STUDY ON THE MOLECULAR BASIS OF INDIVIDUAL VARIATION IN
SPATIAL MEMORY IN RATS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÇİĞDEM GÖKÇEK SARAÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCES

JUNE 2012

Approval of the thesis:

**STUDY ON THE MOLECULAR BASIS OF INDIVIDUAL VARIATION
IN SPATIAL MEMORY IN RATS**

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ABSTRACT

STUDY ON THE MOLECULAR BASIS OF INDIVIDUAL VARIATION IN
SPATIAL MEMORY IN RATS

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June 2012, 159 pages

Despite very extensive studies related to molecular processes underlying memory formation, still little known about the potential differences in the brain biochemistry between "good" and "poor" learners belonging to a random population of young animals. In the present study, an attempt was taken to correlate the individual variation in short- and long-term spatial memory in three different lines of young, healthy rats: inbred Wistar (W), outcrossed Wistar/Spraque Dawley (W/S) and pigmented Long-Evans rats, with hippocampal levels of selected enzymes known as "memory molecules" including neuronal (n), endothelial (e) and inducible (i) NOS, CaMKIIα, PKA and ChAT. Additionally, in order to indirectly estimate the activity of CaMKIIα and PKA, hippocampal levels of their phosphorylated forms (pCaMKIIα and pPKA) were assessed. Rats were classified as "good" and "poor" learners on the basis of their performance in a partially baited 12-arm radial maze. The hippocampal protein levels were measured using Western

Blot technique. In addition to individual variation in animals' learning capacity, strain-depended differences have also been observed. Deficient performance recorded in inbred W rats compared to outcrossed W/S rats, and "poor" learners from both rat groups had predominantly related to the higher frequency of reference memory errors. The results of biochemical assays showed strain-depended differences in the NOS expression. The overall NOS levels were significantly higher in outcrossed W/S rats compared to inbred W rats. In both rat lines, the rate of learning positively correlated with hippocampal levels of nNOS and negatively correlated with iNOS levels. Hippocampal eNOS levels correlated negatively with animals' performance but only in the W rats. These results suggested that all 3 NOS isoforms are implemented in the learning process playing, however, different roles in neural signaling. Experiments carried out on Long-Evans rats did not reveal a significant difference in the basal hippocampal levels of the CaMKIIα, however, the level of the pCaMKIIα, was significantly higher in "good" learners. Also, hippocampal levels of both PKA and pPKA, as well as that of ChAT were significantly higher in "good" as compared to "poor" learners. Taken together, the latter findings indicate that low hippocampal expression of PKA and ChAT as well as low CaMKIIα or PKA activation may cause learning deficits in random population of young rats, and thus, these enzymes can be considered target molecules when looking for cognitive enhancers to treat memory deficits in young subjects.

ÖZ

SIÇANLARDA MEKANSAL BELLEKTEKİ BİREYSEL VARYASYONUN MOLEKÜLER TEMELİNİN ARAŞTIRILMASI

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Haziran 2012, 159 sayfa

Belleğin moleküler temelini araştıran çok sayıda çalışma yapılmasına rağmen, rastgele seçilmiş genç hayvan populasyonu içinde "iyi" ve "kötü" öğrenen bireyler arasında beyin biyokimyasında olabilecek farklılıklar hakkında çok az bilgi mevcuttur. Sunulan çalışmanın amacı, genç, sağlıklı üç farklı sıçan soyunda, inbred (aynı soydan gelen) Wistar (W), outcrossed Wistar/Sprague-Dawley (W/S), pigmentli Long-Evans, kısa ve uzun süreli mekansal bellekteki bireysel varyasyon ile "bellek molekülleri" olarak bilinen bazı enzimlerin, nöronal (n), endotelyal (e), indüklenebilir (i) NOS, CaMKIIα, PKA ve ChAT, hipokampustaki seviyeleri araında ilişki kurmaktır. Ek olarak, CaMKIIα ve PKA'nın hipokampustaki aktivitesini dolaylı olarak ölçebilmek için bu proteinlerin fosforile formlarının (pCaMKIIα ve pPKA) hipokampustaki seviyeleri de ölçülmüştür. Sıçanlar kısmi pekiştirilmiş 12-kollu radyal labirentteki performanslarına göre "iyi" ve "kötü" öğrenen olarak

