Department of Biology and Biotecnhology

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Department of Biochemistry and Biotechnology

«Dietary supplementation of Hericium erinaceus medicinal mushroom: effects on spatial memory and recognition memory in wild-type mice»

«Συμπλήρωμα διατροφής του φαρμακευτικού μανιταριού Hericium erinaceus:επιδράσεις στη χωρική μνήμη και στη μνήμη αναγνώρισης σε ποντίκια αγρίου τύπου»

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ABSTRACT

Hericium erinaceus (Hr, Bull.) Pers. is a medicinal mushroom (MM) with potential neuroprotective effects. The study of Hr has attracted considerable attention in the last 10 years regarding the possibility to use it in diseases like Alzheimer, dementia and motor dysfunctions. Despite an extensive investigation of the preventing action of Hr in cognitive pathological conditions, to date, no studies have investigated the effects of dietary supplementation with Hr in healthy mice. After oral administration for two months with Hr in wild-type mice, we assess the novelty exploration behavior and recognition memory by using Novel object recognition task (NOR test) and Emergence test. Furthermore, to study the effect of Hr in spatial memory we evaluated the effect by using Y-maze and Object Location task (OL).Data presented in the thesis, indicate that after Hr oral supplementation, wild-type mice show increased recognition memory and increased general locomotion activity but we did not record any effect on spatial memory performance. Those data reveal that the effect of Hr oral supplementation in wild-type mice is specifically and selectively addressed to recognition memory but did not affect spatial working memory.

ΠΕΡΙΛΗΨΗ

Το *Hericium erinaceus* (Hr, Bull.)Pers, είναι ένα φαρμακευτικό μανιτάρι (MM) με πιθανές νευροπροστατευτικές επιδράσεις. Η μελέτη του Hr έχει προσελκύσει μεγάλη προσοχή τα τελευταία 10 χρόνια όσον αφορά τη δυνατότητα χρήσης του σε ασθένειες όπως η νόσος Alzheimer, η άνοια και οι κινητικές δυσλειτουργίες. Παρά την εκτεταμένη διερεύνηση της παρεμποδιστικής δράσης του Hr σε γνωστικές παθολογικές καταστάσεις, μέχρι σήμερα δεν έχουν διερευνηθεί οι επιδράσεις του Hr διατροφικού συμπληρώματος σε υγιείς ποντικούς. Ύστερα από χορήγηση Hr εκ του στόματος ,για δύο μήνες, σε ποντίκια άγριου τύπου, αξιολογήσαμε τη συμπεριφορά εξερεύνησης «του νεου» και τη μνήμη αναγνώρισης, χρησιμοποιώντας τη δοκιμασία αναγνώρισης νέων αντικειμένων (test NOR) και το Emergence test. Επιπλέον, για να μελετήσουμε την επίδραση του Hr στην χωρική μνήμη, αξιολογήσαμε το φαινόμενο χρησιμοποιώντας το Y-maze test και την δοκιμασία εντοπισμού της θέσης των αντικειμένων (OL test). Τα δεδομένα που παρουσιάζονται στη πτυχιακή εργασία δείχνουν ότι μετά από τη χορήγηση Hr συμπληρώματος από το στόμα, τα ποντίκια

άγριου τύπου παρουσιάζουν αυξημένη μνήμη αναγνώρισης καθώς και αυξημένη γενική κινητική δραστηριότητα, αλλά δεν καταγράφηκε καμία επίδραση στην απόδοση της χωρικής μνήμης. Αυτά τα δεδομένα αποκαλύπτουν ότι η επίδραση του Hr συμπληρώματος σε ποντίκια άγριου τύπου, απευθύνεται ειδικά και επιλεκτικά στη μνήμη αναγνώρισης και δεν επηρεάζει τη χωρική μνήμη.

CHAPTER 1

Hericium erinaceus

1.1 Introduction

Hericium erinaceus (Hr,Bull.) Pers. (also known as Yamabushitake, Lion's Mane, or Satyr's beard) is an edible and medicinal mushroom which native to North America, Europe and Asia. It is one of the wood-destroying fungi that cause white rot (Mizuno T., 1999). It is a temperate that grows on both living and dead broadleaf trees and requires cool temperatures of 18 ^{o}C to 24 ^{o}C to produce fruit bodies (Fig.1). The nutritional and medicinal properties of *Hericium erinaceus* produced in low temperature conditions are well known and documented in Europe, China and Japan. (Wong et al., 2012)

Fig.1 –*Hericium erinaceus fresh fruit bodies.*

1.2 Natural habitat and origin

Hr is a saprophytic inhabitant on dead trunks of hardwoods, including oak, walnut, beech, maple, sycamore, elm and other broadleaf trees (Fig. 2, 3). The first report on the cultivation of Hr was published in China in 1988. Hr was well-known and treasured in traditional Chinese and Japanese cookery and medicine, for hundreds of years. In China, it is called Houtou, as its fruit bodies look like the head of a baby monkey, and Shishigashira (Lion's Head). It is one of the four famous dishes in China. In Japan, it is called Yamabushitake, it resembles the ornamental cloth worn by Yamabushi- Buddhist monks practicing asceticism in the mountains. It is also called Jokotake (funnel-like), Usagitake (rabbit-like) and Harisembontake (porcupine fish-like) according to its appearance (Wong et al., 2012).

Fig.2 -*Hr fruit body inside fallen hollow beech*.

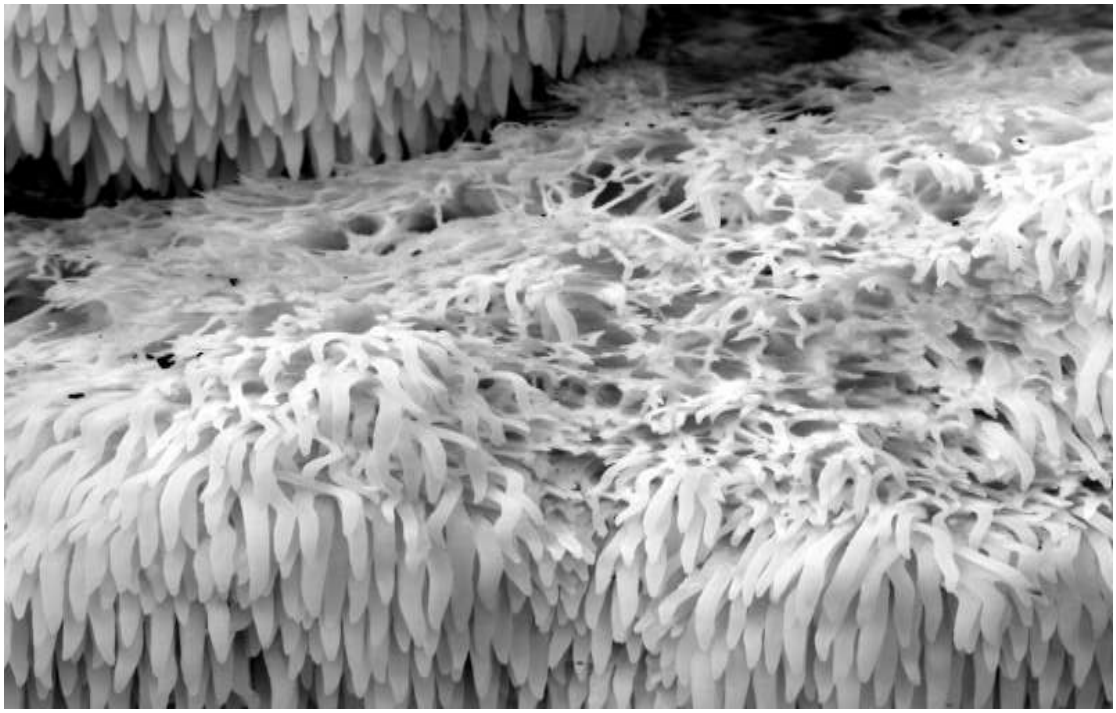

Fig.3 -*Detail of Hr upper surface of fruit body*.

1.3 Medicinal properties

The health benefits of Hr as a curative for problems of digestive tract such as stomach and duodenal ulcers are widely known among Chinese doctors. Ingestion of this mushroom was reported to have a remarkable effect in extending the life of cancer patients. Pills were used in the treatment of gastric and esophageal carcinoma. Further, sandwich biscuits supplemented with the fruit bodies were used in the prevention and treatment of nutritional anemia of preschool children.
Hericenones A and B (cytotoxic phenols) and a novel fatty acid isolated from fruit bodies, exhibited cytotoxity against HeLa cells. Hericenone B had strong antiplatelet activity, and it might be a novel compound for antithrombotic therapy possessing a novel mechanism (Mori et al., 2010).
Fifteen polysaccharides were isolated from hot-water extracts of Hr fruit bodies. Five of them showed antitumor activity and prolonged the longevity of the hosts. Polysaccharides of Hr possess anti-skin-aging activities by enhancing skin antioxidant enzymes, NMP-1 and TIMP-1 activities, and collagen protein levels in aged rats. Methanol extract of fruit bodies was found to have a hypoglycemic effect and reduce elevation rates of serum triglyceride and total cholesterol levels when administered to streptozotocin-induced diabetic rats (Xu et al., 2010). Purified components of Hr act as enhancers to sensitilize doxorubicin (Dox)- mediated apoptotic signaling, and this sensitization can be achieved by reducing c-FLIP expression via JNK activation and enhancing intracellular Dox accumulation via the inhibition of NF-kB activity (Lee JS et al., 2010). These findings suggest that Hr in combination with Dox serves as an

effective tool for treating drug-resistant human hepatocellular carcinoma (Wong et al., 2012).

1.4 Neuroprotective properties

Hr consists of several components, including polysaccharides, proteins, lectines, erinacol and terpenoids, some of whose biological activities have been studied. Hericenone and erinacine have been isolated from the fruiting body and mycelia of Hr, respectively, and hericenone C-H and erinacine A-I, have been shown to stimulate nerve growth factor (NGF) synthesis in cultured astrocytes (Kawagishi et al., 1991, 1994, 1996).
They particularly involved in the survival and organization of cholinergic neurons in the central nervous system (Wong et al., 2012). NGF levels decrease in patients of Alzheimer's disease. In these, the last NGF levels decreases at the frontal cortex. It has been noted that Hr increases NGF secretion, indicating the potential of this fungus in inhibiting the progression of dementia and senile plaques in humans (Friedman et al., 2015). To confirm this, in (Mori et al., 2011), it is reported that mouse models with memory and learning deficits, induced by the accumulation of β-amyloid peptide (25-35) after being fed with Hr powder, show an improvement in cognitive abilities in the recognition test of objects (NOR).
The potential neuroprotective effects of *Hericium erinaceus* in neurodegenerative diseases, including dementia and motor dysfunction, have therefore attracted considerable attention, because both hericenone and erinacine are low-molecular weight, relatively lipid soluble compounds that are able to pass the blood –brain barrier (Hazekawa et al., 2010).
Hericenones are aromatic compounds isolated from the fruting body of Hr. Fresh fruting bodies of the fungus were extracted with acetone. Repeated chromatography of the chloroform-soluble fraction obtained by solvent partitions of the extract with silica gel followed by HPLC with ODS column gave hericenones.
Hericenones A (Fig. 4 structure n^{o} 1), B (Fig. 4 structure n^{o} 2), C, D, E (Fig. 4 structure n^{o} 3, 4, 5), F, G, H (Fig. 4 structure n^{o} 6, 7, 8), ericenes A, B, C(Fig.4 structure n^{o} 9, 10, 11), and hericerin (Fig. 4 structure n^{o} 12) were isolated from the mushroom Hr. Hericenones C, D and E exhibited stimulating activity for the biosynthesis of NGF in vitro (Ma et al., 2010).

