote new learning. In this study, a blocking procedure was used to retard learning to a stimulus that provided no new information (i.e., another cue already successfully predicted an outcome). Although blocking occurred, as expected in the control groups, the authors showed that exciting neurons in mPFC by injecting CNO during the conditioning phase permitted conditioning to the otherwise redundant cue. Importantly, the chemogenetic approach facilitated the activation of a large population of neurons in mPFC, which is not possible with traditional electrical stimulation techniques that affect only a circumscribed area of neurons.

DREADDs were also used to examine the involvement of the dorsal HP and mPFC, alone and in combination, in object and spatial recognition memory consolidation in ovariectomized female mice (Tuscher et al., 2018). In this study, they considered two types of inhibitory DREADDs (hM4Di and KORD) to inactivate the dorsal HP alone, the mPFC alone or both brain regions together, immediately after object training to assess the role of each region in the consolidation of object recognition

and spatial memories, finding that both the dorsal HP and the mPFC are required for the consolidation of object recognition and spatial memories, as suppressing neurotransmission in either brain region impairs performance in tasks like the object placement and object recognition.

To summarize, DREADDs have proved to be a valid tool to manipulate memory, giving the possibility to better manipulate brain circuits involved in memory formation, consolidation and retrieval. This tool has several advantages such as the spatial resolution that, thanks to the viral vectors infection, enables to target specific brain regions or specific cells; they have also a good temporal resolution because, thanks to the CNO's kinetics, DREADDs effect can cover long periods (hours) in order to modulate processes that last for several hours, such as memory consolidation.

AIM

Memory capacity (MC) is the amount of information that can be held in memory for a short period.

Previous studies in our lab, through the use of the *Different/Identical Object Task* (DOT/IOT), showed that the *memory span* in young adult male mice is about of 6 different objects (Sannino et al., 2012), which is very similar to that reported in humans by Miller in 1956.

Furthermore, with this same task, it was possible to show that animals with selective lesions to the dorsal HP were not able to solve the task with a short retention interval when coping with high information load (6-DOT with 1 min delay; Sannino et al., 2012). These findings suggest that the HP regulates object MC not only in LTM but also in STM when the memory load is high, in accordance with data collected previously in humans (Levy et al 2003, 2004) and in primates (Beason-Held et al, 1999).

Remarkably, another study performed in our lab elucidated the molecular mechanisms underlying the HP recruitment in high memory load conditions and short retention interval (Olivito et al., 2014). Using a combination of pharmacological, biochemical and mouse-genetic approaches, it was found that the phosphorylation of the two serines 845 and 831 (S845 and S831, respectively) of the GluA1 subunit of AMPA receptors in the HP is necessary for processing high information load namely to properly perform the 6-DOT.

A common feature of all of these studies is that they were considering only male subjects. Although sex-related differences on memory have been consistently reported (Pauls et al., 2013; Zilles et al., 2012), little it is known on MC in females.

Studying sex-differences in MC becomes crucial during ageing where MC can naturally decrease (Deary et al, 2009) and its decline can be predictive of the onset of some brain disorders such as Alzheimer's disease (AD; Buckner, 2004), which have a major impact in women compared to men (reported by the Alzheimer's Association, Facts and Figures, 2019).

The general aim of this work was to investigate how sex-differences regulate MC in rodents. In particular, we have addressed the following questions and hypothesis:

1. Are there sex-differences in MC?
2. Are females more prone to MC decline with ageing?
3. We previously showed that STM engages GluA1 post-translational modifications in the HP when coping with high memory load request (Sannino et al., 2012; Olivito et al., 2014. Therefore, we hypothesised that the early MC impairment in female mice could be due to a dysregulation in this mechanism.
4. Can exercise training rescue age-related MC deficits in female mice?

To answer all these questions, we combined different *in vivo* and *ex-vivo* approaches. Addressing these issues will be crucial to understand why women, during ageing, are more prone to develop dementia and why they seem to be much more sensitive to the beneficial effects of exercise training.

MATERIALS AND METHODS

Subjects

In all the behavioral experiments of this study we used outbred CD1 adult (3, 6 and 12 months old) male and female mice (Charles River, Italy) housed in groups of 3 – 5 subjects. The housing conditions were maintained at 24 ± 1 °C and 55 ± 5 % relative humidity, with a 12 h light / 12 h dark cycle. Mice were tested during the light phase.

Before every behavioral procedure, the estrous cycle of female mice was checked through a visual method (Byers et al., 2012). This method was chosen because of its low invasiveness in order to avoid any stress for the animals. Female mice were always tested in all the phase of estrous cycle (proestrus, metestrus and diestrus) except for the estrus one, in which there is the lowest peak of estrogens and it is reported that female mice are impaired in memory tasks (Frick et al., 2001; Leal et al., 2018).

All procedures were performed in strict accordance with the European Communities Council directives and Italian laws on animal care.

Experiments

Methods are grouped in five main experiments, as follows:

1. Sex differences in high memory load conditions
2. c-fos brain mapping
3. DREADDs
4. Ageing
5. Exercise training

1. Sex differences in high memory load conditions

Behavioral procedures

Habituation procedure

Before every behavioral procedure, mice were first subjected to a habituation procedure. This was necessary to avoid external factors that can influence mice performance during the task (such as novelty of the experimental room, external distractors, stress due to the experimenter), since it is reported that c-fos protein, as an immediate early gene, can be activated even by light stimuli (Morgan and Curran, 1991; Herdegen and Leah, 1998).

Briefly, the procedure began with the familiarization of mice with the experimenter for 2 – 5 min. Then, mice were allowed to familiarize with their personal waiting cage for 10 min. Finally, mice were habituated, for 2 – 5 min, to other stimuli (except for the exposure to the objects) that they would have faced during the behavioral procedure, such as to be taken and then put back from their personal cage or to be taken for intraperitoneal injections.

The entire procedure must be repeated for at least 1 week.

Different/Identical Object task

The Different and the Identical Object Task (DOT/IOT) were performed as described by Sannino et al., 2012.

Briefly, animals were isolated for 15 min in a personal waiting cage before testing, and then subjected to a familiarization trial (T1, 10 min) in an empty open field (35 x 47 x 60 cm). The habituation period allows to assess for eventual motor impairment or exploratory behavior abnormalities. After 1 min spent in their waiting cage, they were subjected to the study phase (T2, 10 min), during which they were allowed to explore the objects. During this stage animals were allowed to explore the objects until they accumulated 35 sec of total objects exploration for the IOT, or 105 and 210 sec of total objects exploration for the 3- and 6-DOT, respectively. Exploration

was defined as the time in which the nose of the mouse was in contact (less than 2 cm from the object) with the object (Broadbent et al., 2004; Ainge et al., 2006); in this phase, depending on the experiment, mice were allowed to freely explore the objects or their exploration was matched for age or sex (Table 1). In case of exploration matched for age, each young mouse was allowed to explore objects exactly the same time of its older partner; while in case of exploration matched for sex, each female mouse was allowed to explore objects exactly the same time of its male partner.

After a delay of 1 min, for short term memory (STM) or 24 h for long term memory (LTM) task, animals were exposed to identical copies of the familiar objects and to a new object (test phase or T3, 5 min).

Two different new objects were used, and the position of the new object was changed across animals in a random order to avoid any bias linked to the object used or to the position.

The behavior of the animals was recorded by a video-tracking system (Anymaze, Stoelting, USA), analyzed by a trained observer and the results were expressed with the discrimination index, that is time spent exploring the new object as a percentage of the total exploration time (% new object exploration/T3 total objects exploration) (Broadbent et al,. 2004; Ainge et al., 2006).

Experiment	**Sex**	**Age**	**T2 objects exploration**
Sex differences in high memory load conditions	Males and females	3 months	free
c-fos brain mapping	Males and females	3 months	matched
DREADDs	Females	3 months	free
Ageing	Males and females	3, 6, 12 months	matched
Exercise training	Females	12 months	free

Table 1. Summary of sex, age and type of objects exploration for animals used in the experiments.

2. c-fos brain mapping

For this experiment 3 months old CD1 male and female mice underwent the study phase of the 6-DOT (high memory load) and were sacrificed after 1 h in order to ensure Fos protein expression (Guzowski et al., 2001; De Leonibus et al., 2002;

Maviel et al., 2004). Handled naïve sex-matched mice were used as control and for normalization of data. All mice were handled as described in the previous section (habituation procedure) and maintained in their own waiting cage before the behavioral procedure to avoid any aspecific expression of c-fos.

After 1 h from the end of the study phase, mice were deeply anaesthetized and transcardially perfused with 1x PBS followed by 4% paraformaldehyde (PFA; Sigma Aldrich). Brains were collected and post-fixed for 48 h in PFA and 30 μm coronal slices were obtained with the use of a vibratome (Leica VT1000 S) and stored in PBS and sodium azide (0.02%) at 4° C until the histological processing.

c-fos immunohistochemical (IHC) analysis

Five free-floating sections per animal were chosen for each brain region we considered.

After rinsing in 1x PBS, brain sections are pre-incubated with 0.5 % H_2O_2 in 100 % ethanol for 20 minutes to block intrinsic peroxidase activity. Sections were then washed three times with 1x PBS before being pre-incubated for 1 h in a blocking solution made of PBS 0.3% Triton X-100, 1% BSA and 5% normal goat serum (NGS). Blocking solution was then removed and slices were incubated for 3 days at 4°C with a solution containing 2% NGS, PBS 0.3 % Triton X-100 and 1:400 rabbit anti-c-fos antibody (sc-52; Santa Cruz Biotechnology). Sections were washed three times in PBS Triton 0.3% (PBS-T) and then incubated for 2 h with anti-rabbit IgG peroxidase-labeled diluted 1:300 in PBS-T containing 1% NGS. Brain sections were exposed to avidin-biotin complex (Avidin/Biotin Blocking Kit; SP-2001, Vector Laboratories, Burlingame, CA). The staining was visualized by color reaction with 3,3′ diaminobenzidinetetrahydrochloride (DAB Peroxidase HRP Substrate Kit, 3,3'-diaminobenzidine; SK-4100; Vector Laboratories, Burlingame, CA) under microscopic control until optimal staining was achieved (approximately 2 – 5 min). The color reaction was terminated by rinsing the sections in Milli-Q water overnight. Finally, sections were mounted on microscope glass slides and coverslipped with

Mowiol (4-88; Sigma Aldrich). Control sections, which had not been exposed to primary antibody, were processed in parallel. Images (5x magnification) were taken using the microscope Leica DM6000B with Leica digital camera DFC 480 RGB and Leica application Suite X (LAS X) software.

The number of c-fos positive cells were manually counted with ImageJ software (National Institute of Health, Bethsda, MD) in all the brain structures for each slice and averaged for each animal. The resulting values per animal were normalized on naïve average, thus values are expressed as % on naïve expression.

c-fos positive cells were analyzed in the following brain regions: dentate gyrus (DG) and CA1-CA3 subfields of the dorsal hippocampus (HP), ventral hippocampus (vHP), anterior cingulate (ACC), prelimbic (PL) and infralimbic (IL) regions of the prefrontal cortex (PFC); dorsal, intermediate and ventral septum (Sep); perirhinal and entorhinal cortex (PRh and ENT, respectively).

ER-α immunohistochemical (IHC) analysis

The same female subjects used for c-fos IHC procedure were used for this step. The procedure is the same used for the c-fos IHC analysis except for the primary and secondary antibodies used.

Primary antibody was 1:100 mouse anti-ER-α antibody (sc-8005; Santa Cruz Biotechnology) diluted in 2% NGS and PBS 0.3 % Triton X-100 (PBS-T).

Secondary antibody was anti-mouse IgG peroxidase-labeled diluted 1:100 in PBS-T containing 1% NGS.

Images (20 x magnification) were taken using the slide scanner microscope Zeiss Axio Scan and Zeiss Zen Blue software. The number of ER-α positive cells were manually counted in dorsal HP (CA1, CA3, dentate gyrus, stratum lacunosum moleculare, oriens and radiatum) with ImageJ software (National Institute of Health, Bethsda, MD) and averaged for each animal.

3. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Recombinant AAV viral vectors

Plasmids for DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) were amplified and purified using EndoFree Plasmid Mega Kit (Qiagen), following manufacturer's instruction. AAVs were made by the TIGEM AAV Vector Core.

AAV2/5 serotype produced resulted in the following titer (GC/ml): 1.4 x 10^{12} for AAV 2/5-CaMKIIa-HA-rM3D(Gs)-IRES-mCitrine.

Surgery

The surgical procedure was performed as previously described (Giordano et al., 2018). In brief, female mice were bilaterally injected with AAV 2/5-CaMKIIa-HA-rM3D(Gs)-IRES-mCitrine in the dorsal HP. The stereotaxic coordinates used for the dorsal HP were: AP = - 1.8 mm; ML = ± 1.5 mm relative to bregma, DV= - 1,5 mm from the dura, according to the atlas of Franklin and Paxinos (2001). Injection volume was of 0.5 µL/side.

Mice were then allowed to recover for 10-15 days after surgery during which their health was monitored according to standard surgery care procedures.

Behavioral procedure for DREADDs experiment

After the surgery recovery period, a group of female mice was injected intraperitoneally (i.p.) with NaCl 0.9% (saline; control group) and another group of female mice was injected i.p. with CNO (1 mg/kg; Hello Bio) and both groups were tested in the 6 DOT with 24 h delay. Considering the CNO's kinetics (CNO peak in the brain occurs within 40-60 min; Roth el al., 2016), injections were made 30 min before 6-DOT began, in order to obtain the maximum influence of the CNO in the post-training phase (memory consolidation). At the end of study phase (T2), each mouse was left to rest in its own waiting cage for 1 hour, to reduce at the minimum

any eventual interference, after which they were put back in their resident cage. 24 h later mice were submitted to the test phase (T3).

As control experiments, we designed two further groups of female mice; one group was infected with the AAV vector and CNO/saline injections were made immediately post-training, while another group of female mice was not infected with the AAV vector but only injected with CNO/saline pre-training.