sınıflandırılmıştır. Proteinlerin hipokampustaki seviyeleri Western Blot tekniği ile ölçülmüştür. Hayvanlarda öğrenme kapasitesindeki bireysel varyasyounun yanı sıra soya bağlı farklılıklar da gözlenmiştir. W sıçanlar W/S sıçanlara göre öğrenme eğitiminde eksik performans göstermişlerdir. Her iki sıçan grubunda da uzun süreli bellek hata sayıları "kötü" öğrenenlerde yüksektir ve öğrenme performansı ile ağırlıklı olarak ilişkilidir. Biyokimyasal testlerden elde edilen sonuçlar, NOS ekspresyonunda soya bağlı farklılıklar olduğunu göstermiştir. Genel olarak bakıldığında, NOS ekspresyon seviyeleri W/S sıçan grubunda W sıçan grubuna göre anlamlı derecede yüksektir. Her iki sıçan soyunda, mekansal öğrenme kapasitesi ile nNOS seviyesi arasında pozitif, iNOS seviyesi arasında ise negatif ilişki bulunmuştur. eNOS seviyesi ise sadece W sıçanlarda mekansal öğrenme kapasitesi ile negatif ilişkilidir. Bu sonuç, 3 NOS izoformunun nöral iletimde farklı roller oynayarak öğrenme süreçlerinde rolü olduğuna işaret etmektedir. Long-Evans sıçanlardan elde edilen sonuçlara göre, hipokampustaki bazal CaMKIIα seviyesinde belirgin fark yokken, pCaMKIIα seviyesi "iyi" öğrenen sıçanlarda anlamlı derecede yüksektir. Ek olarak, hem PKA, pPKA hem de ChAT'ın hipokampustaki seviyeleri "iyi" öğrenen grupta "kötü" öğrenenlere nazaran anlamlı derecede daha yüksektir. Alınan bu son sonuçlar birlikte değerlendirildiğinde, hipokampustaki düşük CaMKIIα veya PKA aktivasyonunun yanı sıra hipokampustaki düşük PKA ve ChAT ekspresyonunun da rastgele seçilmiş genç sıçan populasyonunda öğrenme bozukluklarına neden olabileceğine işaret etmektedir. Alınan bu sonuç, söz konusu enzimlerin genç populasyonlarda görülen bellek bozukluklarının tedavisine ilişkin bilişsel geliştiriciler arandığında hedef molekül olarak düşünülebileceklerini ortaya koymaktadır.

Anahtar kelimeler: Hipokampus, Mekansal bellek, Kısmi pekiştirilmiş 12-kollu radyal labirent, Western Blot, NOS izoformları, CaMKIIα, pCaMKIIα, PKA, pPKA, ChAT, Sıçan

To My Family,

For your endless support and love

ACKNOWLEDGEMENTS

Gezer for their help and being with me throughout the longer nights during experiments.

Special thank to all my labmates both in Lab B-57 and Lab-144 for their friendly collaboration. Additionally, I also want to say thank to my labmates Ekin Keçecioğlu, Birsen Elibol and Melike Sever for their help.

I would like to thank to my friends Gül Çalışır Açan and Sinan Can Açan, for their help with formatting. I also want to say thank to Onur Baloğlu and Alper Döm for their help with troubleshooting computer problems.

I have very special thank to my friend Kubilay Yıldırım for his invaluable friendship, support and for always being there through my hard times.

I also wish to thank to my friends Pelin Sevinç, Oya Ercan, İlknur Dursun and Süreyya Özcan for their lovely friendship.

I would like to send my warmest thanks to my husband, Serkan Saraç, for his endless patience, support and love.

This thesis is dedicated to my family. No words can express how much your love, wisdom, unfailing support, thrust and encouragement has meant over the years. Without you I couldn't have done this. Thank you for everything.

To the rest of my friends and all staff at Department of Biological Sciences, METU for their support.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

#	Number
°C	Degree celsius
μg	microgram
μl	microliter
Ab	Antibody
Acetyl CoA	Acetyl coenzyme A
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APS	Ammonium per sulphate
Asp286	Aspartic acid on position 286
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
CA1	Cornus ammoni region 1
CA2	Cornus ammoni region 2
Ca^{2+}	Calcium
CA3	Cornus ammoni region 3
CaMKIIα	Ca^{2+}/calmodulin-dependent protein kinase II alpha
cGMP	Cyclic guanosine monophosphate
ChAT	Choline acetyltransferase
cm	centimeter
$CuSO_4$	Copper sulphate
dH_2O	Distilled water
EDTA	Ethylenediamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
ERK	Extracellular signal regulated kinase
g	gram