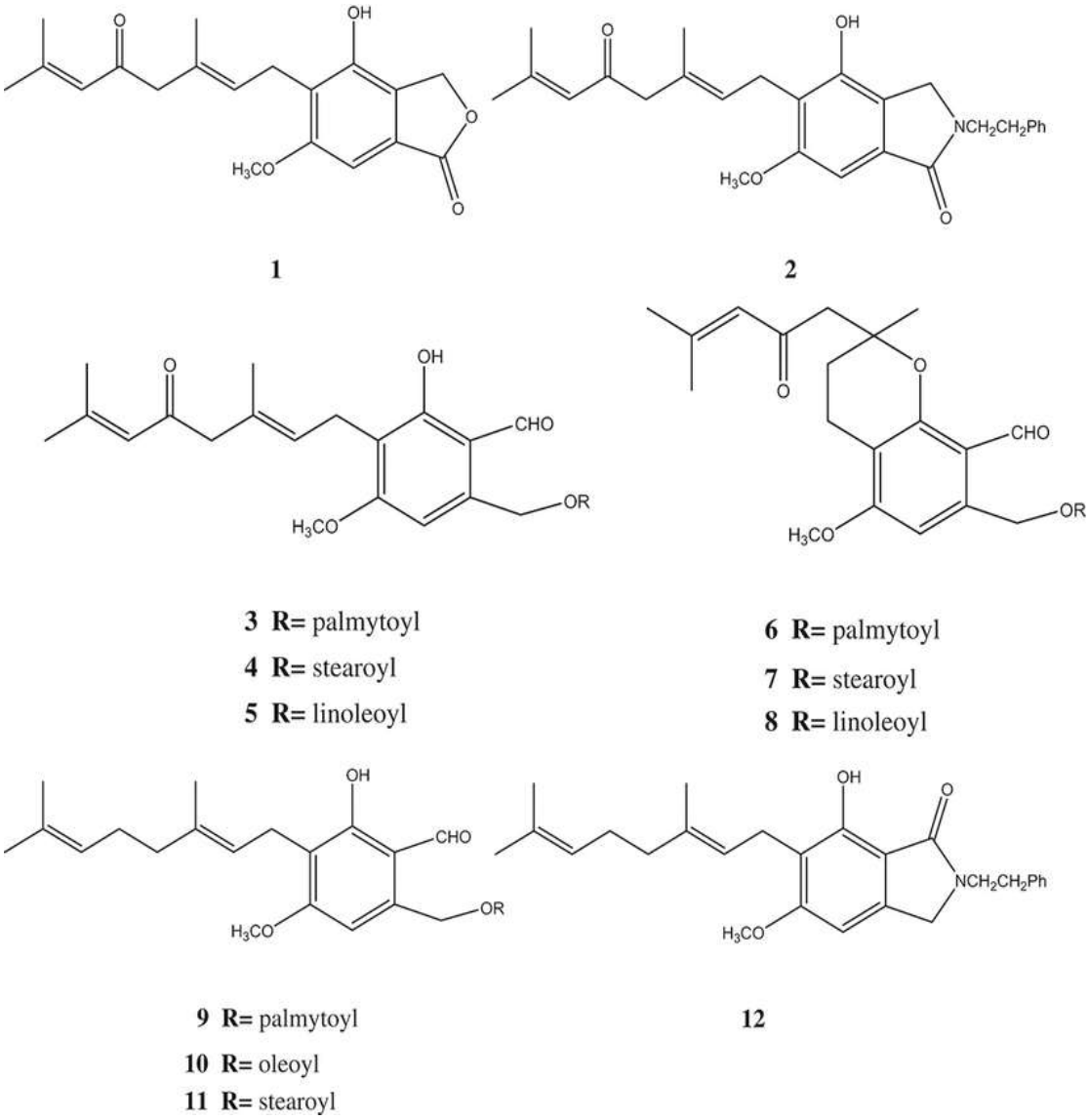

Fig.4 *–Structures of compounds 1-12.*

Erinacerin A (Fig. 5 structure n^{o} 13) and B (Fig. 5 structure n^{o} 14), 3-Hydroxyhericenone F (Fig. 5 structure n^{o} 15), hericenone I (Fig.5 structure n^{o} 16) and hericenone J (Fig. 5 structure n^{o} 17) were also isolated from the fruiting bodies of Hr.

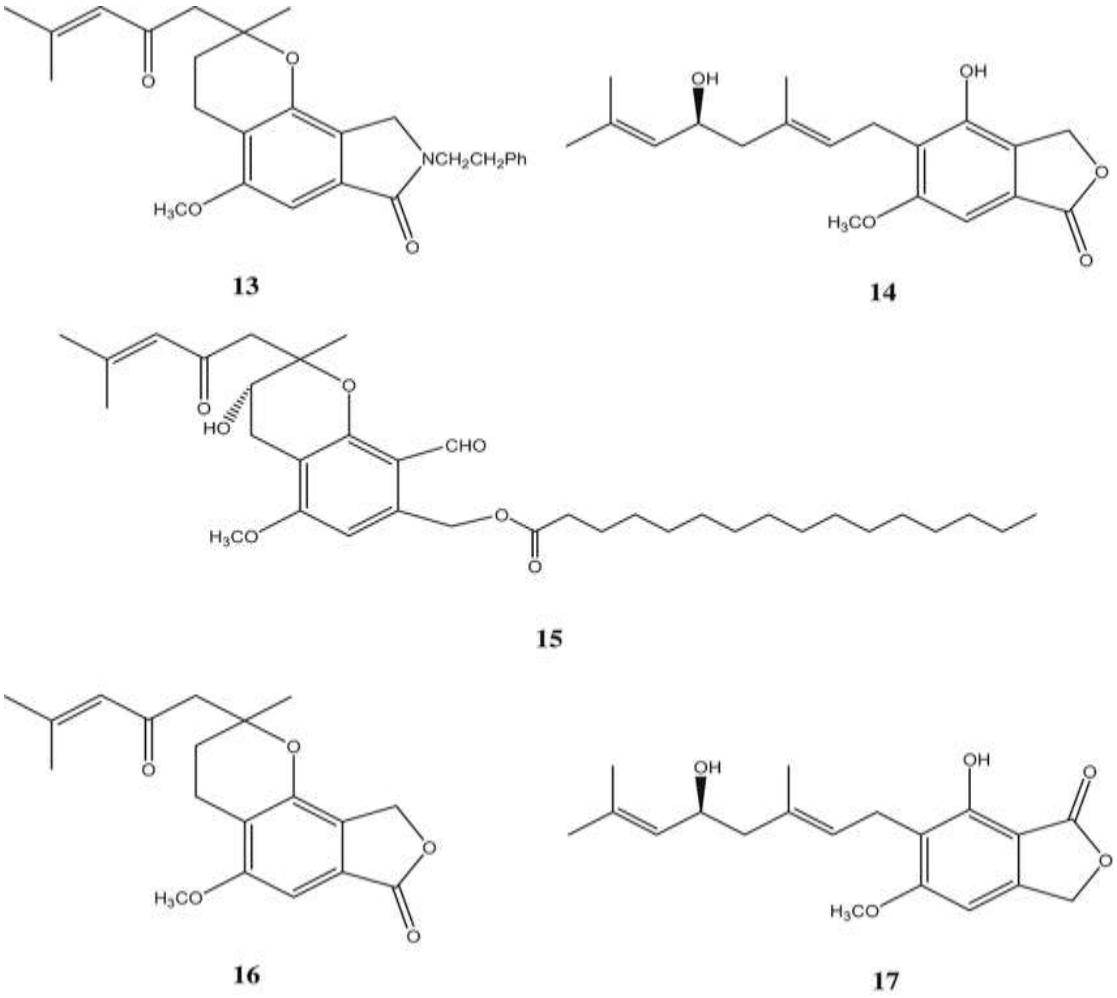

Fig.5 –*Structures of compounds 13-17.*

All erinacines possess a cyathane skeleton consisting angularly condensed five-, six- and seven-membered rings. Erinacines A, B, C (Fig. 6 structure n° 18, 19, 20), D (Fig. 6 structure n° 21), E, F, G (Fig. 6 structure n° 22, 23, 24), H, I (Fig. 6 structure n° 25, 26), P (Fig.6 structure n° 27), Q (Fig. 6 structure n° 28), J, K (Fig.6 structure n° 29, 30), R (Fig.6 structure n° 31) and erinacol (Fig. 6 structure n° 32), isolated from the mycelia of Hr, show stimulating activity for NGF biosynthesis. The fungus was cultivated by shaking at 30 °C for 4 weeks; then the culture was centrifuged and the mycelia were extracted with ethanol. The extract, after concentrating the solvent, was fractionated by solvent partition between ethyl acetate and water. Repeated silica gel chromatography and HPLC of the ethyl acetate extract gave erinacines (Ma et al., 2010).

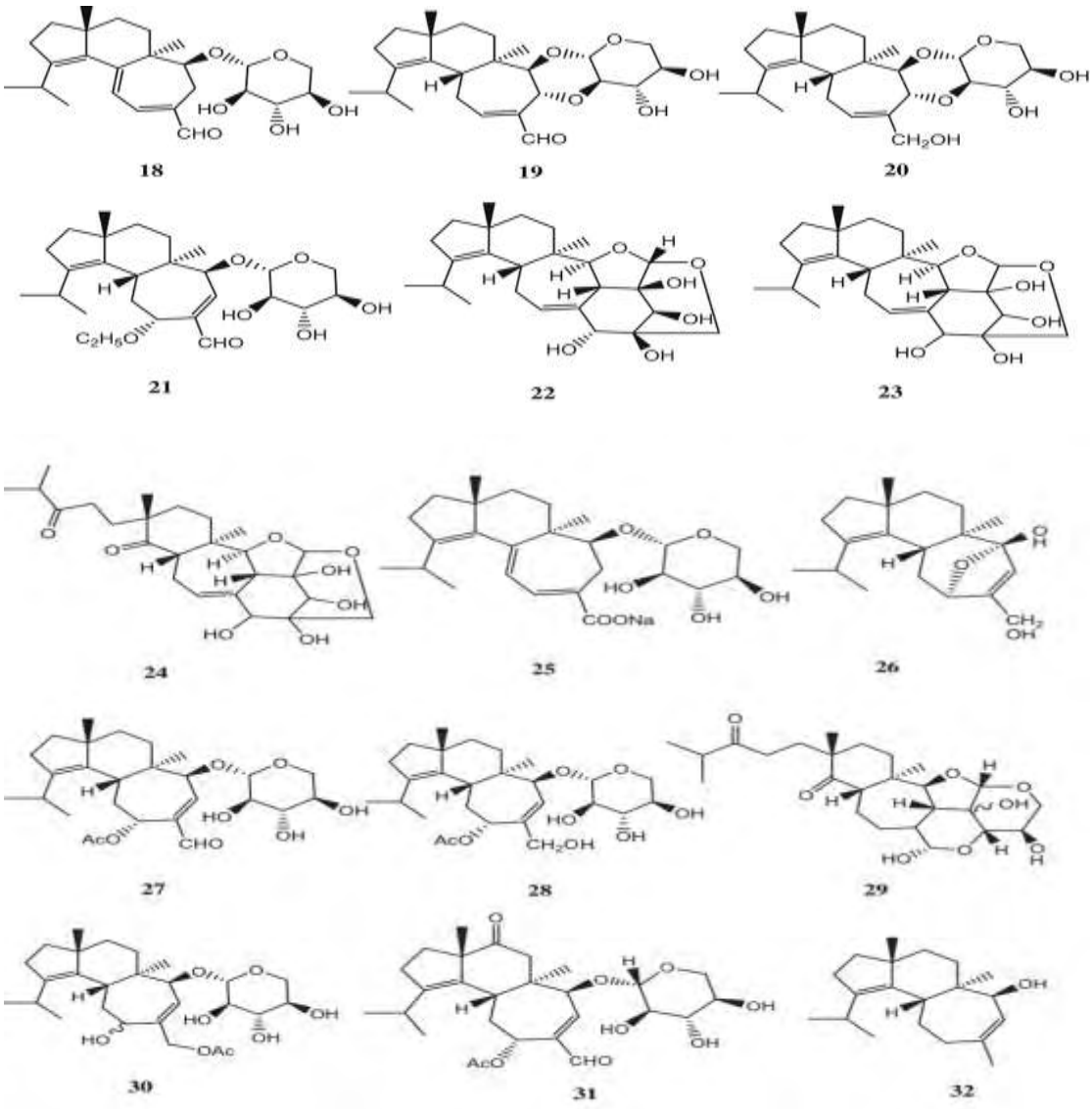

Fig.6 –*Structures of compounds 18-32.*

CHAPTER 2

The Memory

2.1. The Importance of the medial temporal lobe in memory processes

Several brain regions are involved in the storage of memory traces, but not all areas have the same importance (Kandel ER et al., 2003). It has been observed that subjects with temporal lobe damage show deficiency of memory (Ameen-Ali Ke et al., 2015). In this regard, studies on amnesia have been carried out by observing subjects reporting surgical lesions on a temporal lobe. It has been previously noted that the electric stimulation of the lobe produced a "memory of a lived experience". These observations, however, did not convince the scientific community that the temporal lobe could be an important place for memory processes as all the patients examined had epileptic outbreaks in the temporal lobe, and the most effective sites to evoke experience responses were close to these outbreaks. So, patient's responses could, therefore have been the product of localized seizure activity. In addition, these types of responses were obtained in only 8% of all stimuli performed in the temporal lobe. Subsequently, evidence of the importance of temporal lobes in mnemonic processes was provided, around 1950, by the study of patients who had undergone bilaterally removal of the hippocampus and the zones adjacent to the temporal lobe for the treatment of epilepsy (Kandel ER et al., 2003).