To minimize the animals used in the experiments and to test the effect of the two injections on the same individuals, every experimental group of female mice was re-tested 7-10 days after the first 6-DOT exposure, switching the CNO/saline injections order and using a different set of objects.

Immunofluorescence analysis for virus verification

At the end of the behavioral procedure for DREADDs experiment, mice were deeply anaesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA; Sigma Aldrich). Brains were dissected and post-fixed for at least 48 h in PFA 4%, then they were washed in PBS and put in 30% Sucrose for 24 h. 30 µm coronal slices were obtained with the use of a cryostat (Leica CM3050 S) and stored in PBS and sodium azide 0.02% at 4° C until the beginning of the procedure. Several free-floating sections per mouse were chosen for the dorsal HP to evaluate the correct placement and extent of AAV injection.

After rinsing in 1x PBS, brain sections were incubated in blocking solution made of NGS 5% and 0.3% Triton X-100 in PBS for 1 h. Blocking solution was then removed and replaced with primary antibody solution made of NGS 1%, Triton X-100 0.3% and rabbit anti-HA tag (#3724; Cell Signaling) diluted 1:500 in 1x PBS overnight at 4°C. Sections were washed three times in 1x PBS and then incubated for 2 h at room temperature with secondary antibody solution made with NGS 1%, Triton X-100 0,3% and goat-anti-rabbit Alexa Fluor 647 (Merck Millipore) diluted 1:300 in 1x PBS.

After three washes with 1x PBS, sections were incubated for 10 mins with 4′,6-diamidino-2-phenylindole (DAPI), for nucleic acid staining, at room temperature and then washed three times with 1x PBS. Finally, sections were mounted on microscope glass slides and coverslipped with Mowiol (4-88; Sigma Aldrich). Control sections, which had not been exposed to primary antibody, were processed in parallel.

Slices were manually analysed under a Nikon Ni-E fluorescence microscope equipped with Nikon DS-Ri2 camera. Only mice expressing the reporters m-Citrine and HA in the HP were included in the statistical analysis.

Western Blot analysis

At the end of the DREADDs behavioral procedure, after one week, mice were first injected with CNO or Saline and, after 65 min, they were sacrificed through cervical dislocation and their brains were collected. The dorsal hippocampi (HP) were dissected using a brain matrix (Mouse Brain Blocker, Kopf Instruments). HP sections were homogenized with homogenizing buffer composed of 0.32 M sucrose, 1 mM EDTA, 1 mg/mL BSA, 5 mM HEPES pH 7.4, 1 mM PMSF, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1x Sigma protease inhibitor cocktail in distilled H_2O. Protein concentration was determined by Bradford assay (Bio-Rad).

Dorsal HP homogenates aliquots (20 µg/lane) from CNO and saline treated mice were first boiled at 95°C for 5 min and then separated on 10% polyacrylamide gel and transferred on PVDF membranes (Immobilon-P transfer membrane; Merck Millipore). The membranes were blocked with 5% non-fat powdered milk in TBS + 0.01% Tween-20 (TBS-T) for 1 h at room temperature followed by overnight incubation at 4°C with rabbit anti-glutamate receptor 1 (AMPA subtype) phosphoSer 845 (1:500; Abcam) and rabbit anti-glutamate receptor 1 (1:500, Abcam).

Subsequently, blots were washed with TBS-T and secondary antibodies (1:5000; Bio-Rad) were applied for 1 hr at room temperature. Bands were detected by

chemiluminescence and quantified using ImageJ software (National Institute of Health, Bethsda, MD). Data are expressed as % from saline treated samples.

4. Ageing

Synaptoneurosomes preparation and Western blot analysis

At the end of 6-DOT with 1 min delay 3 and 6 months old female mice were sacrificed through cervical dislocation and their brains were collected. The dorsal HP were dissected using a brain matrix (Mouse Brain Blocker, Kopf Instruments). For each age, two HP of two females were independently pooled on the basis of both the total object exploration at the study phase (T2) and the performance at the test phase (T3).

HP were homogenized as described previously and an aliquot was retained and represented the homogenate fraction.

Other fractions were centrifuged at 3000 x g for 10 min at 4°C. Supernatant was recovered and centrifuged at 14000 x g for 12 min at 4°C. Pellets were re-suspended in Krebs-Ringer buffer (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM EDTA, 10 mM HEPES pH 7.4) and Percoll (45% v/v) and centrifuged at 14000 rpm for 2 min at 4°C.

The synaptoneurosomal fraction was collected on the surface of the solution and re-suspended in Krebs-Ringer buffer. Samples were additionally centrifuged at 14000 rpm for 30 sec at 4°C. The synaptoneurosomes were re-suspended in RIPA buffer composed by 50 mM Tris-HCl pH 7.4, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA and 1x protease inhibitor cocktail (Sigma Aldrich). Protein concentration was determined by Bradford assay (Bio-Rad).

HP homogenates and synaptoneurosomes (10 µg/lane) were separated on 10% polyacrylamide gel and transferred on PVDF membranes (Immobilon-P transfer membrane; Merck Millipore). The membranes were blocked with 5% non-fat powdered milk in TBS + 0.01% Tween-20 (TBS-T) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibodies: rabbit anti-

glutamate receptor 1 (AMPA subtype) phosphoSer 845 (1:500; Abcam), rabbit anti-glutamate receptor 1 (AMPA subtype) phosphoSer 831 (1:500; Abcam), rabbit anti-glutamate receptor 1 (1:500, Abcam).

Subsequently, blots were washed with TBS-T and secondary antibodies (1:5000; Bio-Rad) were applied for 1 h at room temperature. Bands were detected by chemiluminescence and quantified using ImageJ software (National Institute of Health, Bethsda, MD). Young (3 months old) tested or naïve to the test mice were used as control and for normalization of data. Data are expressed as % from 3 months or from naïve treated samples.

TBS/TBS-TX protein extraction and native Dot-Blot

Sequential extraction on TBS and TBS-Triton X-100 (TBS-TX) was performed as previously described in Pignataro et al., 2019. Proteins from TBS and TBS-TX fractions were then used for native Dot-Blot. Primary antibodies used were anti-Aβ42 Clone 295F2 (1:1000, Synaptic System) and anti-OC (1:1000, Millipore). Ponceau S staining was used as loading control. Immunoreactivity was detected by chemiluminescence and dots were quantified by densitometry using ImageJ software (National Institute of Health, Bethsda, MD). Data are expressed as % from 3 months treated samples.

5. Exercise training

Behavioral procedure

12 months old female mice were used for this experiment. For 1 month, the cage of a group of female mice was equipped with a rotating wheel provided with a revolution counter (#1850, Ugo Basile) that occupied roughly half of the cage (exercise group), while the control group of females was maintained in a normal cage without rotating wheel (no exercise group) with food and water *ad libitum*. Body weight (for both groups) and wheel total rotations were checked three times a week.

After this period both groups of female mice were tested in the 6 DOT with 1 min delay.

Statistical analysis

All data are expressed as mean ± standard error mean (SEM). An ANOVA analysis followed by a Duncan *post-hoc* analysis, when necessary (if the p-value of the ANOVA was significant), were performed.

DOT/IOT: Distance travelled in the T1 phase, total object exploration in T2 phase and discrimination index at T3 were analysed with a one-way ANOVA. Depending on the experiment, sex (two levels: male, female) for sex differences in high memory load condition experiment; age (two levels: 3 and 6 months or 3 and 12 months) for ageing experiments; treatment (two levels: saline, CNO for DREADDs experiment; two-levels: no-exercise, exercise for the exercise training experiment) were used as between factors.

c-fos immunohistochemical analysis: counting of c-fos positive cells per slices was averaged per animal and data were normalized on naïve average, thus values were expressed as % on naïve expression. A two-way ANOVA for sex (two levels: male, female) and test (two levels: naïve, test) was used. Duncan *post-hoc* analysis followed when the p-value for interaction of sex x test was significant; if this was not the case a one-way ANOVA for sex or treatment separately was used.

Estrogen's receptors immunohistochemical analysis: counting of ER-α positive cells per slices was averaged per animal and data were normalized on naïve average, thus values were expressed as % from naïve expression. A one-way ANOVA for test (two levels: naïve, test) was used.

Western Blot analysis: for DREADDs experiment, a one-way ANOVA for treatment (two levels: saline, CNO) was used to analyse data for GluA1 and p-S845 (% vs saline); for ageing experiment a one-way ANOVA for age (two levels: 3 and 6 months) was used to analyses data for GluA1, p-S845 and p-S831 and data are expressed as % vs 3 months treated samples.

Native Dot-blot analysis: data were normalized on 3 months old TBS or TBS-TX fraction and expressed as % from 3 months treated samples. A one-way ANOVA for age (two levels: 3 and 6 months) was used to analyse data for Aβ-42 and OC.

RESULTS

Sex differences in high memory load conditions

Memory capacity (MC) is the number of elements that can be held in memory for a short period. Several data were published on this topic focusing also the attention on brain circuits regulating MC in different memory load condition both in humans, primates and mice but only considering male subjects (Beason-Held et al 1999; Levy et al, 2003,2004; Sannino et al, 2012).

Remarkably, there are few information about MC in females even if gender differences on memory are reported (Pauls at al., 2013; Zilles et al., 2012). Furthermore, the study of sex differences becomes necessary since women during ageing are more prone to develop dementia compared to men (Alzheimer's Association, 2010).

To evaluate sex differences in MC, we started by testing young (3 months old) CD1 male and female mice in two different memory load conditions (6-DOT for high memory load, and 6-IOT for low memory load) at two different retention intervals (1 min for STM and 24 h for LTM), with no restriction of exploration during the study phase.

In the 6-IOT (low memory load conditions), at both retention intervals, the two sexes showed no differences but with a strong trend for female mice in total objects exploration at T2 for the 6-IOT at 1 min delay [Sex: $F_{(1,34)} = 3.671$; $p = 0.063$ for 6-IOT at 1 min; Sex: $F_{(1,14)} = 0.565$; $p = 0.464$ for 6-IOT at 24 h] (Table 1).

Male and female mice (n=18 for 1 min; n=8 for 24 h for both groups) showed no differences in the discrimination index at T3 [Sex: $F_{(1,34)} = 2.646$; $p = 0.113$ for 6-IOT at 1 min; Sex: $F_{(1,14)} = 1.404$; $p = 0.255$ for 6-IOT at 24 h] (Fig. 2 A, C), suggesting an equal performance in the low memory load condition beside the retention interval.

When we increased the load with 6 different objects (6-DOT, high memory load conditions), at 1 min (n=18 for both groups), the two sexes continued to show no significant differences in the total exploration of the objects at T2 [Sex: $F_{(1,34)} =$

7.687; p = 0.09 for 6-DOT at 1 min] (Table 1), although female mice show a tendency to increased exploration during the study phase. Analysis of the discrimination index at 1 min showed again a similar performance between the two sexes [Sex: $F_{(1,34)} = 0.071$; p = 0.791] (Fig. 2 B), indicating no differences in memory capacity for STM.

Female mice, as well as male mice (Sannino et al., 2012), do not perform the 9-DOT (Fig. 1; n=8 for 9-IOT females; n=12 for 9-DOT females), suggesting that their object memory span is of about 6 objects, while their performance is correct in the control task (9-IOT) [Test: $F_{(1,18)} = 39.820$; $p < 0.0001$].

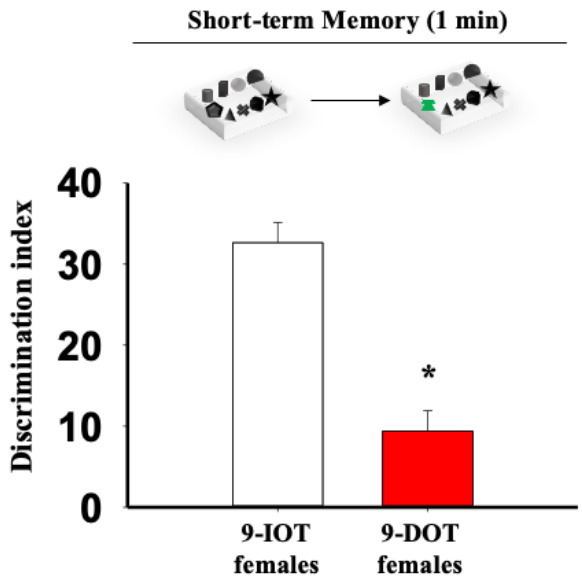

Figure 1. 3 months old female mice showed STM impairment on the 9-DOT task (9-DOT females), with more of 6 different objects, but performed correctly in the 9-IOT task (9-IOT females). * p < 0.05 9-IOT *vs* 9-DOT.

In the 24 h retention interval test, females showed a significant higher exploration of the objects at T2 [Sex: $F_{(1,22)} = 6.724$; p = 0.016] (Table 1).

However, increasing the retention interval to up to 24 h, we found a significant difference in the discrimination index at T3 (n=14 for male mice; n=10 for female

mice) [Sex: $F_{(1,22)} = 5.628$; $p = 0.026$] (Fig. 2 D), suggesting a specific MC impairment in female mice for LTM.

Overall the results of this experiment suggest that female mice are specifically impaired in consolidating objects information into LTM only in conditions of high memory load (Fig. 2 B, D).