GC	Guanyl cyclase
GluR1	Glutamate receptor 1
G-proteins	Guanine nucleotide binding proteins
h	hour
HCL	Hydrochloric acid
HEPES	N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid
Hz	Hertz
ID	Identification
K^+	Potassium
kDa	Kilodalton
L	liter
LTD	Long term depression
LTP	Long term potentiation
M	Molar
mA	miliamper
mg	milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
min	minute
ml	milliliter
mM	milimolar
mm	milimeter
mRNA	Messenger ribonucleic acid
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
Na-K	Sodium-Potassium
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
nm	nanometer
NMDA	N-methyl-D-aspartate

NOS	Nitric oxide synthase
NR2B	N-methyl-D-aspartate receptor subtype 2B
O_2	Oxygen
PKA	cAMP-dependent protein kinase
RNase	Ribonuclease
rpm	revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
SEM	Standard error of the mean
Ser831	Serine on position 831
SPSS	Statistical package for the Social Sciences
TEMED	N-N-N'-N'-tetramethylenediamine
Thr286	Threonine on position 286
V	Volt
W	Wistar
W/S	Wistar-Spraque
$ZnCl_2$	Zinc chloride
α	Alpha
β	Beta
γ	Gama
δ	Delta

CHAPTER 1

INTRODUCTION

1.1. Definition and classification of learning and memory

The brain is the organ that is responsible for what we call the mind. It is the anatomical basis for thinking, feeling, wanting, perceiving, curiosity, as well as learning and memory. In general terms, learning could be defined as the process in which the new information is acquired and memory could be defined as the process in which that knowledge is maintained (Okano *et al*., 2000; Bailey *et al*., 1996; Squire and Kandel, 1999). In behavioral terms, we can define learning as a more or less permanent change in animal's or human's response to a repeated stimulus. Memory is a major mental process and without memory we are only capable of simple reflexes and stereotyped behaviors (Okano *et al*., 2000; Squire, 2004). Learning and memory play a very important role in both human and animal life. Therefore, elucidation of learning processes, anatomy of memory and the molecular mechanisms by which brain codes, stores and retrieves memory traces is one of the most intriguing and challenging issues in the contemporary neuroscience.

Studies have shown that memory can be classified into two general classes: declarative (explicit) memory and nondeclarative (implicit) memory (Figure 1) (Squire and Kandel, 1999; Smith, 2002; Kesner and Martines, 2007). Declarative memory is a conscious recall of the knowledge related to facts and events. Two types of declarative memory are distinguished: episodic memory

and semantic memory (Moscovitch *et al*., 2006; O'Keefe and Nadel, 1978) (see Figure 1). Episodic memory includes information about autobiographic events ("what", "where", "when") while semantic memory stores factual information that is independent of personal experience, i.e. linguistic knowledge. In contrast to this, nondeclarative memory is a nonconscious recall of motor skills and also contains simple associative forms, e.g. classical conditioning and nonassociative forms, e.g. habituation and sensitization (Eichenbaum and Cohen, 2001; Okano *et al*., 2000; Squire and Kandel, 1999; Squire, 2004). It is the memory that is required to use a previously learned motor skills, i.e. riding a bicycle or playing tenis.

The declarative and nondeclarative memory have different anatomical bases therefore there are patients with impaired declarative memory whose nondeclarative memory is completely spared (Bailey *et al*. 1996; Hawkins *et al*., 2006; Okano *et al*., 2000; Smith, 2002; Zola-Morgan *et al*., 1989a; Zola-Morgan *et al*., 1989b). Clinical studies on the patient H.M. (Scoville and Milner, 1957) suggested that declarative memory highly depends on medial temporal lobe structures of cerebrum containing the hippocampal formation. Further clinical and animal studies confirmed this notion (Squire and Zola-Morgan, 1991; Zola-Morgan and Squire, 1993; Suzuki and Amaral, 2004). On the other hand, further studies attempting to elucidate the anatomy of memory indicate that nondeclarative memory requires such brain structures as striatum, cerebellum, and amygdala (Hawkins *et al*., 2006; Bailey *et al*., 1996; Morris, 2003).