2.2 Medial temporal lobe structure

The medial temporal lobe consists of the combination of two regions (Fig. 7): the parahippocampus and the hippocampus and it can be subdivided into the:

- Parahippocampal regions, which include: the perirhinal cortex, the parahippocampal cortex and the entorhinal cortex.
- Hippocampus which includes: the Cornu Ammonis, the dentate gyrus and the subiculum. (Eichenbaum H. et al., 2007)

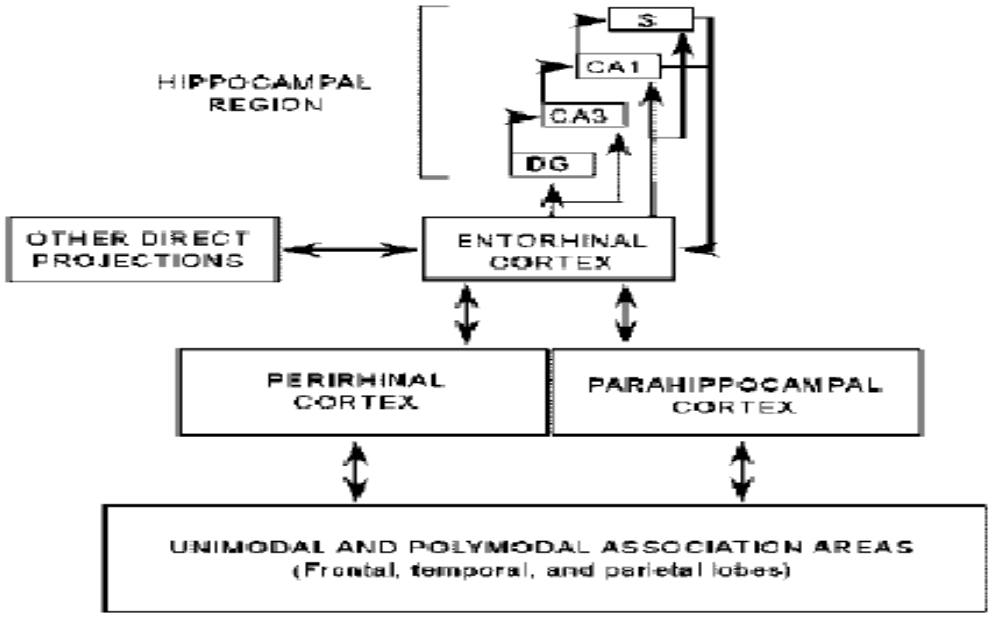

Fig.7 -*A schematic view of the medial temporal lobe structures important for declarative memory.*

2.3 Hippocampus in general

One of the most captivating features of the hippocampus is its neuroanatomy. The relatively simple organization of its principal cell layers coupled with the highly organized laminar distribution of many of its inputs has encouraged its use as a model system for modern neurobiology. The hippocampus proper has three subdivisions: CA1, CA2, CA3 (CA derives from Cornu Ammonis. The other regions of the hippocampal formation include the dentate gyrus, subiculum, presubiculum, parasubiculum and ethorinal cortex.

A common organizational feature of connections between regions of the neocortex is that they are largely reciprocal. As first described by Ramon y Cayal (1983), this is clearly not the case for the connections that link the various parts of the hippocampal formation.

The entorhinal cortex can be considered the first step in the intrinsic hippocampal circuit. Cells in the superficial layers of the entorhinal cortex give rise to axons that project the dentate gyrus. The projections from the entorhinal cortex to the dentate gyrus form part of the major hippocampal input pathway called perforant path. The principal cells of the dentate gyrus, the granule cells, give rise to axons called mossy fibers that connect with pyramidal cells of the CA3 field of the hippocampus. These cells are the source of the major input to the CA1 hippocampal field (the Schaffer

collateral axons). The CA1 field then projects unidirectionally to the subiculum, providing its major excitatory input, but also to the enthorinal cortex (Fig. 8).

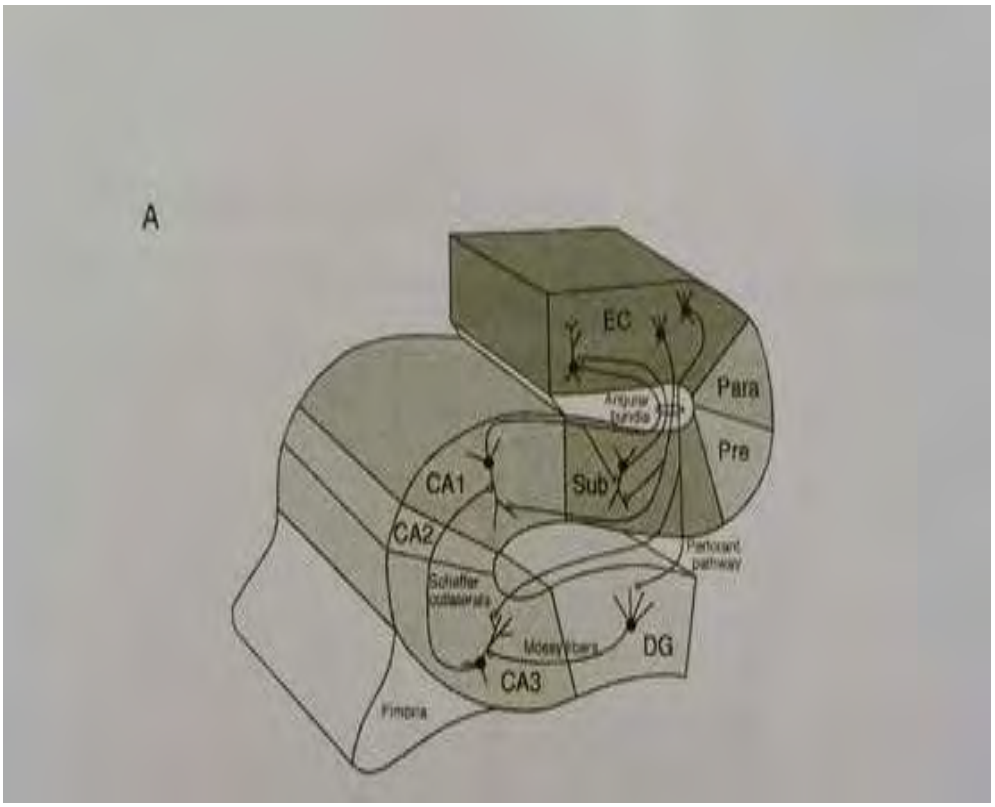

Fig.8 *–The hippocampal structure. Neurons in layer II of the entorhinal cortex project to the dentate gyrus and the CA3 field of the hippocampus proper via the perforant pathway. Neurons in layer III of the entorhinal cortex project to the CA1 field of the hippocampus and the subiculum via the perforant and alvear pathways. The granule cells of the dentate gyrus project to the CA3 field of the hippocampus via mossy fiber projections. Pyramidal neurons in the CA3 field of the hippocampus project to CA1 via Shaffer collaterals. Pyramidal cells in CA1 project to the subiculum. Both CA1 and the subiculum project back to the deep layers of the entorhinal cortex.*

2.4 Information Path in the Medial temporal lobe

Information, such as visual and tactile, from the cortex, enter the medial temporal lobe from different points (Squire LR et al., 2004). The main inputs to the perirhinal cortex are from associative areas that process information on object quality (the "thing") in a unimodal manner. Instead, most of the inputs arriving at the level of the parahippocampal cortex, come from areas that process polymodal spatial information, that is, information on object localization (the "where") (Eichenbaum H. et al., 2007). The parahippocampal cortex receives primarily information from the dorsal areas (Squire R. et al., 2004).

Information about the representation and location of objects ("what" and "where") remain essentially separate: the perirhinal cortex projected mainly at the lateral entorhinal cortex, while the parahippocampal cortex is projected mainly at the medial part of the entorhinal cortex (Fig. 9). Although there are connections between the perirhinal cortex and the parahippocampal cortex and also between areas of the entorhinal cortex, the information proceeds for different pathways to converge subsequently to the hippocampus level (Eichenbaum E. et al., 2007). Hippocampal output generates feedback connections, ie hippocampus information about location and representation of the object returns to the entorhinal cortex, and then re-transmitted to the parahippocampal and perirhinal cortex. Finally, the information returns to neocortical areas, from which the input to the medial temporal lobe.

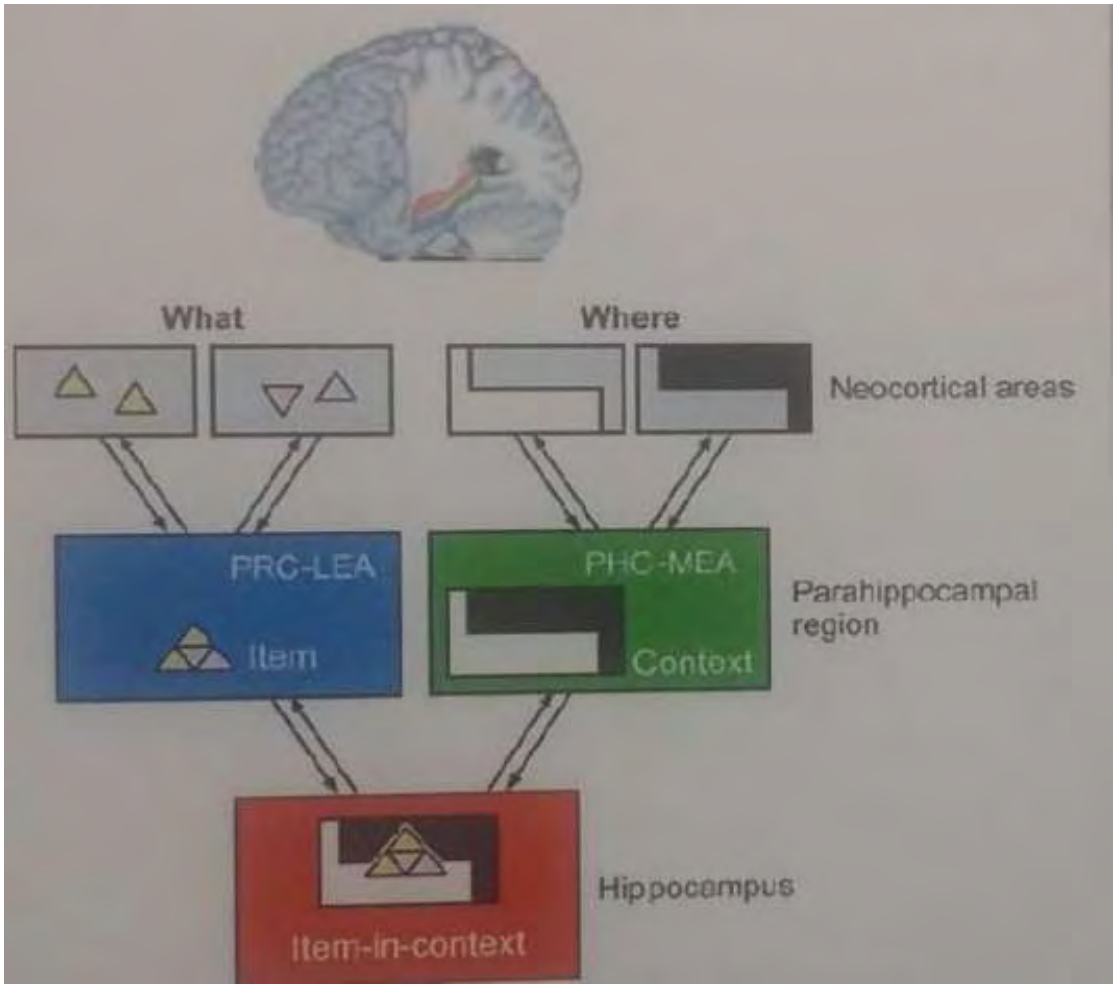

Fig.9 -*Neocortical inputs that carry a representation of objects about "what" converge in the peripheral cortex (PRC) and lateral entorhinal area (LEA).Instead, the information about the location of the objects, the "where," converge to the para-hippocampus cortex (PHC) and the medial entorhinal area (MEA). Such information converges into the hippocampus.*

2.5 Remembrance Experience

One of the crucial issues remains to understand how information and experiences are encoded in the brain. However, the aforementioned anatomical evidence suggests that information is coded and recovered during the storage process. The steps underlying the storage process are of three types:

- Acquisition and encoding: at this stage the stimulus is transposed and translated into memory potentials.
- Consensus and Consolidation: The potential action code is stabilized and held in memory for a certain period of time.
- Retention and Consolidation: The potential action code is stabilized and held in memory for a specified period of time; Recall (retrieval) information is recalled at the level of knowledge by recall or acknowledgment In the first case, a mnemonic direct retrieval of previously stored information occurs. In the second case, information is invoked by conscience by means of an associative stimulus (Baddeley A. et al., 1993).