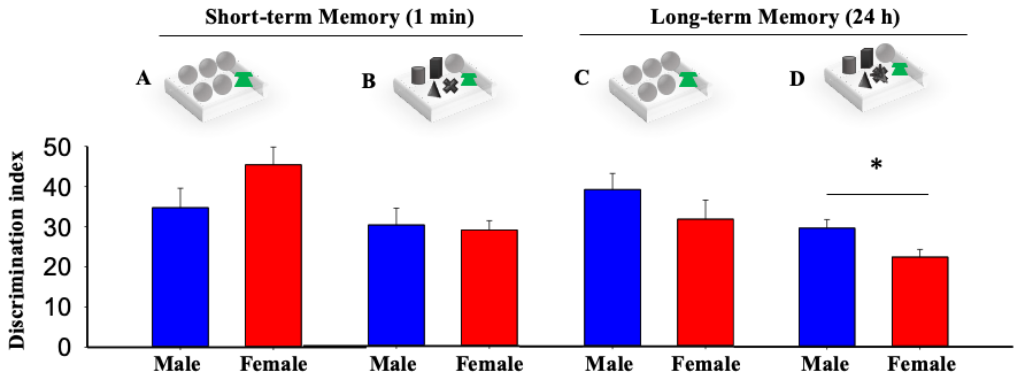

Figure 2. Male and female mice performance in different memory load conditions A-C. 3 months old male and female mice show no differences in the discrimination index in low memory load conditions (6-IOT) at both retention intervals (1 min and 24 h); **B-D.** When increasing the information load up to 6 different objects female mice are impaired at 24 h retention interval. * $p < 0.05$ male *vs* female.

Group	Behavioral task	T2 [objects exploration (s)]
3 months old males	6-IOT 1 min	32.222 ± 0.995
3 months old females	6-IOT 1 min	35.444 ± 1.356
3 months old males	6-IOT 24 h	35.125 ± 0.398
3 months old females	6-IOT 24 h	36.375 ± 1.614
3 months old males	6-DOT 1 min	161.944 ± 9.528
3 months old females	6-DOT 1 min	195.111 ± 7.234
3 months old males	6-DOT 24 h	172.429 ± 9.208
3 months old females	6-DOT 24 h	204.100 ± 6.423

Table 1. Summary of total objects exploration (T2) data of sex differences in high memory load conditions experiment. Data are expressed as mean ± SEM.

Different brain areas are activated in male and female mice by the high memory load task

Data collected previously in our laboratory (Sannino et al., 2012, Olivito et al., 2014) showed that in male mice the dorsal HP recruitment is crucial in high memory load condition, both at short (1 min) and at long delay (24 h).

Here we tested the hypothesis that female mice do not consolidate object information in conditions of high load as a consequence of lower hippocampal recruitment.

To address this hypothesis, we mapped the brain regions activated by young female mice as compared to males after being exposed to high information load (6-DOT). To this aim, we performed immunohistochemical analysis of c-fos (Fig. 3 A), an immediate early gene (IEG) rapidly induced in distinct brain areas following learning experiences. C-fos variations are, indeed, widely used to estimate different patterns of activation of specific brain regions (Guzowski et al., 2001; De Leonibus et al., 2002; Maviel et al., 2004)

The brain regions analyzed were (Fig. 3 B): the dentate gyrus (DG) and CA1-CA3 subfields of the dorsal hippocampus (HP), the ventral hippocampus (vHP), the anterior cingulate (ACC), the prelimbic (PL) and infralimbic (IL) regions of the prefrontal cortex (PFC), the dorsal, intermediate and ventral septum (Sep) and the perirhinal (PRh) and entorhinal (ENT) cortex. Results for each structure are described below.

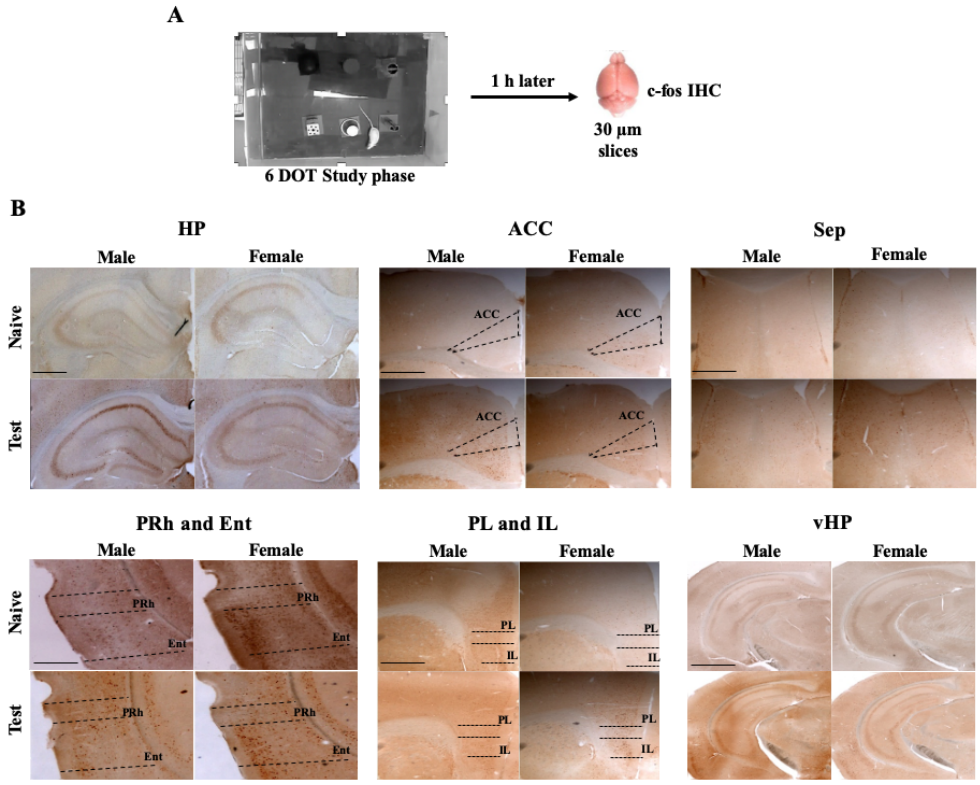

Figure 3. The c-fos brain mapping. A. Experimental design to map the brain region recruited in male and female mice in high memory load conditions (6-DOT); **B.** Representative images of brain areas in which c-fos expression was evaluated; scale bar: 200 µm.

Dorsal Hippocampus (CA1-CA3 and DG): c-fos positive cells were counted in the DG and CA1-CA3 subfields 1 h after exposition to the 6-DOT (Fig. 4 G, H). The results show that c-fos is activated in both sexes in all hippocampal subregions when compared with sex-matched naïve controls. However, male mice present a much higher activation as compared to females after performing the task. No basal differences between sexes was found in control mice (data not shown) [DG (Sex: $F_{(1,27)} = 5.484$; $p = 0.026$; Test: $F_{(1,27)} = 28.024$; $p < 0.0001$; Sex x Test: $F_{(1,27)} = 5.493$; $p = 0.026$; CA1-CA3 (Sex: $F_{(1,23)} = 5.198$; $p = 0.032$; Test: $F_{(1,23)} = 13.490$; $p = 0.013$; Sex x Test $F_{(1,23)} = 5.198$; $p = 0.032$]. Interestingly, Duncan *post-hoc* analysis revealed a significant difference between male (n=7) and female mice (n=7) in CA1-

CA3 (p = 0.003; Fig. 4 G) and DG (p = 0.002; Fig. 4 H). These results indicate a different involvement of the HP in response to high memory load conditions between males and females, thus supporting our working hypothesis that female mice show less activation of the HP during object memory consolidation in conditions of high load.

Anterior cingulate cortex (ACC): the pattern of c-fos activation in the ACC parallels that observed in the HP, as it was found activated in both sexes in response to the task, but the effect was significantly higher in male as compared to female mice, as revealed by the two-way ANOVA [Sex: $F_{(1,27)} = 7.395$; $p = 0.011$; Test: $F_{(1,27)} = 38.347$; $p < 0.0001$; Sex x Test: $F_{(1,27)} = 7.393$; $p = 0.011$]. Duncan *post-hoc* analysis confirmed that males (n=9) express more c-fos than females (n=8) ($p = 0.0007$; Fig. 4 I) in response to the task.

Lateral Septum (Sep): In the Sep, the pattern of c-fos activation is opposite to the one observed in the HP and ACC; indeed, female mice show a much stronger activation of this region, as compared to males, after the 6-DOT [Sex: $F_{(1,26)} = 4.323$; $p = 0.047$; Test: $F_{(1,26)} = 31.954$; $p < 0.0001$; Sex x Test $F_{(1,26)} = 4.323$; $p = 0.047$]. Duncan *post-hoc* analysis confirmed that female mice (n=6) express more c-fos positive cells than male mice (n=10) after exposition to the task ($p = 0.006$; Fig. 4 C).

Ventral Hippocampus (vHP): we also analysed the vHP for the mapping of sex-differences in brain activity in high memory load conditions because it is controversially considered as a functionally separate structure from the dorsal HP (Flore et al., 2016). Recent unpublished evidence in our laboratory have shown an involvement of this structure in spatial memory, however, less is known about possible sex-differences. The results show that the vHP is activated in both sexes as compared to naïve animals [Sex: $F_{(1,18)} = 0.367$; $p = 0.552$; Test: $F_{(1,18)} = 9.170$; $p = 0.007$; Sex x Test: $F_{(1,18)} = 0.367$; $p = 0.552$]. Indeed, one-way ANOVA for the factor Test show a significant difference in c-fos expression between naïve and tested mice

for both sexes [Test: $F_{(1,7)} = 6.116$; $p = 0.042$ for male mice; Test: $F_{(1,11)} = 6.241$; $p = 0.029$ for female mice] (Fig. 4 F).

Prelimbic cortex (PL): in this structure the two-way ANOVA followed by Duncan's *post-hoc* analysis revealed a different expression of c-fos between male and female mice depending on the testing condition [Sex: $F_{(1,23)} = 7.568$; $p = 0.011$; Test: $F_{(1,23)} = 5.023$; $p = 0.035$; Sex x Test: $F_{(1,23)} = 7.568$; $p = 0.011$]; in particular in male mice ($n=9$) there were more c-fos positive cells compared to naïve and female mice ($n=7$) ($p = 0.001$; Duncan *post-hoc*); female mice, indeed, show no differences in c-fos expression compared to naïve mice (Fig. 4 A), thus suggesting that female mice do not recruit at all the PL in the 6-DOT.

Infralimbic cortex (IL): in this brain region the two-way ANOVA showed only an effect for test [Sex: $F_{(1,23)} = 0.311$; $p = 0.582$; Test: $F_{(1,23)} = 4.511$; $p = 0.044$; Sex x Test: $F_{(1,23)} = 0.311$; $p = 0.582$]. The one-way ANOVA for test showed only a strong trend but no significant differences in c-fos expression for males [Test $F_{(1,12)} = 4.245$; $p = 0.061$] and no significant differences in females [Test $F_{(1,11)} = 1.052$; $p = 0.372$], indicating that this region is not recruited in high memory load conditions ($n=9$ for male mice; $n=6$ for female mice; Fig. 4 B).

Perirhinal cortex (PRh): the two-way ANOVA showed again a significant value for the variable Test but not for the Sex one ($n=8$ for male mice; $n=6$ for female mice) [Sex: $F_{(1,22)} = 0.578$; $p = 0.337$; Test: $F_{(1,22)} = 14.020$; $p = 0.011$; Sex x Test: $F_{(1,22)} = 0.338$; $p = 0.567$]. Indeed, one-way ANOVA for Test show a significant difference in c-fos expression between naïve and tested mice for both sexes [Test: $F_{(1,10)} = 13.683$; $p = 0.041$ for male mice; Test: $F_{(1,12)} = 6.843$; $p = 0.022$ for female mice), indicating that in both sexes there is an increase in c-fos expression after exposition to the 6-DOT but no differences between sexes (Fig. 4 D).

Entorhinal cortex (ENT): similar to the PRh, also for the ENT we found only an effect of Test but no difference between sexes, nor interaction between the two factors [Sex: $F_{(1,20)} = 0.965$; $p = 0.337$; Test: $F_{(1,20)} = 7.799$; $p = 0.011$; Sex x Test: $F_{(1,20)} = 0.968$; $p = 0.336$]. The one-way ANOVA for males [Test $F_{(1,9)} = 7.068$; $p = 0.026$] and females [Test $F_{(1,11)} = 5.330$; $p = 0.041$] indicates that the 6-DOT induces an increase in c-fos expression compared to the naïve condition (male mice n=9; female mice n=7; Fig. 4 E).

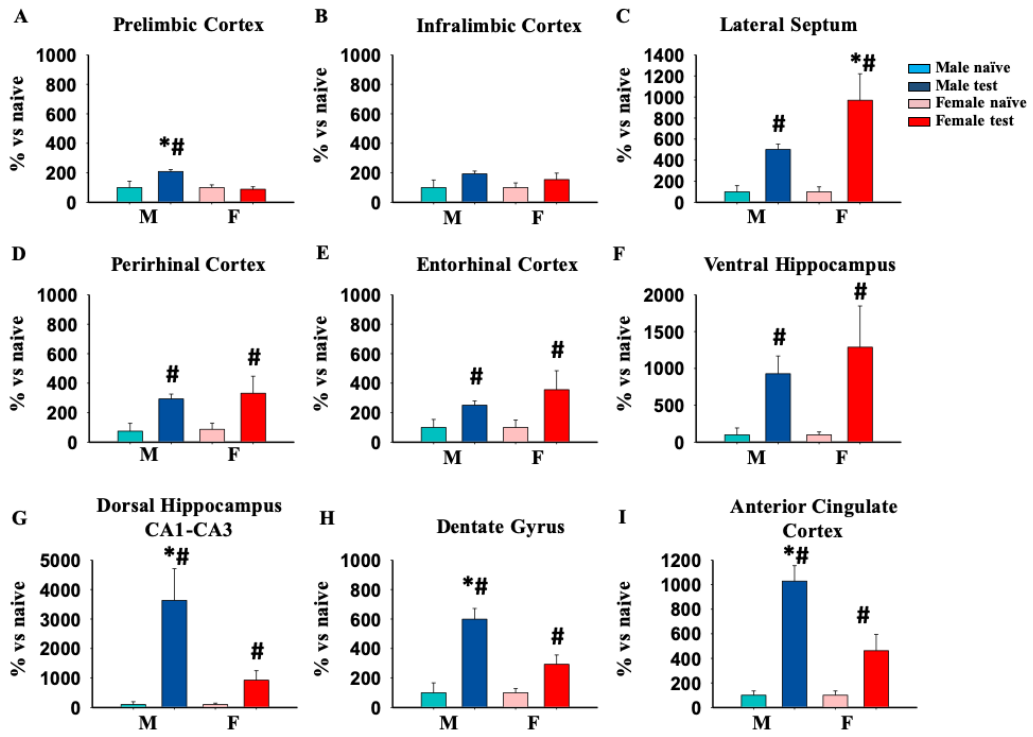

Figure 4. Sex differences in brain region activation 1 hour after exposure to high information load. c-fos expression is significantly increased in both sexes after learning in the 6-DOT, in all brain areas considered, except the infralimbic part of prefrontal cortex (IL; **B**). Female mice showed a lower activation of c-fos compared to male mice in the dorsal CA1-CA3 subfields of the hippocampus (**G**), in the dentate gyrus (DG; **H**), in the anterior cingulate (ACC; **I**) and in the prelimbic (PL; **A**) cortex. No differences between sexes were found in the perirhinal (PRh; **D**) and the entorhinal (ENT; **E**) cortex and in the ventral part of hippocampus (vHP; **F**); male mice show a lower c-fos expression compared to female mice only in the lateral septum (Sep; **C**); Data are represented as % on naïve

expression ± SEM. # $p < 0.05$ naïve *vs* test within group, * $p < 0.05$ male *vs* female; M: males; F: females.