Furthermore, depending on the time span of the memory trace retained in the nervous system, memory is classified as short-term or working memory (STM/WM), which is formed quickly and can last for minutes or hours, and long-term or reference memory (LTM/RM) that lasts from hours to days, weeks, or years. It is generally accepted that STM involves post-translational

modifications of preexisting proteins leading to functional changes such as enzyme activation/inactivation resulting in receptor sensitization or desensitization, masking/unmasking of the receptors, change in ion channels conductance etc., which alter the efficiency of synaptic transmission (Lynch, 2004; Paratore *et al*., 2006). In contrast, LTM formation can be blocked via inhibitors of transcription and/or translation indicating that it relies on *de novo* protein synthesis and gene expression (Hawkins *et al*., 2006). Newly synthesized proteins in the course of memory consolidation can contribute to restructuring synaptic contacts by i.e. fiber outgrowth and new synapse formation, which will alter synaptic efficacy beyond the duration of STM.
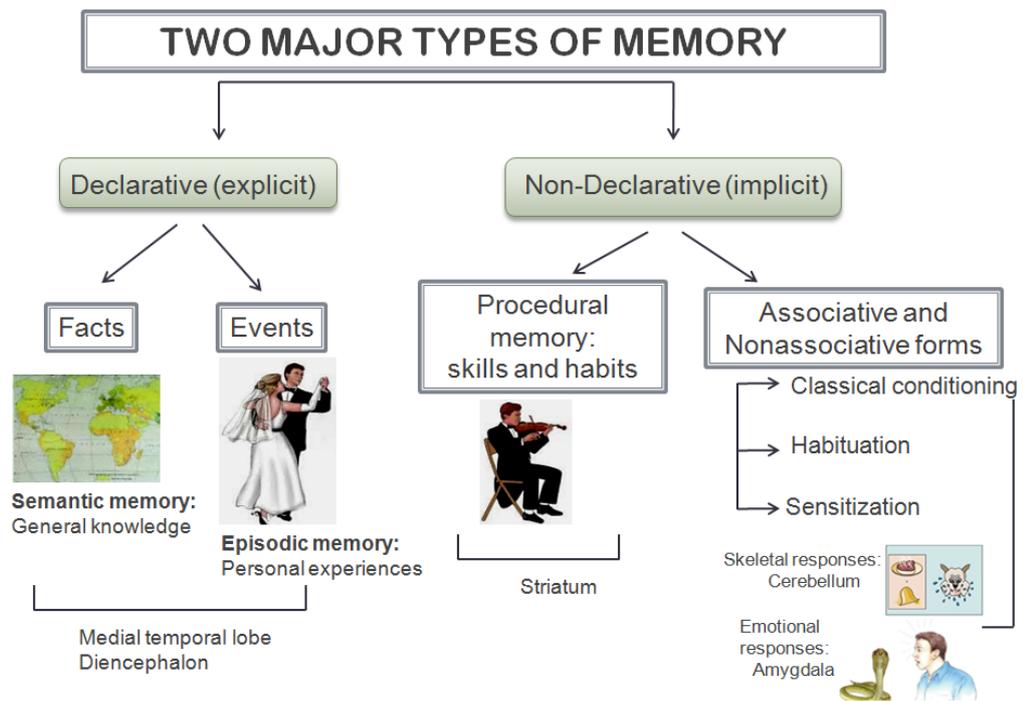
Figure 1 Classification of memory systems.

1.1.1 Spatial memory

Spatial memory is considered a form of episodic declarative memory answering the question "where?". It is related to exploratory behavior which represents the natural curiosity and need to get information about a new environment (Berlyne, 1950; Thinus-Blanc *et al.*, 1996). It could be described as a function of brain that responsible for codifying, storing and retrieving information about the arrangement of objects or specific routes in space (Kessels *et al.*, 2001).

The spatial working memory can be defined as a short-term storage of limited amount of spatial information, which can be used for other cognitive processes and is available for immediate access (Awh and Jonides, 2001). On the other hand, the spatial reference memory, a term first coined by Olton *et al.* (1979), refers to spatial information which is used over and over again and is usually obtained as a result of repeated training therefore showing higher resistance to interference. Spatial learning and memory tasks are the most frequently used experimental paradigms in the studies on learning and memory using animal models. Today, preferential experimental animals used in these studies are small rodents such as rats and mice which demonstrate many anatomical, physiological and behavioral similarities with other mammals including human, but are easily available and easy to maintain. In wild, these animals live in complex labyrinths underground burrows and forage in the open areas of grassland or in the bush. Their life style requires good spatial memory therefore they are suitable subjects for experimental studies on spatial learning and memory (Whishaw, 1991; Whishaw and Tomie, 1997).