During the encoding process, the representation of distinct elements (people, objects, events) occurs at the perirhinal cortex and the lateral entorhinal cortex. Also, during encoding, object information is integrated with contextual information ("where") that are formed at the level of the parahippocampus cortex and in the medial area of the entorhinal cortex. When the object is subsequently reprinted in the retrieval phase, the hippocampus retrieves the contextual information retrieving at the parahippocampal cortex and the medial entorhinal cortex. As the hippocampus is the structure that associates objects to their context, hippocampal processes trigger feedback mechanisms in order to obtain a contextual representation of the information. The hippocampal recovers the representations of the objects associated with the stimuli re-emerged from the perirhinal and lateral entorhinal cortex. The recovery of the association between object contexts is the experience of "remembering" (recollection) (Eichenbaum E. et al., 2007)

2.6 Case of H.M Patient

A patient at the age of 9 had an incident that caused him a cerebral lesion and began to suffer from intermittent epileptic seizures of the temporal lobe that were resistant to any pharmacological treatment. Given the poor quality of life at 27 years the patient H.M. was subjected to a surgery. The surgical lesion, practiced on the HM patient, extended to various brain regions, such as temporal lobe, ventral and medial temporal cortex, amygdala, hippocampus formation(which includes hippocampus properly mentioned, subiculum and dentate gyrus), the entorhinal cortex, the perirhinal cortex and the parahippocampus. After surgery, epileptic attacks were much more pharmacologically controllable but the removal of the medial part of the temporal

lobes resulted in the appearance of a very serious memory deficit. The patient's deficit included:

- Amnesia anterograde, patients fail to remember new events occurring in autobiographical contexts, ie they cannot form new memories (Brown MW et al., 2001).
- Amnesia retrogrades, to a lesser extent, or memory loss for events and events a few years before the intervention.
- Serious deficiencies in spatial orientation (Kandel ER et al., 2003).

Observing the HM patient, it was noticed that not all types of memory had been compromised in the same way and other types preserved.
In particular, they remained:

- Short-term memory (in the order of seconds or a few minutes).
- Long-term memory of events occurring remotely with respect to intervention (adolescent childhood).
- A normal mastery of speech.
- A good intellectual quotient.

The residual mnemonic capacities relate to tasks that have two common characteristics:

1) They engage in the execution of reflections involving perceptual or motile capacities known and repeated over time.

2) Do not require complex awareness or complex cognitive processes such as comparison and evaluation. So, in the face of a very complex problem, the patient may be able to find the resolution as fast as a normal subject, but he will not be able to remember, knowingly, that he had to deal with it before. If you asked the patient how the performance of a task was improved after several days of practice, compared to the first day, the patient might respond to never having performed those tasks before then (Kandel E.R. et al., 2003).

2.7 Classification of memory types

From the observation of what is preserved and what is lost after the injury of the medial temporal lobe, two types of memory are distinguished in the literature (Fig. 10).

- Imputed memory (or non-declarative), is a memory that relates to the mode of Executing an act and being invoked to the mind unconsciously. This kind of memory is generally connected with the performance-based training, motors or perceptions, of a reflex type. It is a kind of rigid memory (not ductile), closely related to the original conditions in which learning took place.
- Explicit (or declarative) memory is a memory that involves knowledge of facts about people, places, or objects. This type of memory is invoked in mind with deliberate and conscious efforts. It is a very flexible type of memory and requires the ability to associate different information together (Kandel E.R. et al., 2003).

The formation of declarative memory depends on the system of connected anatomical structures located in the medial temporal lobe (Clark R. et al., 2000).

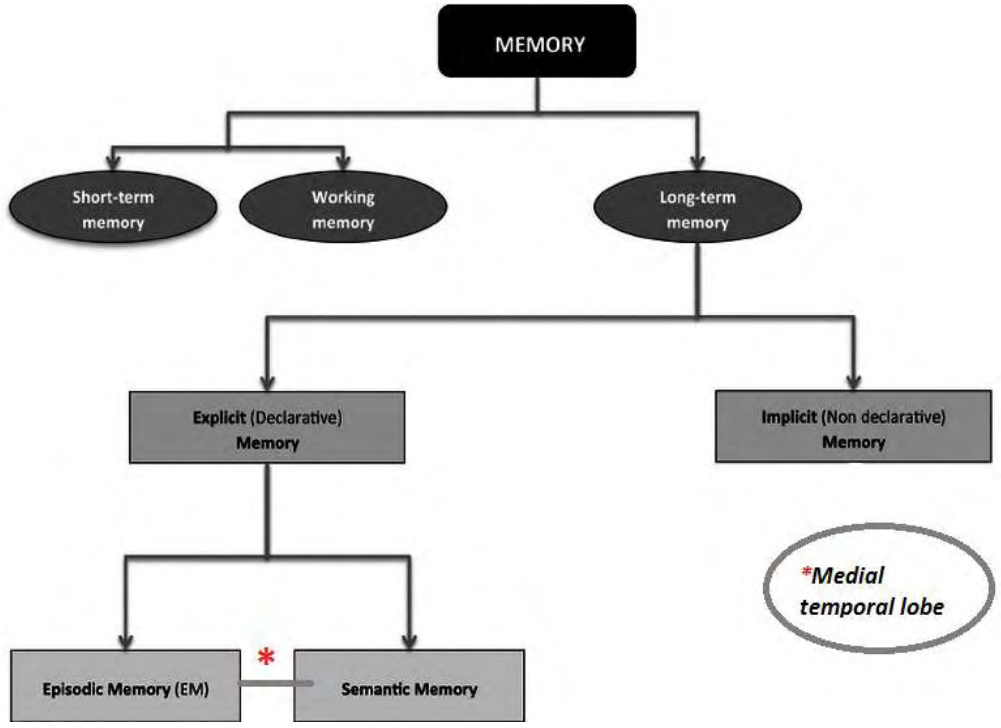

Fig.10 -*Classification of memory.*

2.8 Subdivision of Explicit Memory

The psychologist E. Tulvig first proposed that the declarative (or explicit) memory could be further subdivided into episodic and semantic memory. In 2010, J. Clark proposed that recognition memory is a type of episodic memory.

2.8.1 Episodic Memory

This type of memory concerns autobiographical experiences and often includes timing of events (Ameen-Ali Ke et al., 2015). It consists of the representation of specific events and involves as much as contextual information, and in addition, in order to form episodic memory, it is necessary to "consciously remember or" recharge the event. "So the subject runs to the perception of time to remember the events (Ameen-Ali Ke et al., 2015). Episodic memory is defined as a memory of events personally experienced, or the memory of what is happening in the past of one's life. It has happened, having the consciousness of "where" and "when" events have taken place. It allows the individual mentally to go to his past. This memory was considered to be present only in humans because it is not demonstrable the perception of time in an animal model (Ennaceur et al, 2010).

2.8.2. Semantic memory

Knowledge of facts and notions independent of personal experiences is defined, as a whole, semantic memory. An example is the notions we learned from school or books. It is that kind of memory that includes the knowledge of things, facts and concepts, as well as words and their meaning. They are faculties connected to Semantic memory also remembers the name of things, the definition of words that are heard and the richness of speech (Kandel ER et al., 2003). Semantic memory is defined as the recording of facts and concepts and is independent of the context of space temporal period in which it was acquired (Ennaceur et al., 2010).

2.9 Recognition memory

Recognition memory is a type of episodic memory. Both are explicit forms of memory through which the experiences can be consciously recalled (Clarke J. R. et al., 2010). It defines recognition memory as that neurological process by which subjects identify a stimulus they previously had experience. "Recognition" is the behavioral result of this process (Steckler T. et al., 1997). One of the features highlighted in people with cognitive decline, such as patients suffering from Alzheimer's, is to show difficulties in recognizing. A familiar object, ie previously encountered and the relative difficulty in distinguishing it from a new object (Clarke JR et al., 2010). The memory of "recognition" is a fundamental aspect of our ability to remember and is to identify an event (Brown MW et al., 2001). The recognition process is therefore considered to be at least two components (Fig. 11):

- Discrimination of the family, that is, knowledge of what has been previously encountered (Brown M et al., 2001).

- Ability to remember information about the spatial and /or temporal context in which objects were encountered (Brown M.W. et al, 2001).

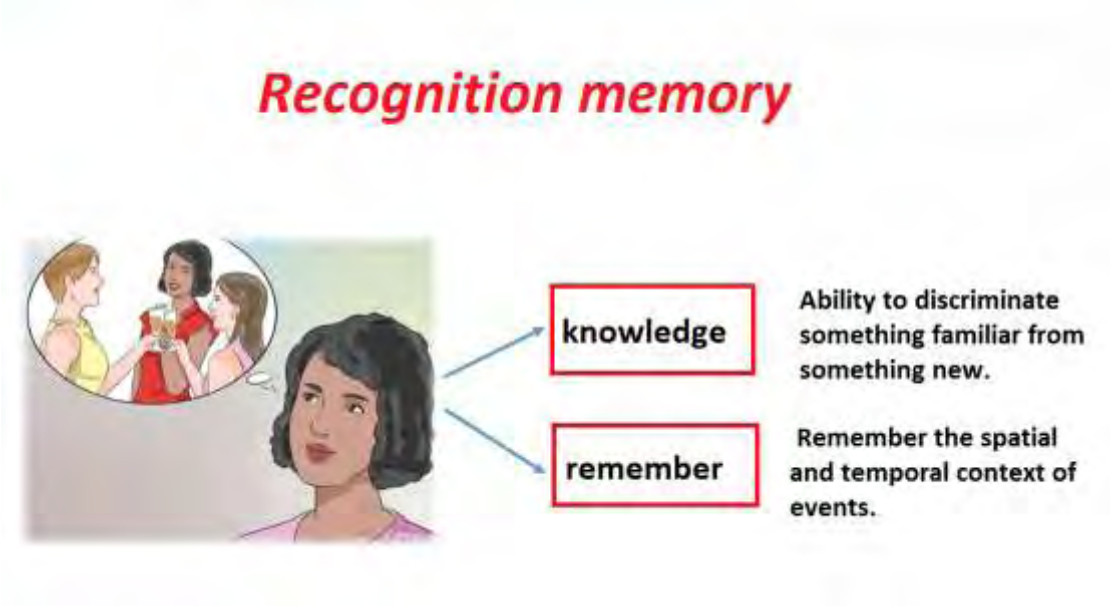

Fig.11 –*Two components of Recognition memory.*

2.9.1. Recognition memory models

Two models for recognition procedures (Brown M.W. et al., 2001) have been hypothesized:

1) Multiple Processes: This model assumes that recognition memory is not a unitary process, but processes within it, that is to say, the two components of the recognition memory: 1. Recognize 2.Recall the context are two qualitatively different processes and therefore separable (Eichenbaum H. et al., 2007).

2) Unique Process: It is the most parsimony model and considers recognition memory as a unitary process. This model assumes that the discrimination of family members and the ability to remember the spatial and temporal context are two processes that differ only quantitatively.

The two models can be explained through an example. By meeting a person on the road, you can recall the information that is relevant to him (such as the name and where we have met before). The two processes are therefore the recognition of the person ("family") and the memory of the previous encounter. The unit model supports the uniqueness of the process. Recognition Memory is an integral part of a class of memories that collectively damage the anterograde amnesia phenomena (Ameen-Ali K.E. et al., 2015).