To summarize the data collected in this brain mapping experiment, we found a sex-independent increase in all brain areas of the cortico-limbic circuit, as compared to naïve animals (indicated with "#" in Figure 4), including the HP, the vHP, the PRh, the ENT, the PL and Sep, but not the IL cortex. We found some interesting differences between sexes in some of the regions analyzed (indicated by "*" in Figure 4). In particular, while in the Sep we found c-fos highly expressed in females compared to males, concerning the PFC (ACC and PL cortices) and the dorsal HP we have found a significant higher increase in males compared to females.
This difference may be the neural substrate responsible for the LTM deficit that we have previously observed in females (Fig. 1). This is, to our knowledge, the first evidence of a sex-related difference in brain areas recruitment for MC.

No modulation of ER-α occurs in female mice in response to high memory load conditions

The 17β-estradiol (E2) is the principal circulating estrogen synthetized in the gonads by the enzyme aromatase from testosterone and, a part of it acting in the brain, is synthetized directly in neurons (reviewed in Rosselli, 2007).
E2 exerts neuromodulatory effects binding, mainly, two kind of intracellular receptors (ERs), ER-α and ER-β, activating several signalling cascades in the cells (reviewed in Ho et al., 2002).
Most of all these signaling cascades, such as the increase of intracellular calcium, the activation of the mitogen-activated protein kinase (MAPK) cascade, including the extracellular signal-activated kinase (ERK1/2) or the activation of the protein kinases A, B and C (PKA, PKB and PKC), may arise also from the influence of other factors.

This issue makes quite difficult to evaluate the real E2 contribution on these cascades.

Since we observed a LTM deficit in young female mice (Fig. 1 D), to verify any estrogens influence in female mice HP in high memory load conditions, we decided to evaluate the expression levels of ERs since this parameter can account for E2 influence (reviewed by Foster, 2012).

To this aim, one hour after exposing female mice to the 6-DOT study phase, we performed only ER-α IHC analysis on brain slices since E2 binds this receptor with more affinity than ER-β, and, moreover, ER-α is more transcriptionally active than ER-β (Bean et al., 2014).

The experimental design was identical to the one used for the c-fos study.

ER-α positive cells were counted in the HP subfields where this receptor is mainly expressed: CA1, CA3, DG, stratum lacunosum moleculare, stratum oriens and stratum radiatum (Rai et al., 2010).

One-way ANOVA for test showed no differences in ER-α expression between the two groups of females in the CA1 [Test: $F_{(1,9)} = 0.013$; $p = 0.911$] (n=6 for tested females; n=5 for naïve females), CA3 [Test: $F_{(1,9)} = 0.051$; $p = 0.827$] (n=6 for tested females; n=5 for naïve females), stratum lacunosum moleculare [Test: $F_{(1,9)} = 1.103$; $p = 0.321$] (n=6 for tested females; n=5 for naïve females), stratum oriens [Test: $F_{(1,9)} = 0.208$; $p = 0.658$] (n=6 for tested females; n=5 for naïve females), stratum radiatum [Test: $F_{(1,9)} = 0.448$; $p = 0.530$] (n=6 for tested females; n=5 for naïve females) and in the DG [Test: $F_{(1,8)} = 4.909$; $p = 0.06$] (n=5 for tested females; n=5 for naïve females).

Since no significant differences were found between the two groups in ER-α expression in the several HP subfields analyzed, we averaged them all.

In the total HP, again, no difference was found in ER-α expression between tested and naïve female mice [Test: $F_{(1,9)} = 0.293$; $p = 0.601$] (Fig. 5 A).

Data collected in this step suggested that the 6-DOT does not affect ER-α expression in the HP.

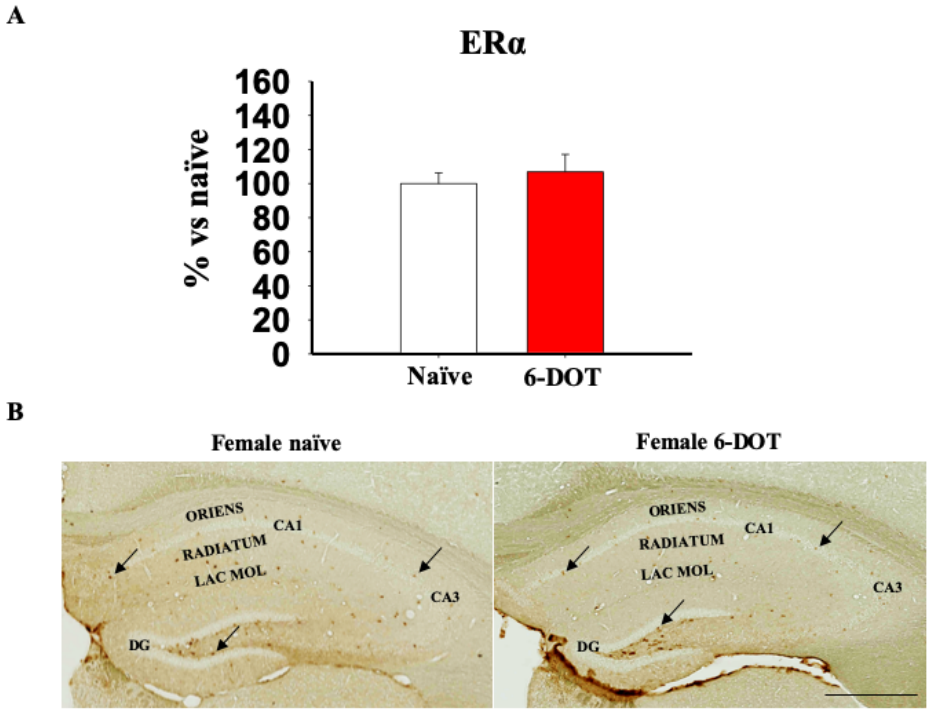

Figure 5. ER-α expression in female mice HP. A. No differences between the two groups of females in ER-α expression were found in the several HP subfields that we considered so we averaged them all. In the total HP, again, we found no differences, suggesting no changes of ER-α expression in high memory load conditions.

B. Representative images (20x magnifications) of tested and naïve female mice are shown. Black arrows indicate ER-α positive cells. Scale bar: 200 µm.

Hippocampal activation rescues female memory consolidation impairment

Based on our previous result of lower hippocampal activation in female than male mice, we hypothesized that the minor engagement of the HP could be responsible of the deficient memory consolidation in female subjects. In order to revert the HP hypoactivation found in females after the 6-DOT in comparison to males, we decided to use adeno-associated viral vectors (AAV) for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), as this chemogenetic approach allows to manipulate neuronal activity for long delays. This was suitable to cover the memory consolidation phase, which lasts at least 1 h post-learning (Lamprecht and Ledoux,

2004; Kandel, 2012), a time needed for *de novo* protein synthesis necessary for memory consolidation.

To this aim, we focally inoculated the AAV2/5-CaMKIIa-HA-rM3D(Gs)-IRES-mCitrine in the dorsal HP to activate the cAMP/PKA pathway and, after 3 weeks, we injected i.p. a group of female mice with saline (NaCl 0,9%; control group; *n* =11) and another group of females with CNO (*n*=11) and we tested them in the 6-DOT with 24 h delay. The CNO injections were performed 30 min before the 6-DOT considering that the peak of CNO activity in the brain occurs in 40-60 min after its systemic injection (Roth et al., 2016). In this way, we obtained the maximum CNO activity in the brain during the consolidation phase.

While we found no differences between the two groups for both locomotor activity at T1 [Treatment: $F_{(1,20)} = 0.013$; $p = 0.910$] (Table 2) and total object exploration during the study phase [Treatment: $F_{(1,20)} = 0.835$; $p = 0.371$] (Table 2), in the test phase (T3) we found a significant increase of the discrimination index in CNO injected female mice compared to the control group (Saline injected) [Treatment: $F_{(1,20)} = 4.388$; $p = 0.049$] (Fig. 6 A).

Interestingly, when the same treatment is performed immediately post training (immediately after the T2 phase of the task) in a different group of females with saline (*n*=12) and CNO (*n*=12), both groups were impaired [Treatment: $F_{(1,22)}= 1.217$; $p = 0.281$] (Fig. 6 B), suggesting that the HP must be activated in the early phase of memory consolidation, as previously reported for other behavioral tasks and for the same task in male mice (Lamprecht and Ledoux, 2004; Kandel, 2012; Olivito et al., 2014). As expected, both groups of mice show no differences for the locomotor activity at T1 [Treatment: $F_{(1,22)} = 0.291$; $p = 0.595$] (Table 2) and for total objects exploration during the study phase [Treatment: $F_{(1,22)} = 0.012$; $p = 0.914$] (Table 2). Only mice showing viral expression in the HP were included in statistical analysis (we checked for the correct AAV expression in HP through an anti-HA immunofluorescence; Fig. 6 C).

In order to confirm the DREADDs-mediated rescue of HP activation, female mice, one week after 6-DOT exposure, were injected again with CNO/Saline and sacrificed 65 min later. After cervical dislocation, we collected brains and dissected the dorsal HP. Then, we performed an anti-GluA1 phosphoSer 845 (p-S845) western blot (WB) analysis in the HP homogenates of CNO (n=7) and saline (n=5) injected mice. We found an increase of p-S845 only in CNO injected female mice compared to the saline ones [Treatment: $F_{(1,10)} = 5.469$; $p = 0.041$] (Fig 6 D) after normalization on GluA1 levels where we found no differences between the two groups [Treatment: $F_{(1,10)} = 0.737$; $p = 0.410$] (data not shown), suggesting that the chemogenetic tools was effective in HP activation.

Finally, since recently the proper inert nature of CNO was questioned (Manvich et al., 2018), to exclude from data described previously that the memory consolidation rescue was to attribute to aspecific effects of CNO, we injected with CNO/Saline an additional group of female mice non-infected with the AAV construct (saline n=9, CNO n=8) 30 min before testing them in 6-DOT with 24 h delay. We found no difference in the performance at T3 between groups in the absence of AAV [Treatment: $F_{(1,15)} = 2.248$; $p = 0.154$] (Fig. 6 E). The WB analysis showed no increase in p-S845 levels in the HP homogenates of saline (n=4) and CNO (n=3) female mice in this case [Treatment: $F_{(1,5)} = 0.731$; $p = 0.431$] (Fig. 6 F) after normalization on GluA1 levels where we found no differences between the two groups [Treatment: $F_{(1,5)} = 3.500$; $p = 0.120$] (data not shown).

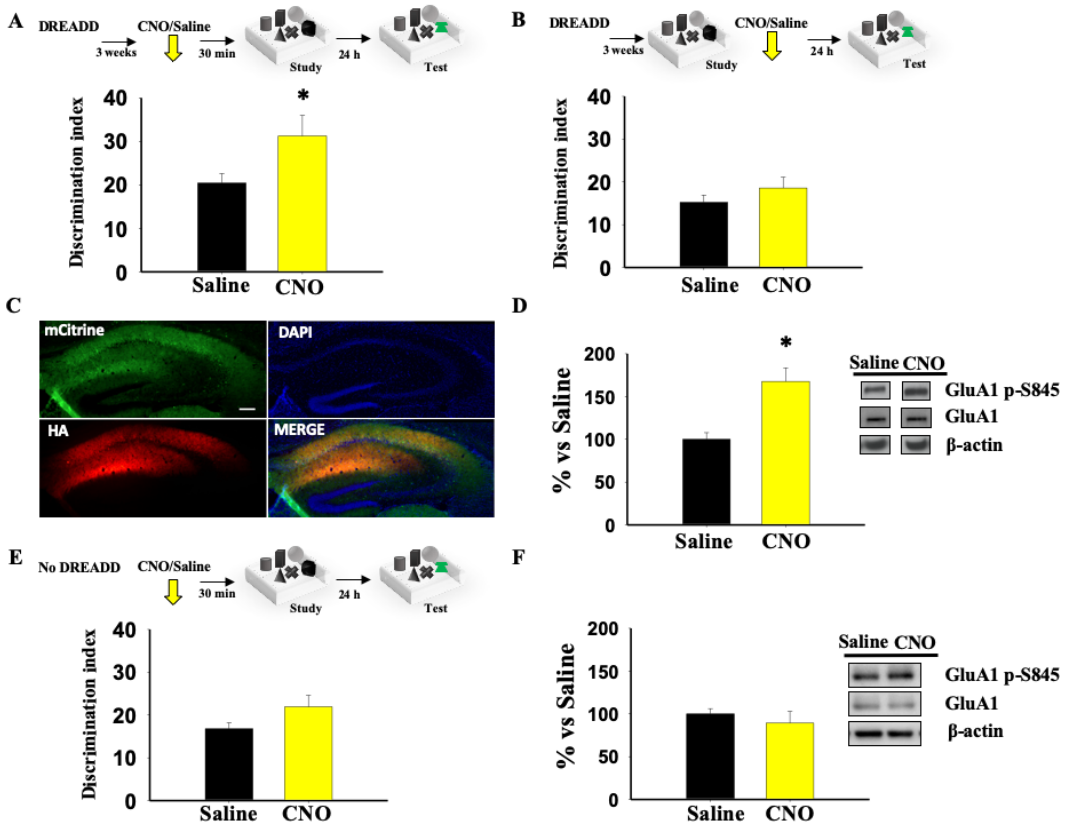

Figure 6. Dorsal HP activation with DREADDs rescues the LTM impairment in young female mice. **A.** HP stimulation through CNO intraperitoneal injection 30 min before testing rescued female mice memory consolidation in high memory load conditions (6-DOT) * $p < 0.05$ saline *vs* CNO; **B.** No effect was found when CNO was injected after the end of training, thus if it reaches the brain after about 40-60 min from the end of the training.; **C.** The expression of the HA-tagged channel rM3D(Gs) was confirmed by mCitrine reporter fluorescence and immunofluorescence for HA in dorsal HP. Scale bar: 50 µm; **D.** Hippocampal activation with DREADDs was further confirmed by the increase in GluA1 phosphorylation at S845, suggesting an increase in PKA activity after i.p. CNO injection. Representative bands for GluA1 p-S845, GluA1 and beta-actin from saline or CNO injected mice are shown on the right. * $p < 0.05$ saline *vs* CNO; **E.** No effect was found injecting saline/CNO in non-infected female mice in the 6-DOT; **F.** No increase in GluA1 phosphorylation at S845 was found in not infected female mice injected with saline/CNO. Representative bands for GluA1-S845, GluA1 and beta-actin from saline or CNO injected mice are shown on the right.