1.1.1.1 Common experimental paradigms used in the studies on spatial learning and memory

Experimental devices commonly used for assessment of spatial learning and memory in small rodents are different types of mazes (labyrinths) (Figure 2). During training, positive reinforcers such as water, sweetened water, food, refuge or the chance to explore new objects may be used in addition to negative reinforcers such as a loud noise, water immersion, wind, intense light or electric shock. The rational for the use of these mazes comes from the notion that subjects are supposed to learn and remember locations of both pleasant and unpleasant events so in the future they can revisit or avoid them. Mazes can be open with multiple route options like Barnes, Morris or Bures arenas (Barnes, 1979; Morris, 1981; Bures *et al*., 1997a) or open with restricted options like radial, T or Y maze (Olton and Samuelsen, 1976; Vorhees, 1987). Researchers have introduced many modifications both to the mazes themselves as well as evaluation protocols to be able to discriminate between different learning strategies used in task solving (i.e. guidance strategy, response chaining, utilization of allothetic versus idiothetic cues), different forms of memory (i.e. short-term working memory versus long-term reference memory), and between the effects of cognitive and non-cognitive factors (motor activity, anxiety level etc.) on animals' performance (Bats *et al*., 2001; Dawood *et al*., 2004; Brandeis *et al*., 1989; Buresova *et al*., 1985; D'Hooge and De Deyn, 2001; Dubreuil *et al*., 2003). However, despite the fact that similar procedures are used in many studies, there are still variables that influence animal performance and result in discrepant results. Among these factors are animals' strain, age, and gender (Jakubowska-Dogru *et al*., 2003; Jakubowska-Dogru *et al*., 2005). Although a great variety of mazes have been designed, the most commonly used are the Morris water maze (Morris, 1981), the Barnes' circular maze (Barnes, 1979) and the radial maze (Olton and Samuelson, 1976) (see Figure 2).

KINDS OF MAZES

Y MAZE
CIRCULAR MAZE
RADIAL MAZE
ARENA MAZE
WATER MAZE
T MAZE
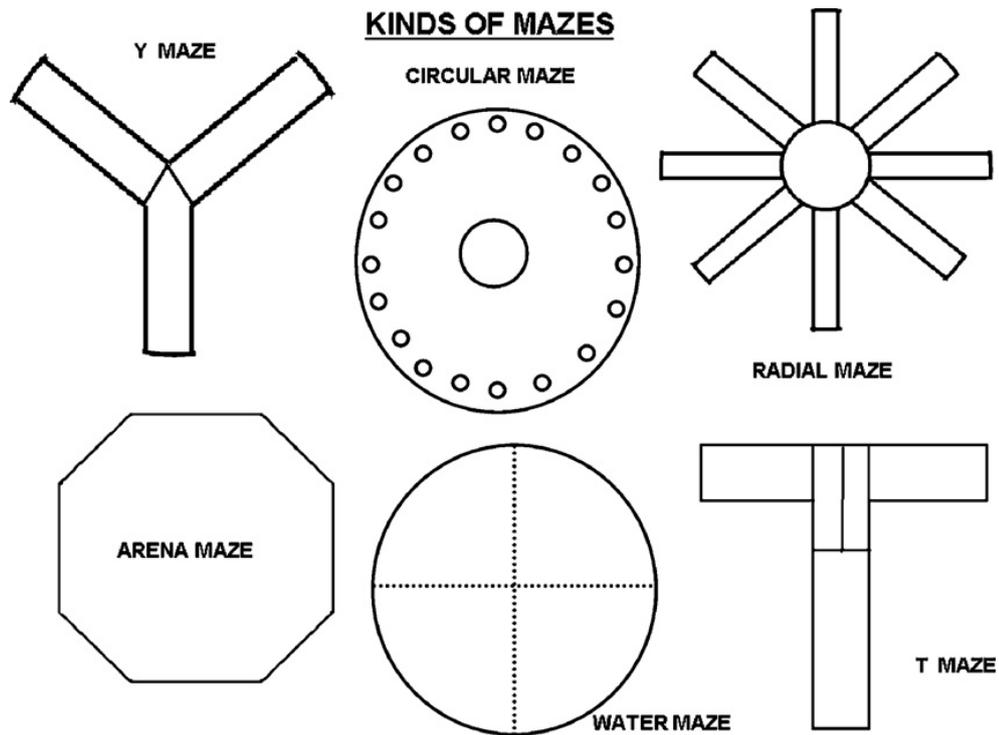
Figure 2 Different types of mazes for the evaluation of spatial memory (Modified after Paul *et al.,* 2009).