On the contrary, the multiple models envisage that one can immediately recognize how familiar the person is, without recalling further information about it as a consequence, in the case of atherogenesis of amnesia; only one of the two processes of recognition memory is compromised. The model that is most empirically supported is the multiple representation models. This model is supported by experimental data relating to amnesic patients with hippocampus showing, or the selective loss of the recognition process or the loss of both, that is, the process of recognition and memory of the same. These different effects on the two components of the cognitive process may in all likelihood depend on the difference in temporal lobe injuries among the different patients. In 1994 Eichenbaum proposed that hippocampus is an essential tool for recalling events of contextual and temporal type information while attributing the role of support to the recognition of family stimuli to the parahippocampal regions. The functional dissociation of recognition memory in its components was further studied by Brown in 2001 and is still a debated subject to date and remains a model to be tested experimentally. If the multi-representation model was accepted by the scientific community, it would be necessary to locate structures in the temporal lobe that selectively mediate these processes and specify the role of the perirhinal cortex and the hippocampus (Ameen-Ali K.E. et al., 2015).

Brown suggested that the perirhinal cortex is part of a circuit involved in the recognition or familiarity of recently encountered stimuli, while the hippocampus was involved in a circuit needed to recall the context. This would allow hypothesis that damage to the parahippocampus would affect the recognition/familiarity process, while damage to the hippocampus should be selectively impacted on the context memory process. According to the theory of the multiple representation processes, hippocampus formation plays a role in the association of memories and relationship between stimuli, while the parahippocampus in the regions allow the memory of individual elements (Ameen-Ali K.E. et al., 2015).

Summing up, therefore, in accordance with this model, the perirhinal and parahippocampal cortex and the medial dorsal nucleus of the hypothalamus are needed for recognition /familiarity.

The hippocampus and the front thalamus are essential for the process of recalling the context.

Scientific literature is still animated on the two models and to date, it is still unresolved if there are different regions of the temporal lobe that contribute, differently, to recognizing the stimulus's familiarity and to remember the context. In particular, there are several demonstrations in the literature for the role of perirhinal cortex in the recognition/ familiarity of an object but not in the memory of the context. Hippocampus, however, seems to play an important role in recalling the context but not in recognizing the object as a family member. However, other studies show that the hippocampus is involved in the object's memory of recognition. In conclusion, regarding the recognition of the context and recognition of familiarity, the role of the hippocampus is still very controversial (Balderas I. et al., 2008).

2.9.2 Anatomical structures involved in NOR and OL test execution

Recognition memory depends on the integrity of the medial temporal lobe. By performing NOR and OL tests in animals with selective lesions in medial-temporal lobe structures, the anatomic basis of recognition memory could be reconstructed as well as investigated by behavioral tests. Specifically, the NOR test shows a preference for the new one but does not run the OL test properly, failing to discriminate the object. These data indicate that hippocampal plays a key role in spatial memory (Mumby D.G. et al., 2002; Steckler et al., 1997; Clark R.E. et al., 2000; Ennaceur A. et al., 1997). The OL Test has shown that it is, therefore "hippocampus-dependent". Conversely, rats with perirhinal cortex lesions successfully test OL (Ameen-Ali K.E. et al., 2015).
It has been described that mice with the lesion of the spine fail to discriminate the position change of the object (OL test but not in discriminating the new object with respect to the familial (Ennaceur A. et al., 1997). The connections between hippocampus and limbic system in a study conducted by Ennaceur in 1997, it was seen that a range of system lesions (sites associated with hippocampus) does not alter NOR's performance even if the test is performed with a latency period of more than 15 minutes, on the contrary, rats with injuries to the central and perirhinal cortex, in the course of the NOR's development, behave indifferently by exploring the new and the familiar object, failing to discriminate between the two objects. Fractured lesion destroyed the ability to identify the familiar object (Ennaceur A. et al., 1997; Balderas et al., 2008).

AIM OF THE WORK

Despite an extensive investigation of the preventing action of *Hericium erinaceus* in cognitive pathological conditions, to date, no studies have investigated the effects of dietary supplementation with H. erinaceus in healthy mice. To approach this point a group of wild-type mice were fed with a dextrin dietary supplementation (dx mice) and another one with Hr dietary supplementation (Hr mice) and we investigated the effect on behaviour in memory skills. We therefore performed in vivo battery of spontaneous behavioural tests (NOR and Emergence test), to assess novelty exploration and recognition memory, in wild-type mice after two months of dietary supplementation with Hr.

CHAPTER 3

Materials and Methods

3.1 Subjects

For the Object Location task (O-L), Spontaneous Y-maze test, Emergence test and Novel Object Recognition task (NOR), blind experiments were carried out in wild type mice (strain C57BL-6J). In order to avoid any potential differences related to the oestrous cycle in females, only males were selected. Each mouse was resided inside its cage in a temperature- and humidity-controlled room.
Wild type male mice divided into two groups: control mice received a normal diet containing 5% dextrin dietary supplementation, for 60 days (dx mice group); the Hericium erinaceus treated mice (Hr mice group) received two months of a diet containing 5% "Micotherapy Hericium" supplement (corresponding to 0.025 g/g body weight). Water was provided ad libitum for both groups.

3.2 Fungal Supplementation

The supplement "Micotherapy Hericium" was provided by A. V. D Reform s.r.l. (Noceto, Parma, Italy).The supplement contains mycelium and fruiting body extract of *Hericium erinaceus* in a ratio 4/1(Table 1). *Hr* (Her. Erin.strain) culture was obtained from the fungal culture bank of MycoMedica d.o.o., Slovenia, and was cultivated in the dark on PDA (Potato Dextrose Agar; Difco, USA) at 24∘C. After 20 days, cultures were transferred onto lignocellulosic substrates and further incubated for 60 days at 24∘C. After incubation, fruiting bodies and fungal biomass were harvested.
Fruiting bodies extractions were performed for three hours using water and ethanol as solvents in a 1:15 extraction ratio (w/v). The remaining extracted liquid as well as mycelium was dried under vacuum at 70∘C and −0.9 bar and further milled by using a UPZ mill (Hosokawa Alpine Aktiengesellschaft, Augsburg, Germany) to obtain particles mostly smaller than 100 μm.
The polysaccharide content of *H. erinaceus* fruiting bodies extract and of *H. erinaceus* mycelium, contained in "Micotherapy Hericium" supplement, was determined using β-Glucan Assay Kit (Megazyme, Ireland) and expressed as total (α plus β) glucan content (Tables 2 and 3).
All experiments were carried out according to the guidelines laid down by the institution's animal welfare committee, the Ethics Committee of Pavia University.

TABLE 1: Nutrient composition of dietary supplements (supplied by A. V. D. Reform, Noceto, Parma, Italy).

Components	Per capsule (mg)
Hericium mycelium	400
Hericium fruiting bodies extract	100
Titled in polysaccharides	38.6

TABLE 2: Nutritional composition of *H. erinaceus* extract.

Analyte	Result	Unit
Calorie	2.23	Kcal/g
Crude proteins	8.25	%wt
Crude fat	0.17	%wt
Crude fiber	5.92	%wt
Polysaccharides/total glucan	>45	%wt
Sodium	0.0146	%wt

TABLE 3: Nutritional composition of *H. erinaceus* mycelium.

Analyte	Result	Unit
Calorie	1.98	Kcal/g
Crude proteins	10.22	%wt
Crude fat	1.02	%wt
Crude fiber	39.2	%wt
Polysaccharides/total glucan	>37	%wt
Sodium	0.0031	%wt

3.3 Apparatus for recording of Behavioral Test

Motor activity was quantified by means of a SMART video tracking system (2 Biological Instruments, Via Leonardo da Vinci, 2, Besozzo VA, Italy). Also, an additional component was used, TRIWISE, which takes as reference point the nose of the mouse. The mouse was filmed by a Sony CCD (PAL) color camera placed above the arena.

3.4 Object Characteristics

As Ennaceur A. (2010) states, the choice of objects to be used during the tests is very difficult since there is no sufficient evidence of perception by animals or objects. Particular attention was paid to the fact that the objects did not reflect the light and each object was well secured on the arena floor so the mice could not move them.

Size of objects.
If the object is "scalable" by mice, allows to the animal to rest the paws or its body on

it. Scalability of an object is a feature that discriminates the explorative ability of mice and should, therefore, be avoided in the behavioral tests.

Object colors and brightness.
Recent studies show that mice discriminate between ultraviolet light and visible light and between the different colors of the blue and green range. Therefore, rodents discriminate between objects that differ in brightness but have difficulty in color discrimination. It is necessary for the colors to recognize that direct light falls on them. Objects with similar brightness, which differ in colors for a human's eyes, may look identical to the eyes of a mouse.

Similarities between objects.
Objects should not be too close to each other because they may be subject to interference.

Smells
It is important to take into account any odors present in the arena, objects, as well as in the hands of the experimenter, as odors may induce the mouse to undue exploratory preferences.

Asymmetric Objects
If the familiar object in the sample phase is characterized by asymmetric properties, it can induce spatial ambiguities in the next choice phase of the test.

3.5 Procedures of Behavioral Test

- ***Emergence Test***

To assess approach and exploratory behavior in rodents, we performed the emergence test, which is a variant of the open-field test that was designed to reduce anxiety by providing a safe enclosure within the open field. This test has been used to test anxiety-like behavior in mice. The arena used is in the shape of an open up parallelepiped, built in white plastic, a biologically neutral material which is easy to clean.
The free exploration test entails housing animals in a compartment prior to giving the animal a free choice between a novel compartment and a familiar one (Griebel et al., 1993). In our experimental conditions, the mouse is situated in a familiar environment (a cage 35 cm long, 25 cm high and 20 cm wide) with a hole in one side through which it can emerge in a larger arena (90 cm long and 60 cm wide) with a laminated floor but without walls. (Fig. 12)

We recorded with a video camera for 8 minutes each mouse (both dextrin mice and mice treated with *Hericium erinaceus*) and then we analyzed the number of times that the mouse puts its front paws on the edge of the cage, the number of complete exits from the cage and the time spent to explore the environment out of the cage.

Fig.12 –*Picture of the cage of the emergency test*.

- ***Novel Object Recognition Task (NOR)***

A test for assessing recognition memory in rodents is the NOR test. The NOR task is used to test novelty exploration and recognition memory in rodents. The test evaluates the rodent's ability to recognize a novel object in the environment. Basically in the NOR task, there are no positive or negative reinforcers, and this methology assess the natural preference for novel objects displayed by rodents. The arena is 60cm long, 20 cm high and 30 cm wide. The used objects were a cylinder of plastic, with base of 3,5cm diameter and height of 3,5cm and another one with different shape; more squared (Fig. 13).

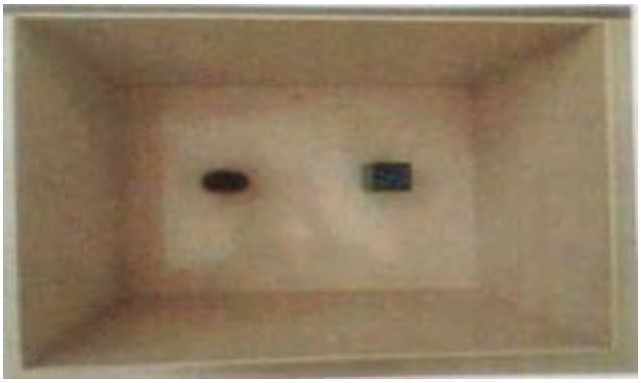

Fig.13 -*Picture of the arena of the NOR test.*

The task consists of three phases: Habituation, familiarization, and the test phase (Fig. 14).

1) In the ***habituation phase***, for the first two days each mouse is given10 minutes to freely explore the open-field arena the absence of objects, after which it is removed from the arena and placed in the holding cage.
2) On the third day, during the ***familiarization phase***, each mouse is placed into the open-field arena and left free to explore two identical objects for 5 minutes.
3) After the *retention phase* has elapsed (15 minutes), in ***test phase*** the mouse is settled back into the open box where it is exposed both to a familiar object, identical to the one previously encountered in the familiarization phase, and to a novel object with a different size and shape (Ennaceur et al., 1992, 2010).

Approaches are defined as nose entries within 2 cm far from the object. The apparatus was wiped clean with alcohol after every trial.