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
Saline (AAV infected) pre-training	6-DOT 24 h	26.939 ± 3.473	155.564 ± 13.070
CNO female mice (AAV infected) pre training	6-DOT 24 h	27.456 ± 2.942	140.968 ± 9.177
Saline (AAV infected) post-training	6-DOT 24 h	29.442 ± 1.152	137.467 ± 11.566
CNO female mice (AAV infected) post-training	6-DOT 24 h	28.150 ± 2.101	135.433 ± 14.572
Saline (no AAV)	6-DOT 24 h	29.046 ± 3.246	96.100 ± 13.158
CNO female mice (no AAV)	6-DOT 24 h	35.722 ± 4.820	121.738 ± 12.432

Table 2. Summary of locomotor activity (T1) and total objects exploration (T2) data of DREADDs experiment. Data are expressed as mean ± SEM.

Memory capacity prematurely decreases with ageing in female as compared to male subjects

It has been already reported that memory capacity (MC) can naturally decrease during ageing (Albert, 1997). Furthermore, MC decline seems to be a form of mild cognitive impairment that reliably predicts the conversion to Alzheimer's disease (AD; Buckner, 2004).

The LTM defect found in young female mice prompts the idea of focusing the attention on MC changes with ageing since, as mentioned before, women are more prone to develop dementia compared to men. Therefore, we decided to characterize both short-term and long-term memory capacity (STMC and LTMC, respectively) of female subjects at different ages, from young (3 months) to middle (6 and 12 months) age in the DOT/IOT.

We started the characterization of STMC testing 6 months old female mice and young controls matched for objects exploration during the study phase (3 months old). We found that 6 months old female mice (n=10) perform the 6-IOT at 1 min similarly to young subjects (n=10) [Age: $F_{(1,18)} = 0.120$; $p = 0.733$] (Fig. 7 A). No differences were found at this age for the locomotor activity at T1 [Age: $F_{(1,18)} = 0.478$; $p = 0.498$] (Table 3 B) and for total objects exploration at T2 [Age: $F_{(1,18)} = 1.357$; $p = 0.259$] (Table 3 B). However, when challenged with high memory load conditions (6-DOT, 1 min delay), 6 months old female (n=12) mice showed an impaired performance as compared to young matched controls (n=12) [Age: $F_{(1,22)} = 5.315$; $p = 0.030$] (Fig. 7 B), indicating that a memory load-dependent defect is

already detectable at this stage; also in this case, the two groups of female mice showed no differences for the locomotor activity at T1 [Age: $F_{(1,22)} = 0.319$; $p = 0.578$] (Table 3 A) and for total objects exploration at T2 [Age: $F_{(1,22)} = 0.192$; $p = 0.665$] (Table 3 A).

To establish the severity of the STMC impairment at 6 months in female mice, we considered a new group of 6 and 3 months old female mice and tested them in the 3-IOT/DOT (3-DOT: intermediate memory load) at 1 min delay (n=8 for IOT and n=7 for DOT for both groups of age). In this case, no impairment was found in 6 months old female mice as compared to matched 3 months old female mice [Age: $F_{(1,14)} = 0.094$; $p = 0.763$ for IOT; $F_{(1,12)} = 0.122$; $p = 0.732$ for DOT] (Fig. 7 C, D), indicating that the deficit observed in the 6 months old females (Fig. 7 A) is specific for high memory load conditions. Also in the 3-IOT/DOT, female mice show no differences for the locomotor activity at T1 [Age: $F_{(1,14)} = 0.038$; $p = 0.847$ for 3-IOT; Age: $F_{(1,12)} = 0.055$; $p = 0.817$ for 3-DOT] (Table 3 C, D) and for total objects exploration at T2 [Age: $F_{(1,14)} = 0.870$; $p = 0.366$ for 3-IOT; Age $F_{(1,12)} = 0.028$; $p = 0.870$ for 3-DOT] (Table 3 C, D).

Based on these results we have also characterized STMC in 12 months old female mice, hypothesizing a progressive deterioration of MC could occur. Considering that 6 months old mice were already found impaired in the 6-DOT, we tested 12 months old in the 6-IOT to evaluate possible worsening of memory performance. We tested 3 and 12 months old female mice (n=9 for both groups) in the 6-IOT at 1 min (low memory load condition) matching them for total objects exploration. We found that 12 months old female mice are able to perform the task similarly to 3 months old female mice [Age: $F_{(1,16)} = 0.213$; $p = 0.651$] (Fig. 7 E), finding also no differences between the two groups for locomotor activity at T1 [Age: $F_{(1,16)} = 0.009$; $p = 0.927$] (Table 3 E) and total objects exploration at T2 [Age: $F_{(1,16)} = 0.00003$; $p = 0.995$] (Table 3 E).

To summarize this part, we found that, at 6 months, female mice memory impairment is not only age- but also load-dependent as they are not anymore able to solve the 6-DOT (high load) at a short retention interval (1 min). These data suggested a STMC decrease in female subjects. Indeed, they are not impaired both in the control task (6-IOT; low load) and with an intermediate load (3-DOT), and the performance is stable until 12 months of age where, as described below, we performed only the 6-IOT because 12 months old female properly perform the 3-DOT at long retention interval (24 h). On the other hand, male mice performance in high memory load condition is not significantly affected until 12 months of age (Fig. 7 F). Indeed, parallel experiments in our laboratory demonstrated that only a small percentage of 12 months old male mice are impaired in the 6-DOT at 1 min delay (data not shown).

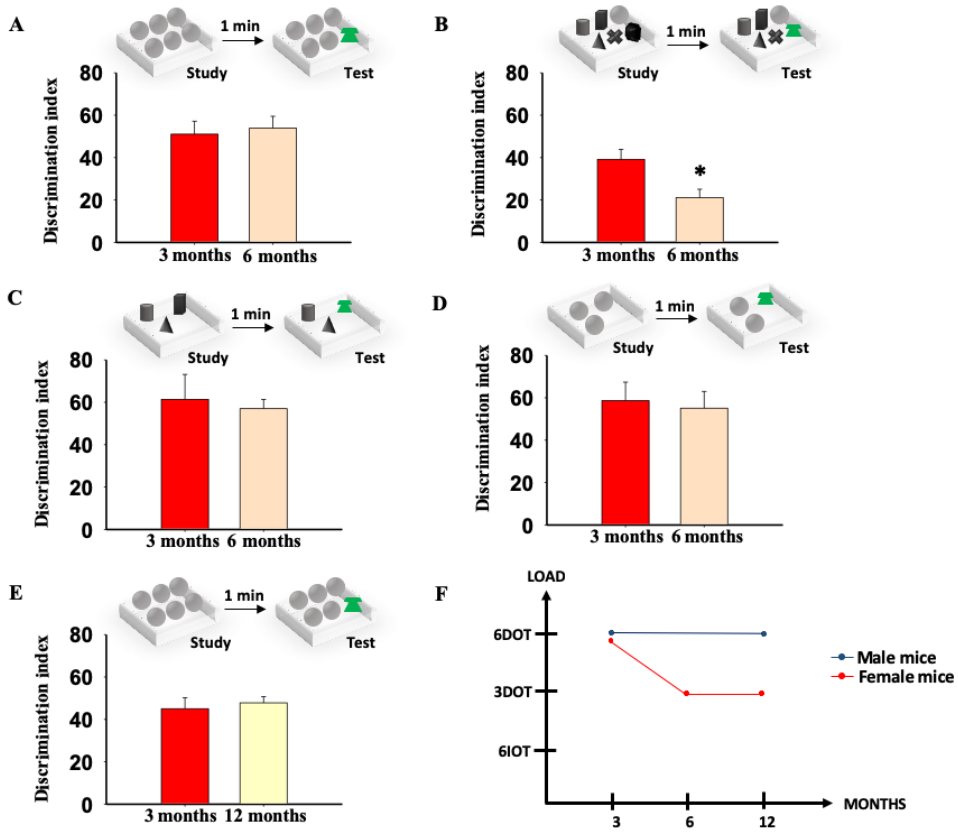

Figure 7. Age-dependent decline in STMC in female mice. Female mice 6 months old underwent the 6-IOT/DOT or the 3-IOT/DOT and were tested after 1 min delay (**A-D**). 3 months old female

mice were used as control matched for exploration time with 6 months old mice during the study phase; **A.** 6 months old female mice can discriminate the new object in condition of low memory load (6-IOT); **B.** However, they did not explore the new object in conditions of high memory load (6-DOT), showing reduced STMC; **C-D.** Same age mice can still perform the intermediate, 3-DOT (**C**), and the low, 3-IOT (**D**), memory load task; **E.** Older female mice, 12 months, were tested in the 6-IOT with 1 min delay, but no further decrease in MC was observed; **F.** Schematic summary of the STMC course considering the progression of both information load and months. * $p < 0.05$ 3 months *vs* 6 months

A

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	6-IOT 1 min	35.144 ± 2.670	34.940 ± 0.118
6 months old females	6-IOT 1 min	40.870 ± 7.838	35.080 ± 0.025

B

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	6-DOT 1 min	30.101 ± 3.141	65.725 ± 4.867
6 months old females	6-DOT 1 min	35.646 ± 9.309	69.017 ± 5.716

C

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	3-IOT 1 min	33.499 ± 3.272	34.900 ± 0.173
6 months old females	3-IOT 1 min	34.269 ± 2.173	35.063 ± 0.018

D

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	3-DOT 1 min	37.043 ± 13.204	58.400 ± 14.083
6 months old females	3-DOT 1 min	41.229 ± 11.916	61.743 ± 14.369

E

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	6-IOT 1 min	37.032 ± 2.382	33.100 ± 1.292
12 months old females	6-IOT 1 min	36.496 ± 5.244	33.111 ± 1.305

Table 3. Summary of locomotor activity (T1) and total objects exploration (T2) data of ageing STMC experiments. Data are expressed as mean ± SEM.

Concerning the LTMC characterization, we tested 6 months old female mice directly in intermediate memory load condition (3 DOT) with 24 h delay, reasoning that if 6 months female mice were already impaired in the 6-DOT with 1 min delay they would have been also impaired in the 6-DOT at 24 h delay (which represents an augmented memory capacity condition).

We found that 6 months old female mice (n=7) show a similar performance to the 3 months old ones (n=7) for LTM in the 3-DOT [Age: $F_{(1,12)} = 2.415$; $p = 0.146$] (Fig. 8 A). Again, both groups of female mice showed no differences for locomotor activity [Age: $F_{(1,12)} = 1.444$; $p = 0.252$] (Table 4 A) and for total objects exploration [Age: $F_{(1,12)} = 0.004$; $p = 0.947$] (Table 4 A). This result let us deduce that if they are not impaired in the intermediate load (3-DOT) at 6 months, they would not be also with a lower load (6-IOT).

Thus, we directly tested 12 months old female mice as compared to 3 months old-matched females (n=8 for both groups) in the 6-IOT with 24 h delay (Fig. 8 B), considering their ability to perform the same task correctly when the delay is of 1 min (Fig. 7 E). Also in this case, 12 months old female mice were able to perform the task similarly to 3 months old female mice [Age: $F_{(1,14)} = 0.156$; $p = 0.699$] and both groups of females show no differences for locomotor activity at T1 [Age $F_{(1,18)} = 1.102$; $p = 0.307$] (Table 4 B) and for total objects exploration at T2 [Age: $F_{(1,18)} = 0.0002$; $p = 0.986$] (Table 4 B). Thus, we evaluated how 12 months old female mice perform in the 3-DOT at 24 h delay, thus with a higher memory load condition then the 6-IOT. Again, we found that both groups of females showed no differences in locomotor activity at T1 [Age: $F_{(1,9)} = 0.427$; $p = 0.529$] (Table 4 C) and in total objects exploration at T2 [Age: $F_{(1,9)} = 0.012$; $p = 0.915$] (Table 4 C), and that aged female mice (n=6) perform the task like the 3 months old female mice (n=5) [Age: $F_{(1,9)} = 3.11$; $p = 0.111$] (Fig. 8 C). This result also justified why in the previous set of experiments related to the STMC for the 3-DOT we did not test the 12 months old mice as the lack of impairment in LTMC with the 3-DOT makes highly unlike to find an impairment in the same task using a shorter delay.