1.1.1.1.1 Radial-arm maze

The radial-arm maze was first built up by Olton and Samuelson in 1976 for evaluation of spatial working memory in rats. The original device was composed of eight linear arms merging in the center. According to an original "win-shift" experimental protocol, at the beginning of a daily trial, a food pellet is placed at the end of each arm; soon after the animal earlier subjected to food-deprivation is placed in the maze, on the central platform, and is allowed to choose freely between the arms. The optimum strategy in such a paradigm is to visit each arm only once and to retrieve all food pellets with minimum energy spent. To remember the places already visited, animal may use either extramaze (allocentric or allothetic) cues (usually visual stimuli belonging to

the room) or intramaze cues (i.e. odor, visual, tactile), if such cues are available. If the experiments are run in darkness or when extra- and intramaze cues are eliminated, animals may use kinesthetic and vestibular (egocentric or idiothetic) signals to orient themselves in the space. Another experimental paradigm which can be applied to study working spatial memory using radial maze, is the "win-stay" paradigm based on delayed matching-to-place strategy. In this paradigm, on a first trial (sample trial), only one door leading to the baited arm is open and animal is forced to choose a preselected arm containing food reinforcement. After a delay which may be gradually increased from 10s to i.e. 30 min, animal is again introduced to the maze but this time, entries to all arms are open and the animal is supposed to make only one free choice (choice trial). It will win another pellet if enters the previously baited arm (Jakubowska-Dogru *et al*., 2003).

The partially baited version of a radial maze serves evaluation of both working and reference spatial memory. In this version, the number of arms is usually increased, and the reinforcement (i.e. food pellet) is placed only in the half randomly selected arms. In this latter case, the optimal strategy on a single trial is not to visit the arms which are never baited, and not to re-visit the baited arms already visited. Visiting arms which are never baited is counted as a reference memory error (RME), while revisiting baited arms counted as working memory error (WME).

1.2 Anatomy of memory and hippocampal circuitry

The studies on the anatomy and physiology of memory in mammals have been carried mainly on mice, rats and monkeys. Process of memory formation is interlocked with perceptual and attentional processes and the mnemonic processes themselves include several distinct stages such as encoding memory traces, memory consolidation, and memory recall or retrieval. Specially

designed experimental paradigms, however, to a certain degree allow investigators to separate the effects of different manipulations (i.e. brain lesions and/or drug administration) on these different aspects of mnemonic functions. Early theories related to memory formation (Arbib *et al*., 1972) postulated that experiences and input of sensory information set up persistent electrical activity in the central nervous system. These so called reverberating circuits, or populations of continuously active neurons, were postulated to form a basis for information storage in some coded form. When the active neural processes cease, forgetting occurs, and that bit of information is lost.

The results of more recent behavioral studies combined with electrophysiological, lesion, pharmacological, and imaging techniques e.g. fMRI (functional magnetic resonance imaging) and PET (positron emission tomography) indicate that in the mammalian brain, there are several, more or less independent circuits which acquire and store different types of information to influence behavior, thus, represent different kinds of memory (Alkon *et al*., 1991; Granon *et al*., 1994; McDonald *et al*., 1993; Mishkin and Appenzeller, 1987; Mishkin, 1993; Packard *et al*., 1989; Sherry and Schacter, 1987; Shuterland *et al*., 1988; Thompson and Krupa, 1994; Weiskrantz, 1990; White, 2005; Zola-Morgan and Squire, 1993). Declarative memory seems to depend on temporal lobe structures and diencephalon. These structures are sometimes referred to as **"Extended Hippocampal Circuit"** and include hippocampal complex (entorhinal cortex, hippocampus and subiculum), retrosplenial cortex, part of cingulate gyrus, anterior thalamic nuclei, and mamillary bodies (MacLean, 1948). In this circuit, an important role is assigned to the hippocampus. The hippocampus is formed by two interlocking sheets of cortex and in cross-section has a very defined laminar structure with distinct layers where rows of principal neurons (pyramidal and granular cells) are arranged. The main pyramidal cell layers are in the Cornu Ammonis CA1-4 regions (mainly CA1 and CA3 regions)**,** and granular cell layer in the dentate gyrus

(DG)**.** The connections within the hippocampus generally follow this laminar format and, as a rule, are uni-directional. They form well-characterised closed loops (Amaral and Witter, 1989; Amaral, 1993; Ishizuka *et al*., 1990).