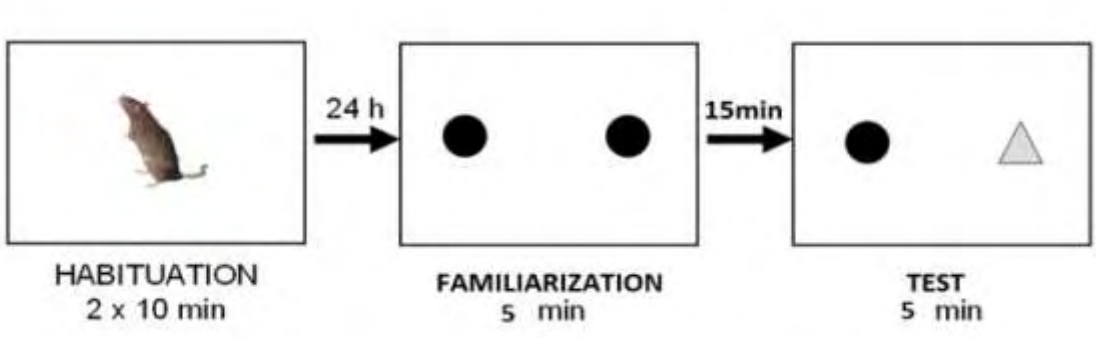

Fig.14 -*The total procedure of the NOR task.*

We explore a possible effect of *Hericium erinaceus* on spatial memory by using two different behavioral tasks:

- ***The Object Location Task (OL)***

The O-L task is a variant of the spontaneous object recognition task (NOR) and allows to evaluate the memory for the spatial location of objects to be investigated. This test is performed within an open box-arena. It has two big advantages: It is not required to learn any rules and only a short training is sufficient. Also, it is not necessary to use food as reinforcement, so there is not another factor that changes the performance of the test, like a change in dietary appetite (Dix S.L. et al., 1998).

The used objects were cylinder of plastic, with base of 3,5cm diameter and heights of 3,5cm.The arena was the same with this one in NOR test (Fig. 15).

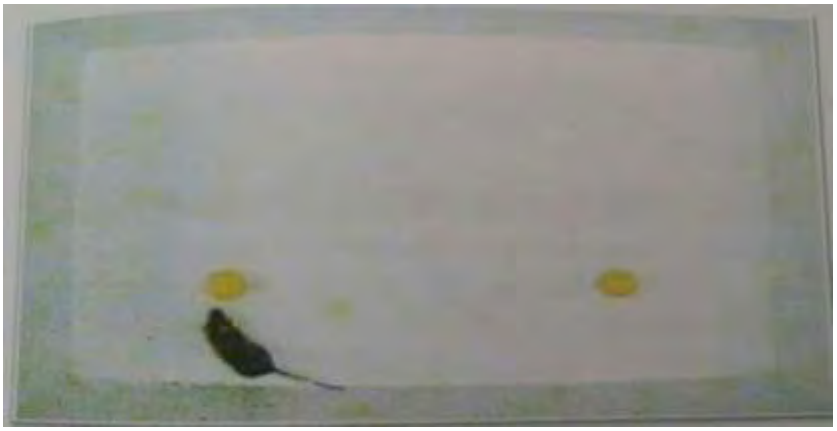

Fig.15 -*The arena of the OL test.*

Like the NOR, OL test consists of three phases: habituation, familiarization (sample phase), and test phase (Fig. 16).

1) In the **habituation phase**, for the first two days each mouse is given 10 minutes to freely explore the open-field arena in the absence of objects, after which it is removed from the arena and placed in the holding cage.
2) On the third day, during the **familiarization phase (sample phase)**, each mouse is placed 10 cm away from the side wall, into the open-field arena containing two identical objects for 5 minutes. During the familiarization phase, the mouse is free to explore two identical objects for the same amount of time. To prevent coercion to explore the objects, the mouse is placed against the center of the opposite wall with its back to the object.
3) After that *the latency-retention phase* has elapsed (15 minutes), we cleaned the arena and objects with alcohol and we attached the mouse back into the open box, for 5 minutes **(test phase)**, where it is already been exposed to both

familiar objects that are identical to the ones previously encountered in the sample phase, but one of these is repositioned in a new place within the box. An approach is defined such that nose entries within 2 cm of the object (Ennaceur et al., 2004).

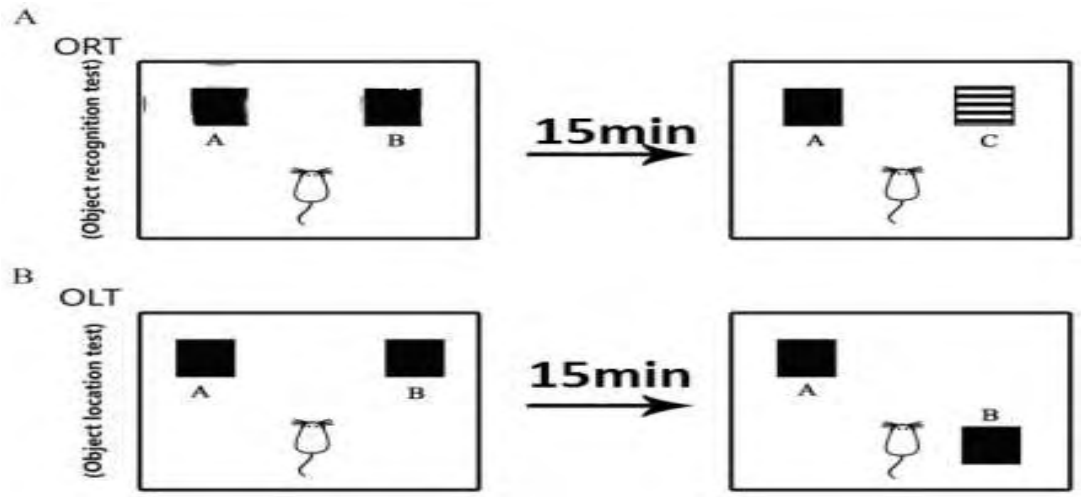

Fig.16 -*The total procedure of the OL task (B), comparable to the NOR task (A).*

- ***Y-maze Test.***

The "Spontaneous Y-maze", is a simple, automatable and quick test that assesses the willingness of rodents to explore new environments, spatial working memory and general locomotion activity. As the behavior is not reinforced with external reward or punishment is called "spontaneous". Free-running Y-maze has the obvious advantage of avoiding excessive stressful handling of mice as well as providing an additional useful measure of locomotion activity by counting the frequency of arm entries Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited (Gerlai 1998; Gerlai 2001; Stewart et al., 2011; Hughes 2004).

The Y-shaped maze (floor and walls) was constructed with three symmetrical grey, opaque acrylic plastic arms at 120° angle from each other. Each arm is 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top, and converges at an equilateral triangular central area that is 4 cm at its longest axis. (Fig. 17)

Fig.17 *–Picture of the Y-maze*.

Each mouse was placed at the center of apparatus (Fig. 18). It was allowed to freely explore the three arms for 8 minutes (Huang et al., 2006; Faizi et al., 2011; Maurice T. et al., 1994). In the Y-maze task, an arm entry was defined as all four limbs within the arm. A triad was denied as a set of three arm entries, when each consecutive entry was to a different arm of the maze (ABC, ACB, BAC, BCA, CAB and CBA). The maze was cleaned with 10% ethanol between sessions to eliminate odor traces. A failure is defined as the entry is defined as the entry repeated in the same arm (ABA, ACA, AAB, AAC, BAB, BCB, BBC, BBC, CBC, CAC, CCB, CCA) (Mori et al., 2011, 10).

The number of arm entries and the number of triads were measured. The alternation percentage value was generated by dividing the number of triads by the number of possible alternation (number of entries -2) and then multiplying by 100.

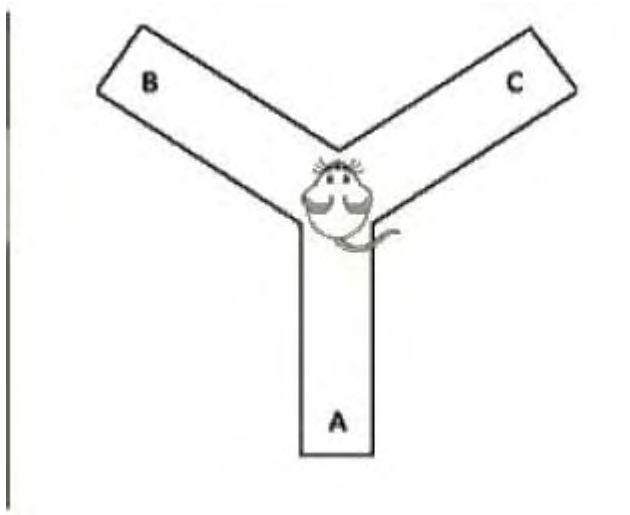

Fig.18 –*Representation of the Y-maze.*

3.6 Behavioural Test Analysis

3.6.1 Parameters

Emergency Test

The software collected the following:

1) The number of times a mouse completely emerged from the cage with all four limbs.
2) The amount of time a mouse spent exploring the large environment outside the cage.
3) The latency before the first exit from the cage.

Novel Object Recognition Task

The software collected the following:

1) The number of approaches.
2) Total duration of approaches.

3) Average duration of an approach.
4) Latency of the first approach.

Object Location Task

The software collected the following:

1) Number of approaches (frequency of approach): this measure refers to the number of time when an animal had approached the two objects.
2) Total duration of approaches: this measure refers to the amount of time spend by an animal in exploring the object.
3) Average duration of an approach: this measure refers to the amount of time spent by an animal in exploring the object divided by the frequency of approaches.
4) Latency of the first approach: this measure refers to the amount of time spent by an animal to approach any of the two objects.

Y-maze Test

The software collected the following:

1) The frequency of arm entries: an arm entry was defined as all four limbs within the arm.
2) The number of triads: a triad was defined as a set of three arm entries, where each consecutive entry was to a different arm of the maze (ABC, ACB, BAC, BCA, CAB and CBA).
3) The alternation percentage value was generated by dividing the number of triads by the number of possible alternations (number of entries -2) and then multiplying by 100.

3.6.2 Statistics

After using Bartlett's test for Homogeneity of Variances, two way ANOVA repeated measures were used, where the parameters: number of approaches and total duration of approaches or latency of first approaches, were the dependent variable, the within subjects factor was "familiar object" or "novel object" and the between subjects factor was "dextrin" or "Hericium" treatment. For emergency test a one way ANOVA test was used.
Descriptive statistics, expressed as data, are reported as means ± standard error of the mean (SEM), and statistical comparisons were made by using Student's t-test. Before applying the Student t-test and the Shapiro-Wilk test was performed for each pool of

data to confirm a normal distribution.
In figures symbols indicate *P <0.05, **P <0.01, ***P <0.001. NS means non statistically significant.

CHAPTER 4

Results

4.1. Emergence Test

We first investigated the effect of the oral supplementation with *Hericium erinaceus* on novelty exploration behavior in healthy mice by using the emergence test.
We tested 12 dx mice and 22 Hr mice and measured the number of complete exits, the exploring time and the latency to the first exit in the Hr mice group compared to the dx mice group.
The results show that the Hr mice had a higher frequency in the number of complete exits (9.5 ± 0.8 versus 4.1 ± 0.8). The difference is statistically significant $P < 0.001$ (Fig. 19a).
Furthermore, Hr mice spent more time exploring the new environment compare to dx mice (129.6 sec ± 14 versus 59.2 sec ± 15.8).The difference is statistically significant $P < 0.01$, (Fig. 19b)
Hr mice showed a lower latency before the first exit (13.1 ± 2.6 sec versus 31.7 ± 10.3 sec). The difference is statistically significant $P < 0.05$ (Fig. 19c).
It has been recognized that several parameters of a task need to be recorded to support the validity and interpretation of the data of a behavioral experiment (Ennaceur A. et al., 2005).
Overall, the number of complete exits (2.3- fold increase), the exploring time (2.2 times longer), and the decreased latency to the first exit (2.4 times shorter), all of them indicate an increase in novelty-seeking behavior after the oral supplementation with *Hericium erinaceus* for two months.