In parallel, we also characterized the LTMC trajectory of male mice (Fig. 8 D, E); differently from female mice, 6 months old male mice (n=10) tested in 6-DOT at 24 h delay continue to show no differences as compared to 3 months old ones (n=10) [Age: $F_{(1,18)} = 2.143$; $p = 0.160$] (Fig. 8 D) showing also no differences for locomotor activity at T1 [Age: $F_{(1,18)} = 1.591$; $p = 0.223$] (Table 4 D) and total objects exploration at T2 [Age $F_{(1,18)} = 0$; p = //] (Table 4 D). Instead, male mice are impaired at 12 months compared to young controls in the 6-DOT with 24 h delay (n=10 for both groups) [Age: $F_{(1,18)} = 18.063$; $p = 0.0005$] (Fig. 8 E). Also in this step, both groups of male mice show no differences for locomotor activity at T1 [Age: $F_{(1,18)} = 0.095$; $p = 0.761$] (Table 4 E) and for total objects exploration at T2 [Age: $F_{(1,18)} = 0.010$; $p = 0.920$] (Table 4 E).

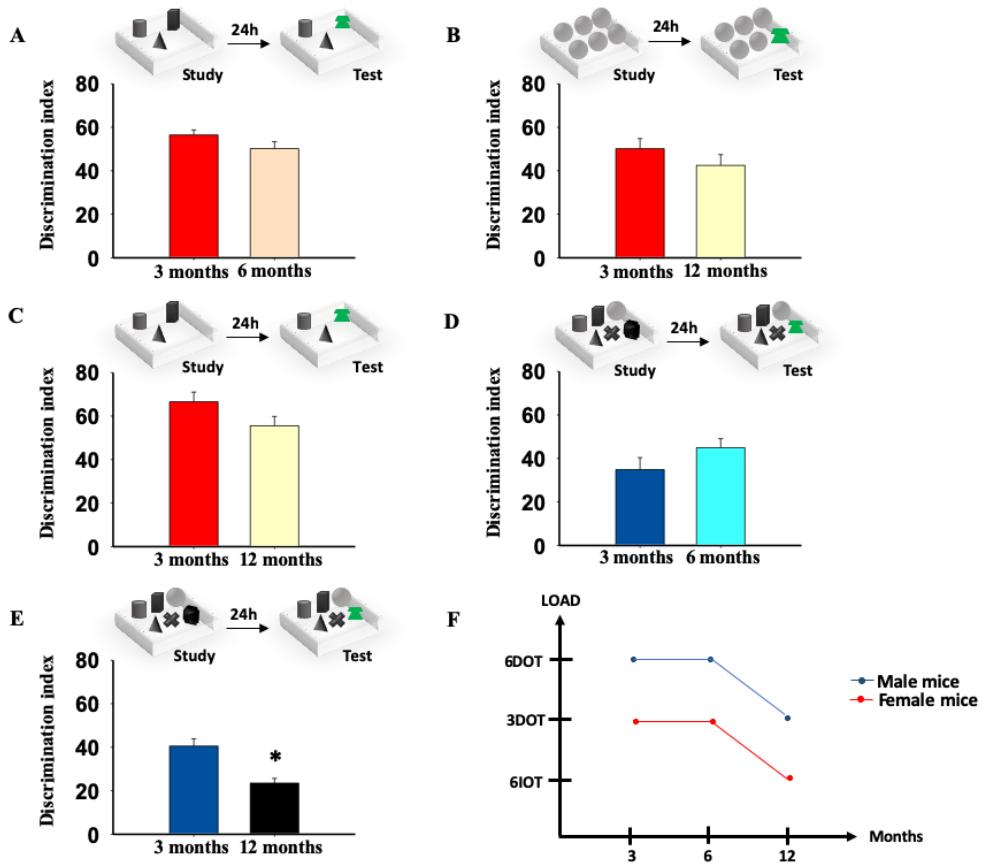

Figure 8. Age-dependent decline in LTMC in female and male mice. 6 and 12 months old female mice underwent the 3-DOT or 6-IOT and were tested after 24 h delay, while 3 months old female

mice were used as control and matched for exploration time during the study phase; **A.** 6 months female mice continue to solve the task also at long delay with intermediate load of information (3-DOT); **B-C.** 12 months old female mice demonstrated no deficit at 24 h delay test with low (**B**) and intermediate (**C**) memory load; **D.** As opposite to 6 months old female mice (Fig.7 B), male subjects show no impairment in the 6-DOT (high memory load) with 1 min delay at the same age; **E.** However, 12 months old male mice showed impaired ability to recognize the new object at 24 h test of the 6-DOT, indicating an ageing effect on performance. **F.** Schematic summary of the LTMC course considering the progression of both information load and months. * $p < 0.05$ 3 months *vs* 12 months

The graph in Figure 8 F is a schematic representation of the progressive age-dependent decline of LTMC in male and female mice to show that females manifest an earlier LTMC decline compared to male ones.

The data collected, considering male and female ageing mice, evidence that female have an earlier STMC and LTMC decline compared to male mice, since they showed a more precocious (at 3 months of age) MC decline that was delay-dependent (female mice are impaired in the 6-DOT at 24 h); in early ageing (6 months) this MC decline become also load-dependent (female mice are impaired in the 6-DOT at 1 min) and the performance remained stable until 12 months of age. Indeed, male mice showed the impairment in high memory load condition and long retention interval (6-DOT 24 h) only at 12 months of age (Fig. 7 F and 8 F).

A

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	3-DOT 24 h	31.149 ± 8.812	49.929 ± 2.413
6 months old females	3-DOT 24 h	58.473 ± 20.962	49.700 ± 2.426

B

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	6-IOT 24 h	47.667 ± 10.001	34.500 ± 0.416
12 months old females	6-IOT 24 h	36.835 ± 2.538	34.490 ± 0.429

C

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old females	3-DOT 24 h	48.188 ± 15.436	44.600 ± 6.881
12 months old females	3-DOT 24 h	37.047 ± 8.839	45.567 ± 5.694

D

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old males	6-DOT 24 h	31.499 ± 3.079	65.740 ± 3.370
6 months old males	6-DOT 24 h	26.539 ± 2.446	65.740 ± 3.370

E

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
3 months old males	6-DOT 24 h	34.404 ± 2.480	43.630 ± 5.729
12 months old males	6-DOT 24 h	33.136 ± 2.787	42.800 ± 5.817

Table 4. Summary of locomotor activity (T1) and total objects exploration (T2) data of ageing LTM experiments. Data are expressed as mean ± SEM.

Synaptic deficits associated to age-dependent memory decline in female mice

Previous data published by our lab showed that in young male mice (3 months old), the phosphorylation of the two serines 845 (S845) and 831 (S831) of GluA1 subunit of AMPA receptors, by PKA and CaMKII respectively (Olivito et al., 2014), in the HP is necessary for STM maintenance in high memory load condition (6-DOT with 1 min delay).

The MC deficits that we have found in young (3 months old) and early aged (6 months old) female mice seems to suggest a different regulation of this pathway in the HP in high memory load conditions.

To verify this hypothesis, we performed a Western Blot (WB) analysis on HP homogenates (homo) and synaptoneurosomes (syn) fractions extracted from 3 and 6 months old females exposed to the 6-DOT (for details see the section “Materials and methods”). In particular, we investigated GluA1, S845 and S831 phosphorylation

levels in young (n=3 for homo; n=2 for syn), and early aged (n=5 for homo and n=4 for syn; Fig. 9 A, B) female mice tested in the 6-DOT with 1 min delay.

Data were normalized on young female mice and expressed as % from 3 months.

In the homo fraction, the comparison between young and early aged female mice showed no differences in GluA1 levels [Age: $F_{(1,6)} = 0.136$; $p = 0.724$] (Fig. 9 A) and in the p-S845 [Age: $F_{(1,6)} = 0.015$; $p = 0.906$] and p-S831 [Age: $F_{(1,6)} = 0.095$; $p = 0.768$) levels (data not shown).

In contrast, in the syn fraction we found a strong and significant decrease in GluA1 levels in 6 months old female mice compared to the 3 months matched-young mice [Age: $F_{(1,4)} = 19.220$; $p = 0.011$]; no differences between the two groups were found both in p-S845 [Age: $F_{(1,4)} = 0.188$; $p = 0.686$] and in p-S831 [Age: $F_{(1,4)} = 0.005$; $p = 0.946$] levels after normalization on GluA1 levels (Fig. 9 B).

Overall, the results of this experiment showed that no differences were found in GluA1 levels in the homo fraction both for 3 and 6 months old tested female mice (Fig. 9 A); considering the syn fraction of the HP, in high memory load condition (6-DOT with 1 min delay) there is a strong and significant decrease in GluA1 levels in early aged tested female mice compared to young ones (Fig. 9 B).

These data suggest that the memory impairment in 6 months old female mice might be due to impaired translational modification of GluA1 receptors in the HP.

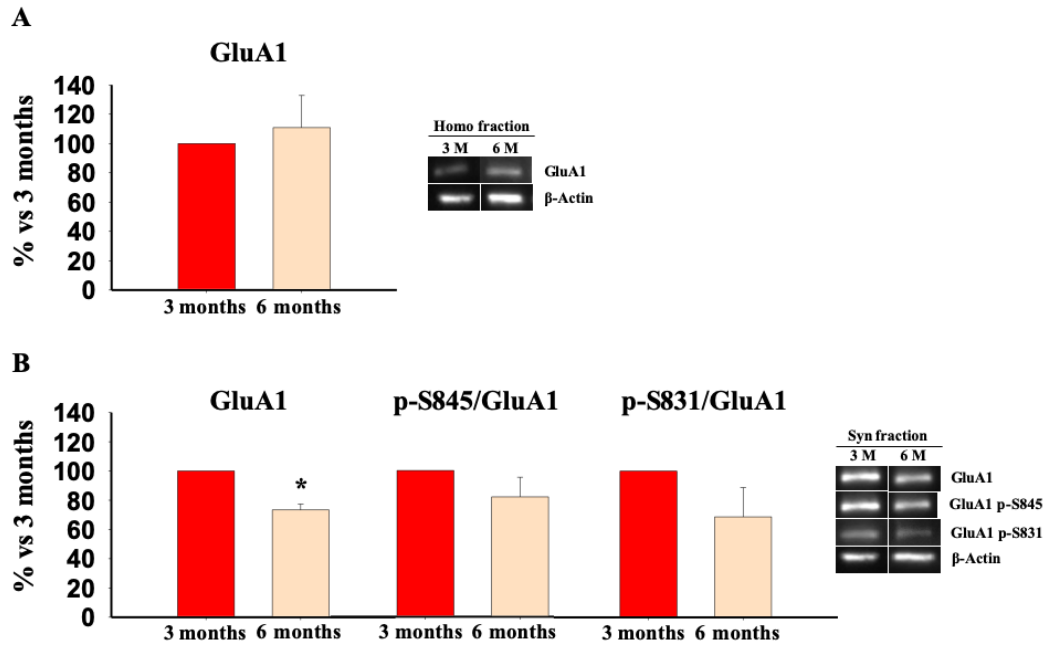

Figure 9. **GluA1 receptors expression impairment associated to the age-dependent memory capacity decline in female mice. A.** Tested 3 and 6 months old female mice HP homo fraction showed no differences in GluA1 levels. **B.** Tested 6 months old female mice HP syn fraction showed a significant decrease in GluA1 levels compared to the 3 months old tested females. No differences, indeed, were found in the p-S845 and p-S831 levels between the two group of female mice. 3 and 6 months old female mice homo (**A**) and syn fractions (**B**) representative bands for GluA1, p-S845 and p-S831 are shown on the right (3 M: 3 months; 6 M: 6 months); * $p < 0.05$ 6 months *vs* 3 months.

Aβ load evaluation in early ageing female mice

In a parallel study performed in our lab, it was observed that, in a 12 months old population of male mice, subjects impaired in the 6-DOT with 1 min delay showed an increase in misfolded proteins levels that pinpointed for a dysregulation on synaptic hippocampal mechanisms associated with HP recruitment in high memory load conditions (De Risi et al.,*Ageing in Cell,* under revision).

Considering the behavioral and biochemical data collected in female mice until now, we aimed at verifying, through native Dot-Blot analysis, whether the impaired MC in 6 months old female mice was correlated to increased Aβ load. We analysed β-

amyloid (Aβ-42) levels together with amyloid fibrils marked with the OC antibody levels on TBS and TBS-TX (corresponding, respectively, to the extracellular and intracellular) fractions extracted from young (3 months old; n=5) and early aged (6 months old; n=4) female mice exposed to the 6-DOT with 1 min delay.

Data were normalized on TBS or TBS-TX fraction of 3 months old female mice and expressed as % from 3 months.

We found a strong increase of Aβ levels in early aged female mice both in TBS [Age: $F_{(1,7)} = 15.491$; $p = 0.005$] and TBS-TX [Age: $F_{(1,7)} = 17.815$; $p = 0.003$] fractions compared to young ones (Fig. 10 A).

Similarly, detection with the OC antibody showed in early aged mice a strong increase of amyloid fibrils in the TBS-TX fraction [Age: $F_{(1,7)} = 7.084$; $p = 0.032$) compared to young ones with no differences in the TBS fraction [Age: $F_{(1,7)} = 0.321$; $p = 0.586$] (Fig. 10 B).

Overall, native Dot-Bot analysis data showed that in early aged (6 months old) female mice there was a strong intracellular accumulation of amyloid fibrils compared to young (3 months old) female mice and an accumulation of Aβ-42, which can be considered as a general increased Aβ load.