Hippocampus, a phylogenetically old part of rhinencephalon, is connected with subcortical limbic structures (amygdala, septum, mammillary bodies, anterior thalamus, hypothalamus, striatum) but also with several neocortical areas including prefrontal/orbitorfrontal cortices, cingulate cortex, piriform cortex, entorhinal, perirhinal and postrhinal cortices, A series of cortico-cortico connections conveys neocortical input through the cortico-hippocampal-cortical loop. The major afferent input hippocampus receives via the perforant path (PP) from enthorhinal cortex which is a gateway from the multitude of other cortical areas. Axons from layers II/IV project to the granule cells of the dentate gyrus (DG) and pyramidal cells of the CA3 region, while those from layers III/V project to the pyramidal cells of the CA1 and the subiculum (Sb) (see Figure 3). Another extrinsic input to the hippocampus constitutes of cholinergic afferents originating mainly from the medial septum-diagonal band (MS-DB) complex. It has been demonstrated that dorsal and ventral hippocampus receive separate cholinergic inputs. It was found that the dorsal hippocampus receives cholinergic input mainly from the rostral half of the MS-vertical limb of the diagonal band (VDB) and the core part of the horizontal limb of the diagonal band (HDB), whereas the ventral hippocampus largely from the caudal half part of the MS-VDB and the marginal part of the HDB (Yoshida and Oka, 1995). The medial septal area is considered a pacemaker region for the hippocampal theta rhythm (7.4Hz). Acetylcholine is an important neuromodulator involved in cortical arousal in mammals. Cholinergic modulation is involved in conscious awareness and attention processes required for learning and memory formation and provides intercommunication between different cortical regions. Such communication is achieved in part through temporal re-structuring of neuronal activity by population rhythms.

Hippocampal intrinsic circuitry includes the mossy fibres pathway and Schaffer Collateral/Associational Commissural (SC/AC) Pathway. Mossy fibers (MF) are the axons of DG granule cells terminating on CA3 pyramidal cells and forming their major input. Schaffer Collaterals and Commissural Fibers are the branching axons of CA3 pyramidal neurons projecting to the ipsi- and contra-lateral hippocampal CA1 regions (see Figure 3). Hippocampal efferent projections go back to the septum (lateral septal nuclei), and from CA1 region through subiculum out of hippocampus on to the medial and lateral enthorhinal cortices (LEC) and via, respectively, post- and perirhinal cortices back to the subcortical structures of the extended hippocampal circuit, and to other neocortical areas (Burwell *et al.*, 1995; Suzuki and Amaral, 2004).
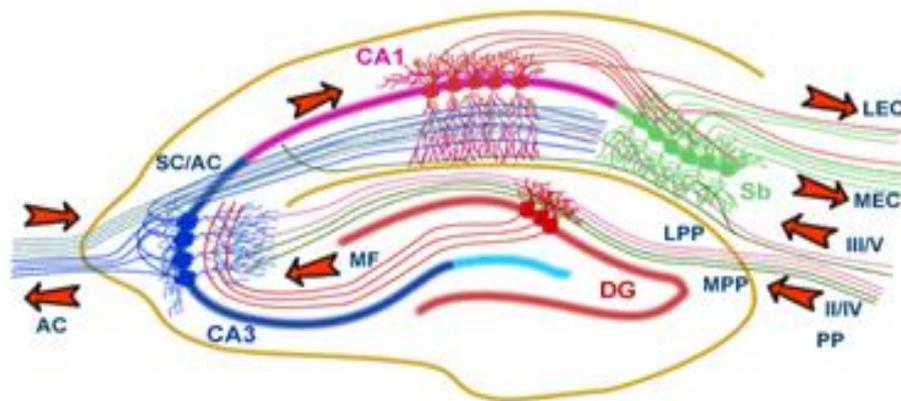
Figure 3 The hippocampal pathway (www.bristol.ac.uk/synaptic/pathways/).

The hippocampus receives via perirhinal and enthorhinal cortex inputs from a cascade of uni- and polymodal association cortices. It was demonstrated that hippocampal circuit contributes to the acquisition and to some extent also to the retrieval of recent and remote episodic memories in human and context–dependent memories in animals. In rats, such context-dependent memory is spatial learning but not when animal is navigating towards a specific and