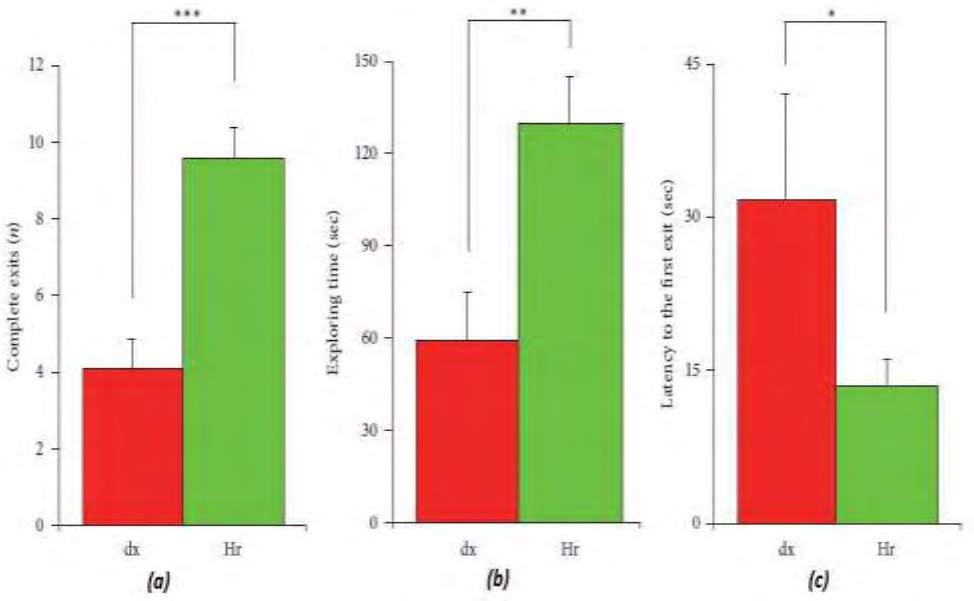

Fig.19 -*Emergence test in dx (n=12) and Hr-dietary supplemented mice (n=22). Histograms show the number of complete exits(a), duration of exploring time (b) and the latency of the first exit(c) in Hr mice (green color) compared to dx mice (red color).*

4.2. Novel Object Recognition Test

Previous studies showed that *Hericium erinaceus* oral supplementation in mice with learning and memory deficits induced by intracerebroventricular administration of β(25-35) amyloid peptide prevented cognitive deficits in a memory recognition task but not in a spatial working task (Hazekawa et al., 2010). Based on these findings, we decided to test the effect of Hr on recognition memory in wild-type mice by using the NOR task.

- First of all we consider the behavior of mice in dx experimental condition between familiar and novel object. From the mice's behavior towards the novel object compared to the familiar object, we took the along results: the number of approaches is approximately the same (13.9 ± 1.7 sec versus 14 ± 0.8) (Fig. 20a), the total duration of the approaches is higher (45.4 ± 7.9 sec versus 33 ± 5.6 sec) (Fig. 20b) and the average duration of an approach to the novel object did not reach the significance (3.2 ± 0.5 sec versus 2.3 ± 0.4 sec) (Fig. 20c). The difference is not statistically significant in any of these cases. However, the comparison of behavior of dx mice towards the novel versus the

familiar object expressed by a longer latency of the first approach to the novel object (42.7 ± 9 sec versus 17.6 ± 5.7 sec, $F_{3,46}$ = 6.47; (Fig 20d)).

- Then, we consider the behavior of mice toward the familiar object in dx and Hr experimental condition. When we compared the behavior of dx and Hr mice toward the familiar object the only parameter that was statistically significantly lower in the latter was the latency for the first approach (17.6 ± 5.7 sec versus 6.7 ± 2.4 sec), P< 0.05, indicating that Hr mice and dx mice approach a familiar object in the arena in fairly similar ways (Fig. 20d).
- Then, we consider the comparison of the behavior of dx mice and Hr mice toward the novel object revealed that the number of approaches (13.9 ± 1.7 versus 18,9 ± 1.5; $F_{3,46}$= 3.24 and P< 0.05) (Fig. 20a) and the total duration of approaches (45.4 ± 7.9 sec versus 65,4 ± 5.7 sec; $F_{3,46}$= 8.86 and P< 0.05) (Fig. 20b), were significantly different, with the Hr mice showing 36% and 44% increases, respectively, in exploratory behavior toward the novel object. Furthermore, the latency of the first approach (42.7 ± 9 sec versus 17.6 ± 5.7 sec; $F_{3,46}$ = 6.47 and P< 0.05) is significantly lower (54% and 31.4% decrease) in Hr mice (Fig. 20d).
- As expected, three out of four parameters that we measured in the behavior of Hr mice with the familiar and novel objects were significantly different, with the total duration of approaches (31.5 ± 3.6 sec versus 65.4 ± 5.7 sec; $F_{3,46}$ =8.86 and $P < 0.001$) (Fig. 20b) ,the average duration of an approach (1.9 ± 0.2 sec versus 3.5 ± 0.3 sec, $F_{3,46}$ = 8.86 and $P < 0.001$, Fig. 20c), and the latency of the first approach (6.6 ± 2.4 sec versus 19.5 ± 5.9 sec, $F_{3,46}$ = 6.47 and $P < 0.05$, Fig. 20d) all being significantly higher.

We conclude that, after Hr dietary supplementation, mice spent more time exploring the novel object and increased the latency of the first approach compared with the familiar object.

To evaluate the discrimination between novel and familiar objects in dx and Hr mice, we calculated the mean novelty discrimination index (NI) by using the following formula: NI= (n – f) / (n + f) (Silvers et al., 2007), where *n* is the average time with the novel object, and *f* is the average time with the familiar object. This index ranges from -1 to 1, where -1 means complete preference for the familiar object, 0 means no preference, 1 means complete preference for the novel object. The NI index was 0.15 for the dx mice and 0.35 for the Hr mice.

In conclusion, Hr mice displayed very different behavior compared with dx mice specifically when exploring novel objects, not familiar objects. The data are concordant and indicate that Hr mice show increased recognition memory performance.

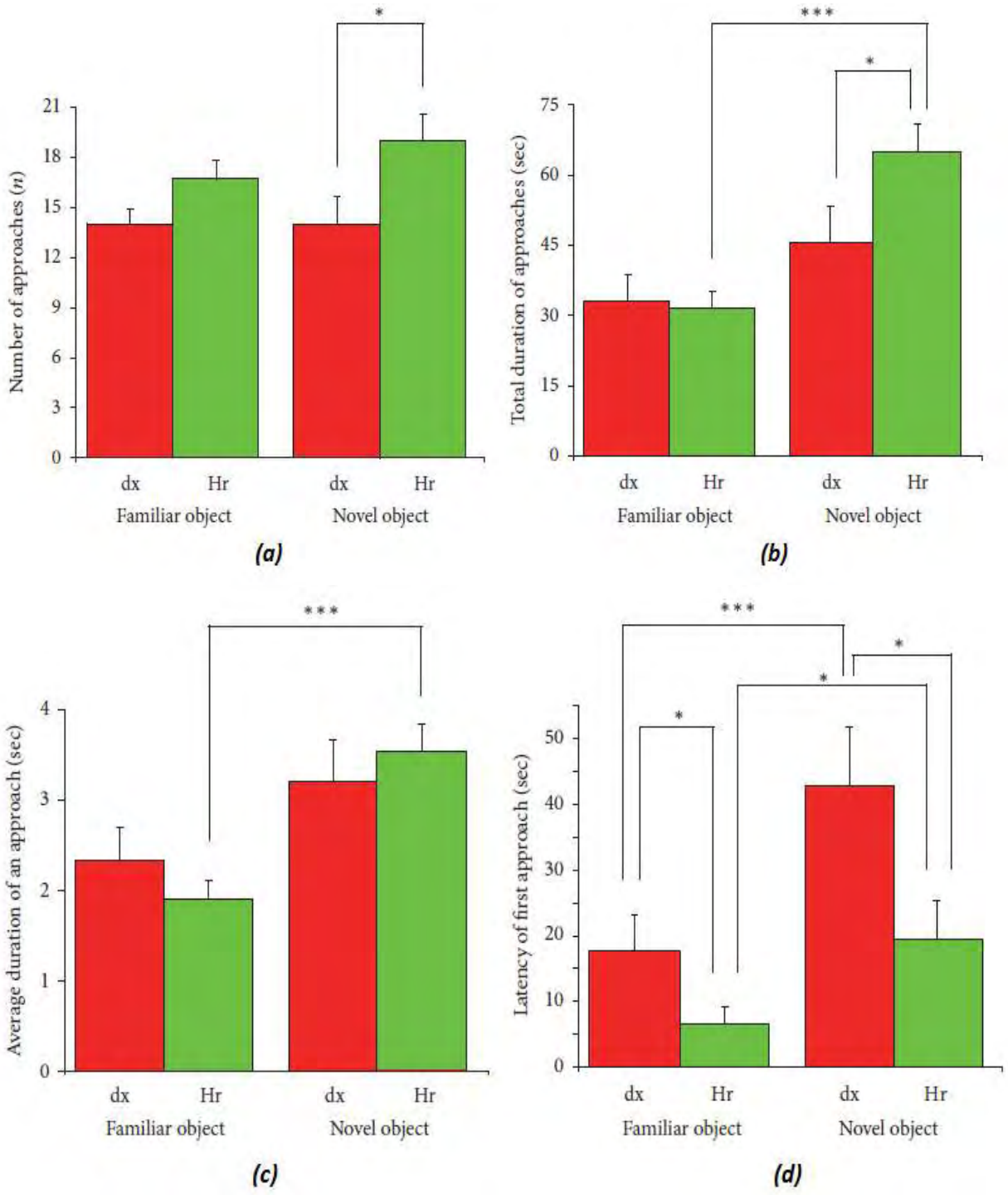

Fig.20 -*Novel object recognition test in dx (n=10) and Hr -dietary supplemented mice (n=15). Histograms show the number of approaches to the familiar and novel objects(a), the total duration of approaches (b), the average duration of an approach (c), the latency to the first approach (d), comparing the Hr mice(green color) and dx mice (red color).*

4.3 Object Location Task

- From the comparison between the dx control mice (n=12) towards the two objects (familiar versus novel) reveals that the number of approaches (13.7 ± 2 versus 15.5 ± 2.3) (Fig. 21A), the total duration of approaches (28.9±4.3 sec versus 30.9± 5.4 sec) (Fig. 21B), the average duration of an approach (2.7±0.6 sec versus 2.6±0.6 sec) (Fig. 21C), the latency of the first approach (7.5± 1.9 sec versus10± 2.6 sec) (Fig. 21D), are not statistically significant.
- In Hr treated mice (n=18) the comparison between the two objects (familiar versus novel) shows that the number of approaches (10 ± 0.9 versus 11.6± 1.2 (Fig. 21A), the total duration of approaches (18.9 ± 2.5 sec versus 19.6± 2.5 sec)Fig. 21B), the average duration of an approach (2.2 ± 0.4 sec versus 1.8± 0.2 sec) (Fig. 21C), the latency of first approach (8.8 ± 2.1 sec versus 12.5 ± 2.1 sec) (Fig. 20D) are not statistically significant.

In object location task when the familiar object is repositioned in a different place, none of the measured parameters were statistically different between dx and Hr mice. We can conclude that after Hr supplementation the animal explore the familiar and the repositioned object for a comparable time, suggesting that Hr supplementation has no effect on spatial memory task.

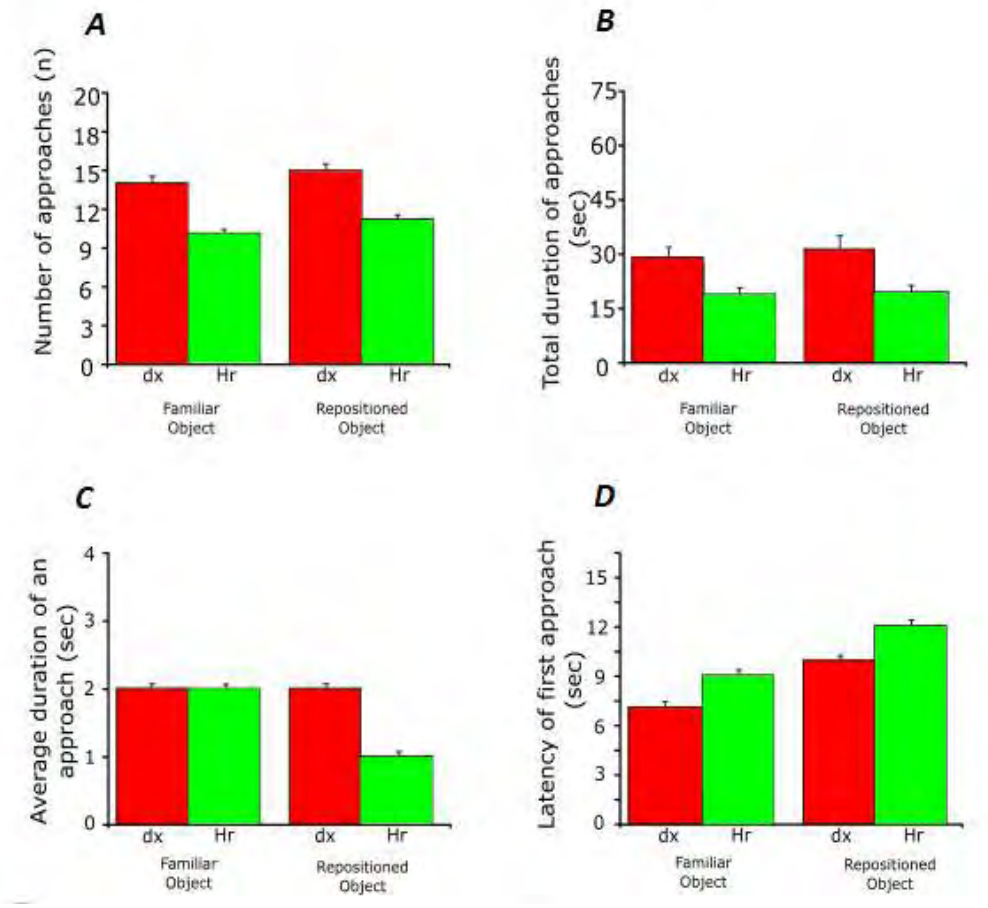

Fig.21 -*Object location task in dx (n=12) and Hr-supplemented mice (n=18). Histograms showing the number of the approaches on the familiar and novel objects (A), the total duration of approaches (B), the average duration of an approach (C) and the latency of first approach (D*), comparing the Hr mice(green color) and dx mice (red color).