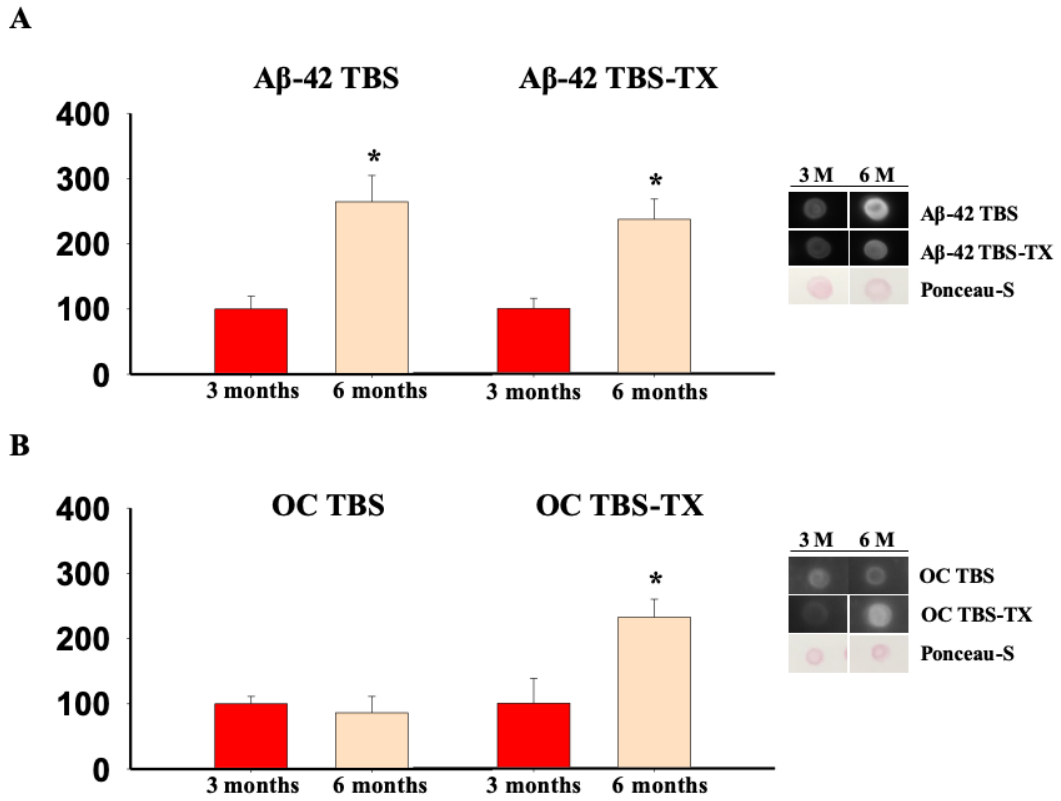

Figure 10. Increased Aβ load in early aged female mice; A. Native dot blot analysis of 6 months old female mice showed increased levels of Aβ-42 both in the TBS (extracellular) and in the TBS-TX (intracellular) fraction compared to 3 months old females. **B.** Early aged females showed also increased levels of amyloid fibrils detected with OC antibody in the intracellular (TBS-TX) fraction. Representative dots for Aβ-42 and OC are shown on the right; 3M: 3 months, 6M: 6 months. * $p < 0.05$ 6 months *vs* 3 months.

Exercise training as a tool to favor hippocampal use in female mice

In young female mice we were able to rescue the LTMC impairment and the HP hypoactivation through a chemogenetic approach (DREADDs, Fig. 6). Considering the data collected in ageing female mice and, above all, the short-term memory-load dependent deficit found in early aged female mice, we decided to test the effect of exercise training (ET) as a different rescuing strategy to improve the hippocampal activation in response to the 6-DOT. Indeed, ET has been largely demonstrated to

induce beneficial effects on animal cognition and physiology (Cotman and Berchtold, 2002) and stimulate hippocampal plasticity (van Praag et al., 1999a; 1999b; Kempermann et al., 1997). Furthermore, ET is a more translational approach than DREADDs, because its effects were confirmed in human studies, where it has been shown that it can increase the hippocampal size and ameliorate memory, with more efficiency in women (Colcombe and Kramer, 2003).

We tried to replicate this approach in mice by submitting a group of 12 months old female mice in a cage equipped with a running wheel (exercise females; *n=8*) and another group of 12 months old female mice in a normal cage (no-exercise females; control group, *n*=9) for 1 month (Fig. 11 A). This kind of ET constitutes a protocol of voluntary ET, which has been shown to improve the hippocampal-dependent performance also in mouse models of dementia (Nichol et al., 2009).

During this period, we measured the total rotations performed in the wheel cage and the body weight for both cages, three times a week for 1 month and finally we tested both groups of females in the 6-DOT with 1 min delay.

Considering only the first and the last day in which the body weight was measured, the two-way ANOVA for repeated measures show a significant interaction Body Weight x Treatment (Body Weight x Treatment: $F_{(1,15)} = 24.439$; $p = 0.0002$) but not for both Body Weight (Body Weight: $F_{(1,15)} = 0.014$; $p = 0.907$) and Treatment (Treatment: $F_{(1,15)} = 2.094$; $p = 0.168$). Indeed, in the last day, Duncan *post-hoc* analysis revealed that the difference between the two groups is almost significant ($p = 0.06$; Fig. 11 B).

In the 6-DOT, we found no differences for locomotor activity between the two groups of female mice at T1 [Treatment: $F_{(1,15)} = 0.959$; $p = 0.343$] (Table 5).

Concerning the test phase (T3), we found a significant increase in the discrimination index in mice from the ET group compared to controls [Treatment: $F_{(1,15)} = 7.932$; $p = 0.013$] (Fig. 11 C), even if the no-exercise group of female mice explored the objects during the study phase more than the exercise group [Treatment: $F_{(1,15)} = 6.502$; $p = 0.022$] (Table 5).

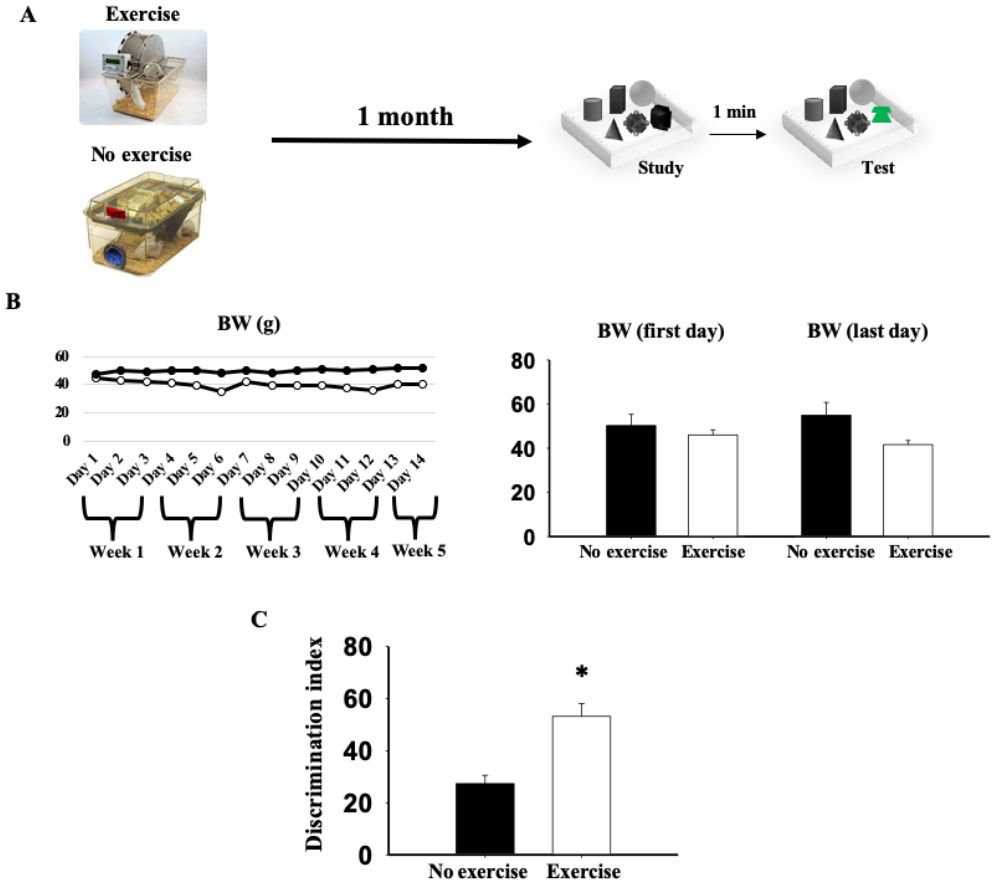

Figure 11. The exercise training approach in ageing female mice rescue their STM impairment in high memory load conditions (6-DOT). A. The experimental scheme of exercise training adopted in ageing female mice; **B.** For 1 month, the body weight was measured three times a week finding, on the last day, an almost significant difference between the two groups. **C.** After 1 month, ET increases the memory performance in the 6-DOT; * $p < 0.05$ no exercise *vs* exercise.

Group	Behavioral task	T1 [Distance (m)]	T2 [objects exploration (s)]
No exercise females	6-DOT 1 min	41.904 ± 14.835	57.089 ± 5.804
Exercise females	6-DOT 1 min	60.901 ± 12.006	28.400 ± 10.015

Table 5. Summary of locomotor activity (T1) and total objects exploration (T2) data of exercise training experiment. Data are expressed as mean ± SEM.

Overall these data demonstrate that one month of ET can improve memory performance in 12 months old mice in the 6-DOT. The hypothesis we are currently testing is that ET improves the hippocampal responsivity to the task in aged mice.

DISCUSSION

It is known that memory is the ability to retain, store and recall information collected during experience and/or through senses, and it can be classified with different criteria.

Among them, there is the one based on how long information can be stored, where we can distinguish *short-* and *long-term* memory (STM and LTM, respectively). STM, as suggested by Miller in 1956, has a limited capacity, while LTM is potentially an unlimited storage of information.

In this study we used a modified version of the *Spontaneous Object Recognition Task* developed by our lab, the *Different/Identical Object Recognition Task* (DOT/IOT; Sannino et al., 2012) that allows to study memory capacity (MC) considering both the information load and the duration.

Specifically, this task was successfully used to define the memory span in adult male mice (Sannino et al., 2012), that, similar to humans, is of 6 different objects.

Furthermore, it was useful to observe that the HP, a medial temporal lobe (MTL) structure generally recruited in regulation of MC in LTM, is recruited also in STM when the information load is high (Sannino et al., 2012). These data parallel previous clinical findings in patients showing reduced memory span for items after hippocampal lesions (Levy et al., 2003, 2004).

However, the peculiarity of all of these studies is that they only consider male subjects.

Indeed, few information about the neurobiology of MC in females have been reported in the literature, even if gender differences in memory abilities are generally amply investigated (Pauls et al., 2013; Zilles et al., 2012).

Studying sex-differences in MC becomes crucial for ageing, where MC can naturally decrease (Deary et al., 2009; Albert, 1997) and its decline can be predictive of the onset of some neurological disorders such as Alzheimer's disease (AD; Buckner, 2004). This makes the assessment of MC and its brain correlates a valid marker of early ageing in clinics (Cerami et al., 2015). The relevance of studying sex-differences in MC comes from the fact that, as reported by the Alzheimer's

Association (*Facts and Figures*, 2019), AD have a major impact in women compared to men. However, the neurobiological mechanisms of this major incidence in women are not known.

The aim of this work was to investigate, in rodents, the influence that sex can have on MC in youth and whether male and female subjects show a similar course of MC decline with ageing.

We first challenged 3 months old CD1 male and female mice in the DOT/IOT with two retention intervals (1 min for STM; 24 h for LTM). The two sexes showed no differences in the performance when they were tested with low information load (6-IOT) at both retention intervals, or increasing the information load up to 6 different objects (6-DOT; high memory load condition) with 1 min retention interval. However, with the same information load, increasing the retention interval up to 24 h, female mice showed an impairment. This data indicates a specific LTM memory deficit for female mice when coping with high information load.

To our knowledge, there are no studies in literature where male and female mice have been tested with the combination of different memory load and retention interval conditions, thus our results represent the first comparison of male/female MC.

What is known from the literature, both from humans' (Kennedy et al., 2005; Shao et al., 2014; Schwartz and Baldo, 2001; Leisman et al., 2016) and animals' studies (Gaulin et al; 1988; Voyer et al 1995; Saucier et al, 2002; Lacreuse et al, 2005) is that in female subjects there is a lower recruitment of the parietal-hippocampal circuit, which favors male subjects performance in tasks recruiting this neuronal pathway. However, women tend to be more performative in tasks requiring the frontostriatal system activation.

This led us to hypothesize that male and female mice could recruit different neuronal circuits for the 6-DOT.

To assess this hypothesis, we conducted a brain mapping experiment to measure which brain areas are actively recruited in high memory load conditions in both sexes

through a widely used approach in neurobiology that is c-fos immunohistochemical (IHC) analysis (Guzowski et al., 2001, De Leonibus et al., 2002; Maviel et al., 2004). We evaluated c-fos expression in almost all brain areas of the cortico-limbic system and found that there is a general c-fos activation almost in all regions analyzed, except the infralimbic cortex (IL) where there is no activation. This lack of activation might be due to the fact that this ventral part of the prefrontal cortex (PFC) is mainly involved in processing explicit-rewards or more biological relevant stimuli, such as social stimuli (De Leonibus et al., 2006) or to the fact that it is not involved in the consolidation of objects-related information. Interestingly, there is only one brain region exclusively activated in male mice, which is the prelimbic cortex (PL), lying just above the infralimbic area. In line with this finding is also the fact the anterior cingulate cortex, which is the associative part of the PFC, is activated in both sexes, but much more activated in male as compared to female mice, thus suggesting that frontal associative areas are underrecruited in female mice during consolidation of objects-related information. Notably, it is known that these frontal areas communicate with the HP through bilateral projections, which are involved in the processes of consolidation and retrieval of memories (as reviewed by Dudai in 2012). In line with these findings, we have found c-fos activation in the dorsal hippocampus was much lower in female as compared to male mice. This different pattern of fronto-hippocampal activation probably accounts for female mice ability to properly perform the task at short delay, but not to store them in LTM (as they are impaired with 24 h delay in the 6-DOT).

The only brain region where we found an opposite result was the Sep, which was more activated in females compared to males. The Sep is widely known to be an important site in processing of sensory information, memory, learning, consolidation and retrieval of passive avoidance response (PAR), reference memory and working memory, long-term potentiation (LTP), theta rhythm, fear, anxiety, stress, emotions, aggression, arousal, motivation, and vegetative function (De Paula et al., 2012;

Ashabi et al., 2011; Gutierrez-Guzman et al., 2011; Klinkenberg and Blokland, 2010; Klinkenberg et al., 2010; Lamprea et al., 2010; Roland and Savage, 2009).
We speculate that female mice might solve the STM task by using the Sep instead of using the HP as we have previously demonstrated for male mice (Sannino et al., 2012). Further studies are necessary to address this issue.
These data are, to our knowledge, the first evidence of a sex-related difference on brain circuitry recruitment in high memory load conditions.

We focused our attention on the hypoactivation of the HP in female mice, because the differential HP recruitment compared to males, may be the neural substrate responsible for the LTM deficit that we have previously observed, and because it has been previously demonstrated that the HP activation is functionally required in male subjects to perform the 6-DOT (Sannino et al., 2012; Olivito et al., 2016).
Before investigating the functional role of the HP hypoactivation in LTM capacity in females, we wanted to test the possible involvement of estrogens in this mechanism. Although we always tested female mice avoiding the estrus phase where, generally, female mice resulted impaired in memory tasks (Frick et al., 2001; Leal et al., 2018), we tested the eventual influence of estrogens modulation in the HP in response to high memory load request. The 17β-estradiol (E2) is the principal circulating estrogen synthetized in the gonads and a part of it, acting in the brain, is synthetized directly in the neurons (reviewed in Rosselli, 2007). E2 exerts its neuromodulatory effects binding, mainly, two kind of intracellular receptors (ERs), ER-α and ER-β, activating several signalling cascades in the cells (as reviewed in Ho et al., 2002).
Most of all these signaling cascades, such as the increase of intracellular calcium, the activation of the mitogen-activated protein kinase (MAPK) cascade, including the extracellular signal-activated kinase (ERK1/2) or the activation of the protein kinases A, B and C (PKA, PKB and PKC), may arise also from the influence of other factors; this makes quite difficult to evaluate the direct contribution of E2 on the

activation of these molecular cascades. Therefore, we verified the estrogens influence in the HP in high memory load condition by evaluating the expression levels of ER-α, which reflects E2 activity (reviewed by Foster, 2012). We evaluated only ER-α expression since E2 binds this receptor with more affinity than ER-β, and because ER-α is more transcriptionally active than ER-β (Bean et al., 2014).

Thus, if the exposure to the 6-DOT was acting on the estrogens pathway, we expected a change first in the ER-α levels.

Through IHC analysis, ER-α positive cells were counted in tested and naïve female mice in the HP subfields where this receptor is mainly expressed: CA1, CA3, DG, stratum lacunosum moleculare, stratum oriens and stratum radiatum (Rai et al, 2010). No differences were found in ER-α expression between naïve and tested females both considering all the HP subfields separately or averaged together. This result suggests no modulation of the estrogens pathway in response to the 6-DOT. Although, further biochemical assays are necessary to reach a final conclusion of the effects of high load task on estrogens receptors activation, these preliminary findings suggest that circulating estrogens do not completely account for female mice c-fos lower HP expression as compared to male mice and likely for the LTM deficit found in the 6-DOT.

Although, it has been widely reported that estrogens can exert neuroprotective effects (Henderson et al., 2000) and can promote mechanisms underlying memory formation and consolidation (Brinton et al., 1997; Woolley et al., 1993), to our knowledge, these findings for the first time explore estrogens influence in relation to memory load.

However, c-fos data are correlative; to prove that prompting HP activation could rescue their memory consolidation impairment in condition of high load (6-DOT) and long retention interval (24 h), we used DREADDs that have been widely used to modulate behavior and specifically memory processes (Whissell et al., 2016). Furthermore, DREADDs were already used to observe the concomitant recruitment of the dorsal HP and PFC in object and spatial recognition memory through

inhibiting those structure separately or in combination (Tuscher et al., 2018). However, this study considered only ovariectomized female mice tested without considering different memory load conditions.

The results of this experiment clearly showed that CNO (which is supposed to activate G_s pathway expressed through AAV 2/5-CaMKIIa-HA-rM3D(Gs)-IRES-mCitrine injections) rescued memory only when active during early memory consolidation, but not when acting 2 h later, or acting in the absence of the AAV injection. Previous findings have shown that this is the temporal window in which in the HP there is *de novo* proteins synthesis that leads to new dendritic spines formation that is a crucial for memory consolidation (Lamprecht and Ledoux, 2004; Kandel, 2012).

Western blot (WB) analysis to evaluate GluA1 phosphoSer 845 (p-S845) expression in the HP homogenates of CNO and Saline injected mice identified an increase of p-S845 levels only in CNO injected mice compared to the Saline ones, suggesting that the chemogenetic tool used efficiently activated GluA1 receptors in the HP. The activation of this serine of GluA1 subunit of AMPA receptors is mediated by PKA activation, which is a downstream target of G_s-pathway activation, and phosphorylation of S845, together with the serine 831, is necessary to cope with high memory load conditions in male mice (Olivito et al., 2014).

Recently, the proper inert nature of CNO was questioned (Manvich et al., 2018). However, CNO alone did not modify p-S845 levels and did not have effects on memory performance in female mice. These last data confirm that the rescue effect that we obtained with DREADDs was not due to an aspecific action of CNO.

As mentioned at the beginning of this section, MC can naturally decrease during ageing and its decline can be predictive of some brain disorders such as AD. The LTM defect found in young female mice prompt the idea of focusing the attention on MC changes with ageing since women are more prone to develop dementia compared to men. We reasoned that, if female mice have naturally the tendency to a

lower HP recruitment, this would weaken the HP and cause an earlier ageing compared to males, in a sort of mechanism of "use it or lose it".

To this aim, we first characterized the progression of STMC decline and found that at 6 months of age, female mice could recognize up to 3 different objects but not 6, at 1 min delay. We then characterized LTMC and, surprisingly, we have found that the LTMC defect in the 6-DOT remains stable until 12 months.

In summary, data collected for STMC characterization showed that, at 6 months, female mice memory impairment was not only time- but also load-dependent as they are not able to solve the 6-DOT (high load) at short period (1 min). These data suggested an early but specific STMC decrease since they were not impaired both in the control task (6-IOT; low load) and with an intermediate load (3-DOT), and the performance was stable until 12 months of age.

Previous findings collected in our lab demonstrated that 12 months old male mice population began to show a variability in performing the 6-DOT with 1 min delay (38%; De Risi et al., *Ageing in Cells,* under revision). This suggests that STMC in female mice is much more sensitive, as compared to male mice, to age-dependent decline.

Concerning LTMC, surprisingly, at 12 months of age, both male and female mice were impaired exclusively in the highest memory load conditions. This finding suggests that the STMC and the LTMC in female mice might be due to different neuronal regulation.

Our data concerning STMC and LTMC characterization in female mice constitute a new finding in the literature because, as in the majority of studies, aged female mice have not been tested considering different memory load conditions (Fan et al., 2010; Fortress et al., 2014). Moreover, while we tested adult (6 months old) and middle aged (12 months old) mice, other studies consider mostly older female mice (Frick et al., 2000; Harburger et al., 2007), highlighting the sensitivity of the DOT/IOT to recognize early memory impairments.

Previous data published by our lab showed that, in young male mice (3 months old) in high memory load conditions (6-DOT with 1 min delay), the phosphorylation of the two serines 845 (S845) and 831 (S831) of GluA1 subunit of AMPA receptor, by PKA and CaMKII respectively (Olivito et al., 2014), is required in the HP.

Generally, age-related cognitive decline is caused by alterations in synaptic number and function in brain regions responsible for memory-related tasks, such as the HP (reviewed in Burke, 2006). Aged animals may show lower levels of LTP induction and increased LTD compared with young animals (Tombaugh et al, 2002).

As reviewed by Henley in 2013, these changes in HP synaptic plasticity can be attributable to defects in AMPA receptors (AMPARs) trafficking.

Overall, our data suggested that in early aged female mice the integrity of the hippocampal synapses is compromised, and it seems to be related to the lower expression of GluA1 subunit of AMPARs in HP and AMPARs dysfunction is typical of Alzheimer's disease (AD; Henley et al, 2013).

Furthermore, disruption of AMPAR trafficking by soluble amyloid beta (Aβ) oligomers is a hallmark of synaptic dysfunction in AD (reviewed in Walsh, 2007). Indeed, accumulation of Aβ42 and OC+ amyloid fibrils has been previously observed in genetic animal model of AD (Rocchi et al., 2017) and ageing (Cruz et al., 2018).

In a parallel study performed in our lab, it was found that 12 months old male mice with impaired STMC showed an increase in Aβ load and impaired GluA1 functional activation in the HP as compared to age-matched cognitively intact mice or to 3 months old mice (De Risi et al., *Ageing in Cells,* under revision).

Using a similar approach, we show here for the first time that impaired STMC in 6 months old female mice is associated to a strong intracellular accumulation of amyloid fibrils compared to young (3 months old) females and an increase in monomeric Aβ-42.

Our biomarkers data collected in female mice, in line with the studies reported above, showed a decreased GluA1 in the synaptosomal fraction of the HP that, in

concomitance with the increased levels of soluble Aβ, could account for the STM deficit found in early aged female mice and represents a condition of early ageing.
These data, together with the behavioral ones, remarked that age-related cognitive decline is anticipated in females compared to male mice, in line with the major propensity of women to develop dementia during ageing compared to men.

In the last part of this work, since in young female mice we were able to rescue the LTMC impairment and the HP hypoactivation through a chemogenetic approach (DREADDs), we decided to try a more "natural" approach to rescue the memory deficit also in aged female mice, namely voluntary exercise training (ET).
ET has been largely demonstrated to induce beneficial effects on animal cognition and physiology (Cotman and Berchtold 2002) and stimulate hippocampal plasticity (van Praag et al., 1999a; 1999b; Kempermann et al., 1997). Since our working hypothesis was that the memory impairments found in female mice may be mainly hippocampal dependent, we tested the effect of ET on STMC in middle aged female mice, which are generally impaired at this age. Choosing the ET meant to use a more translational approach than DREADDs, because its effects were confirmed in human studies, where it has been shown that it can increase the hippocampal size and ameliorate memory, with higher efficiency in women (Colcombe and Kramer, 2003).
We used a protocol of voluntary ET which has been shown to improve the hippocampal-dependent performance also in mice models of dementia (Nichol et al., 2009), and we found a significant increase in the discrimination index in mice from the ET group compared to controls.
This data, that, obviously, is only behavioral and must to be confirmed with further analysis, was very striking.
As future aim for this part, we want to evaluate whether ET in female mice, beyond the mnemonic performance, can also rescue the Aβ accumulation together with the decreased synaptic GluA1.

SUMMARY AND CONCLUSIONS

In this study, we aimed at exploring sex-related differences on MC using the DOT/IOT, developed in our lab in 2012, which was already useful to determine memory span in male mice and to demonstrate the HP recruitment in regulating STM, and not only LTM, in high memory load conditions (Sannino et al., 2012; Olivito et al., 2014). Furthermore, we aimed also to use this task to identify sex-differences in MC during ageing, an idea originated from clinical findings showing that women are more prone to develop dementia compared to men.

We first collected data in young (3 months old) male and female mice, where we found for female mice a long-term memory-load dependent deficit. Specifically, female mice were impaired when coping with high information load and long retention interval (6-DOT with 24 h delay) compared to male mice.

This led us to hypothesize that male and female mice could recruit different neuronal circuits when coping with high information load. Brain mapping through c-fos IHC analysis showed, mainly, that female mice, in high memory load conditions, activated the HP to a lesser extent compared to male mice. Furthermore, we observed null estrogens influence in female mice HP in response to high memory load.

Next, we traced the sex-differences on MC course during ageing where female subjects showed an earlier MC decline compared to males both in STM and in LTM. Behavioral and c-fos data suggested us to better understand which was the HP recruitment in female mice evaluating those pathways necessary for HP recruitment in high memory load condition.

Through WB analysis we found, in early aged female mice (6 months old), that the integrity of the hippocampal synapses was compromised, as demonstrated by the lower expression of GluA1 subunit of AMPARs in the HP.

Following Dot-Blot analysis revealed, in early aged female mice, a strong accumulation of Aβ fibrils compared to young subjects that may account for the abnormal integrity of synaptic mechanism underlying the HP recruitment in high memory load conditions. These data were very similar to those found in parallel in

our lab considering only male mice. The very peculiarity of these data is that the features described above are strongly anticipated in females.

Finally, in this work we aimed to rescue memory impairments found in young and early aged (3 and 6 months old, respectively) female mice using two different strategies.

In young female mice, using a chemogenetic approach (DREADDs) we were able to rescue the LTM deficit in the 6-DOT by stimulating the HP activation.

In early aged female mice, we adopted a more translational strategy, the ET, that, as it has been reported in human studies, ameliorates the performance in hippocampal-dependent tasks and showed major benefits in female compared to male subjects.

As expected, in the exercised group of female mice we were able to rescue the high memory load STM deficit. However, this data needs to be integrated with the evaluation of the possible rescue of the synaptic mechanisms underlying the HP recruitment in high memory load conditions.

Overall, our data suggested that there are relevant sex-differences on MC that can be accounted to a different usage of brain areas between sexes, above all concerning the HP.

Specifically, in young mice experiments, we observed, with the c-fos data, a lower HP activation in females compared to male mice that worsened with ageing where we found an early dysregulation of those mechanism underlying the HP recruitment in high memory load conditions in female mice, together with a strong accumulation of Aβ fibrils in early ageing.

This probably suggests that in females subjects the less usage of the HP can make this structure more sensible to ageing insults and it can account, by shifting the focus to humans, for the major propensity of women to develop some kind of dementia such as the AD compared to men, through a sort of “use it or lose it” mechanism.

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