4.4 Y-maze Test

As shown in figure, Hr mice showed a significantly bigger rate of spontaneous number of entries and alternation triplets (63.7 ± 3.7 versus 50.1 ± 5.5). The difference is statistically significant $P<0.05$ (Fig. 22A).
The average number of alternation triplets is bigger for the Hr group (34.1 ± 1.8 versus 27.3 ± 3.5).The difference is statistically significant $P<0.05$ (Fig. 22B).
By dividing the number of triads by the number of possible alternations (number of entries -2) we calculate the alternation percentage (Fig. 22C). The percentage number of alternations was approximately the same in Hr group and dx group (56.2 ± 1.9% versus 55.4 ± 3.5%). The difference is not statistically significant.
Taken together our data reveal an increase of spontaneous crosses in arms in the group of mice treated with oral supplementation of Hr compared to the group of mice treated with dx but not an improvement in spatial working memory as indicated by the percentage number of alternations.

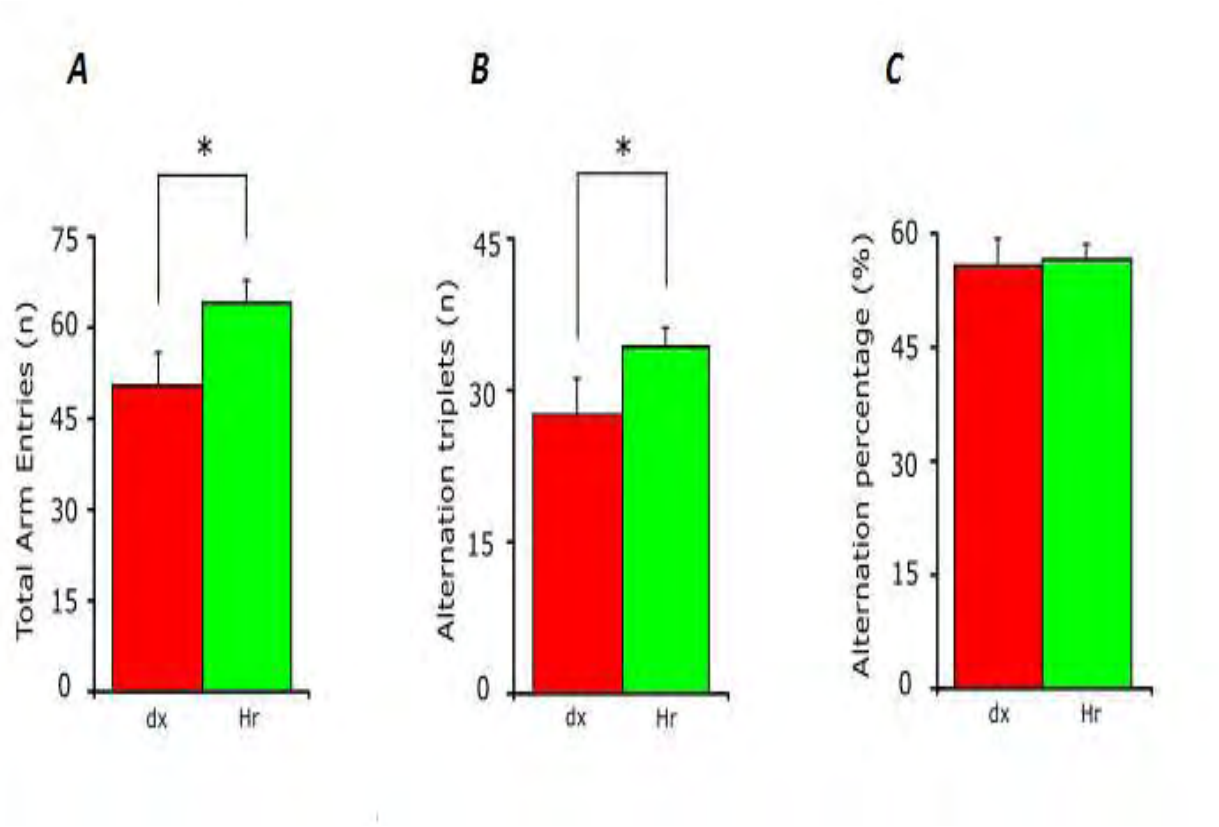

Fig.22 -Y-maze test in dx (n=10) and Hr supplemented mice (n=19). Histograms showing the total number of arm entries (A), the number of alternation triplets (B) and the alternation percentage (C) in Hr mice (green color) compared to dx mice (red color).

CHAPTER 5

Discussion

Recognition memory, a form of declarative memory, can generally be defined as the ability to discriminate the novelty or familiarity of previous experiences by identifying when something (e.g., an object or an environment) has already been encountered.

To explore the role of Hr in recognition memory and exploration behavior we evaluated the effect of two months oral administration in wild-type mice by using two spontaneous behavioral tests, the NOR and the Emergence test.

Data presented in the thesis demonstrate that wild-type mice supplemented with *Hericium erinaceus* increased their recognition memory and the exploration of a novel environment and a novel object.

The ability to cope with novelty is essential in all mammal species (Cloninger CR. 1987). Novelty-seeking has been identified as one of the six major human personality dimensions, whereas neophobia describes hesitancy to engage with novel objects and places and can be considered a risk factor for anxiety disorders. A low level of exploratory activity towards novelty is interpreted as a sign of anxiety-like behavior, whereas a high level reflects less anxiety (Stedenfeld K. et al., 2011).

Furthermore, reduced novelty-seeking and, in turn, increased neophobia can be considered core symptoms of depression; these behaviors are closely related to rigid evaluative patterns and reduced coping flexibility that also characterize the depressive state. A recently published paper described the reduction of depression and anxiety by 4 weeks' intake of *Hericium erinaceus* dietary supplementation in 30 female subjects (Nagano M. et al., 2010). As we did, several studies have used the novelty approach as a measure of anxiety and the parameters measured were the latency, the frequency, and the duration of approaches (Treit D. 1985; Wesierska M. et al., 2003).

Accordingly, spatial memory can be viewed as a subset of declarative memory, with the idea that this broader category is the province of the hippocampus and related structures is responsible for recording information about one's environment and spatial orientation. Research indicates that there are specific areas of the brain associated with spatial memory.

To explore the role of Hr in spatial memory we evaluated the effect of two months oral administration in wild-type mice by using two spontaneous behavioral tests, the OL and Y-maze test. The OL involves multiple items and contextual associations and Y-maze reflects spatial memory capacity, relying on the ability of animals to enter an arm of the Y-maze that was not entered in the previous choice.

In the O-L task, all parameters measured, number, total duration, average duration of approaches and latency of first approach are not statistically significant suggesting that the oral supplementation with Hr in wild-type mice has no effect on spatial working memory.

This data was confirmed in Hr supplemented mice performing Y-maze test that reveals that the alternation percentage, a measure of the mouse performance in choosing different arm at each inspection did not change, while there is in supplemented mice an increase in general locomotion activity as revealed by the number of total arm entries.

Researchers have long been interested in the mechanisms underlying recognition memory and currently there is a general agreement that two distinct cognitive operations, recollection and familiarity, contribute to performance on recognition memory tests (Turriziani et al., 2008). Ongoing debate which is central to our understanding of recognition memory function is whether recollection and familiarity reflect different memory processes that can be dissociated anatomically and functionally (dual-processes model) or it is a single unitary process expression of memory traces of different strength in the context of a unitary declarative memory system (unitary-strength models, Turriziani et al., 2008; Ameen-Ali et al., 2015; Ranganath and Ritchey, 2012).

According to some, recognition memory is supported by two functionally distinct processes mediated by different structures in the medial temporal lobe; the parahippocampal region, is part of a circuit involved in familiarity and recognition of individual items (associated with a feeling of "knowing", Tulving E., 1985; Brown et al., 2001) and the hippocampus, supporting recollected associations and relationships amongst stimuli (associated with a feeling of "remembering", for a review, see Ameen-Ali et al., 2015). Studies involving human amnesic patients with hippocampal damage have provided useful insight into this debate, with some reporting selective recollection impairment with spared familiarity processing (Turriziani et al., 2008), offering support to the dual-process model (Squire and Wixted JT., 2011; Vann SD et al., 2011).

In animals, lesion studies using the spontaneous novel object recognition task (NOR) and object-location task (O-L) have provided a useful insight into the anatomical basis for recognition memory. The perirhinal cortex is critical for successful performance on NOR (Barker et al., 2007; Barker and Warburton, 2011; Norman and Eacott, 2004; Winters et al., 2004), while lesions of hippocampal or fornix have no detrimental effect on NOR test (Barker and Warburton, 2011; Mumby et al., 2002; Winters et al., 2004). Conversely, rats with dorsal hippocampal lesions cannot successfully perform the OL, while perirhinal cortex lesions have no effect on OL task performance (Barker and Warburton, 2011).

The results of the NOR task indicate that the Hr mice spent more time approaching the novel object than the familiar object; the decreased latency of the first approach and the increase in the frequency of approaches, combined with the longer duration of approaches, further support a state of increased novelty exploration behavior. Conversely, the NOR test revealed no differences between Hr and dx mice in the exploration of the familiar object, indicating that the increase in exploratory activity is specifically oriented to the novel object.

The lack of effects with O-L and Y-maze tasks that has been described to be

hippocampal-dependent and, on the other side, the increase in recognition memory obtained with NOR and emergence tasks that has been described parahippocampal-dependent, strongly suggests that Hr supplementation has a selective role in increasing parahippocampal cognitive performances.

The effects of Hr supplementation in wild-type animals strongly suggest that the recognition memory is a dual-process implying different behavioral mechanism and/or different anatomical structures. Furthermore, Hr supplementation exercises selective effect, determining recollection memory with spared familiarity processing. (Mori et al., 2011) examined the effect of Hr oral supplementation on amyloid β(25-35) peptide induced learning and memory deficits in mice, resulting in a significant reduction of alternation behavior in the Y-maze test and of the discrimination ratio in the novel-object recognition test. According to us, Hr does not prevent the cognitive deficits induced in Y-maze test, but prevented the induced impairment in NOR.

In conclusion, those data suggest that the hippocampus formation is not directly involved in the improvement of cognitive performance observed in Hr mice and strongly embraces the hypothesis of the dual processes model in recognition memory, where the perirhinal cortex region supports the recognition of individual items as part of a circuit involved in familiarity toward an encountered stimulus, whereas the hippocampus supports recollected associations and relationships between stimuli (for a review, see Ameen-Ali et al., 2015). Hr oral supplementation is specifically addressed to parahippocampal region increasing glutamatergic synaptic drive novelty exploration behavior and recognition memory.

Our study on the effects in wild-type mice of *Hericium erinaceus* oral supplementation on brain yielded several key findings that we hope will pave the way for new studies in healthy humans and bridge the gap between the millenary Eastern medicine and our Western medicine (Wasser S.P., 2014; Khan M.A., 2013).

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