distinct land mark but only when there is no such a land mark and it must navigate creating in his brain cognitive map of the external environment basing on allothetic cues. This notion was supported by the discovery of the hippocampal "place cells" (O'Keefe and Dostrovsky, 1971; Markus *et al*., 1995; Moser *et al*., 2008). The discovery of "place-cells" by O'Keefe and Dostrovsky (1971) was the key phenomena that later led John O'Keefe and Lynn Nadel (1978) to propose that the hippocampus was the locus of a "cognitive map" of the environment. Hippocampal "place-cells" are probably the principal cells in each layers of hippocampus (mainly CA1 area) which fire in complex bursts when an animal moves towards a specific location in an environment. The region in which a cell fires the most is that cell's "firing-field" or "place-field" (O'Keefe and Dostrovsky, 1971; O'Keefe, 1976). Inside its field, a place cell increases its firing frequency to 20Hz or more, while outside its field, a place cell may fire less than 1 spike every 10 sec (.1Hz). If a sufficient number given, place cells and their fields might cover or "map" any given environment. Therefore, evidence from place cells verifies involvement of hippocampus in spatial mapping (O'Keefe and Nadel, 1978). O'Keefe and Nadel further postulated in their book, "*The Hippocampus as a Cognitive Map*", that the function of the map was not only to allow spatial navigation throughout an environment, but also to play as a memory framework upon which the significant items and episodes of experience could be superimposed. This memory framework is encoding information about what, when and where happened (for detail see htpp://www.cognitivemap.net; Fuhs, 2006; Leutgeb *et al.,* 2005; Moser *et al.,* 2008; and also the works by Bures *et al*., 1997; O'Keefe and Nadel, 1978; Olton *et al*., 1979; Morris *et al.*, 1982).

The extensive connections of the entorhinal cortex with the hippocampus and the neocortex point to this region as a major interface in the hippocampal-neocortical interactions underlying memory. In the recent studies by Moser group (Steffenach *et al*., 2005) it has been demonstrated that in rats, after

training in a Morris water maze, fiber-sparing lesions in the dorsolateral band of the entorhinal cortex, which mediates much of the visuospatial input to the dorsal hippocampus, memory retention and learning of a new task were entirely disrupted. On the other hand, spatial learning and memory were spared by lesions in the ventromedial band of the enthorinal cortex, which connects primarily with the ventral hippocampus and amygdala. These lesions, however, reduced defensive behavior on an elevated plus maze, mirroring the effects of damage to ventral hippocampus.

It is postulated that place recognition, and thus memory of spatial location of visual, olfactory and somatosensory stimuli depends on so called *dorsal stream* beginning in the relevant primary sensory cortex and going via the temporal lobe structures (medial entorhinal and postrhinal cortices) to the posterior parietal cortex, and further to the dorso-lateral prefrontal cortex. On the other hand, there is an evidence that object recognition and thus memory of stimulus quality depends on so called *ventral stream* beginning in the relevant primary sensory cortex and going via medial temporal cortex (lateral entorhinal and perirhinal cortex) to the ventromedial and cingulate prefrontal cortices (monkey studies) (Mishkin and Murray, 1994).

As mentioned earlier, nondeclarative procedural memory seems to depend largely on the integrity of *striatum and cerebellum,* while classical conditioning and especially rapid acquisition of behaviours based on biologically significant events with affective properties on *amygdala* but also *cerebellum*. Amygdala, another important structure of the temporal lobe, is connected by ventral amygdaloid pathway with dorsomedial thalamus (parsa magnocellularis), which in turn sends projection to orbitofrontal cortex (monkey studies) (Carmichael and Price, 1995). Thus, frontal cortex and especially the limbic prefrontal cortex receives inputs from both important for memory limbic structures: hippocampus and amygdala. It is not surprising then, that prefrontal

cortex is also implemented in mnemonic processes. Prefrontal cortex with its dopaminergic afferentation seems to be implemented in the inhibitory control over the non-adaptive behaviors (Dabrowska, 1971) and also seems to be important for the short-term working memory (Goldman-Rakic, 1992).

1.3 Cellular and molecular basis of learning and memory formation

Today, it is generally accepted that memories are encoded as dynamic spatio-temporal patterns of cellular activity within widespread neural networks. This reverberating activity results in altered patterns of connectivity among co-activated neurons (Jungerman *et al*., 2007). Increase or decrease in the strength of neural connections depends on plastic changes occuring at synapses due to repeated synaptic activation (Hebb, 1949; Kandel, 2006). At the basis of an increase (potentiation) or a decrease (depression) in neuron's responses to synaptic activation are biochemical processes including often complex intracellular signal transduction pathways and molecular cascades leading to transient or sustained functional and/or structural changes in activated neurons. Inheritance of complex characteristics such as learning and memory depends on polygenic systems which have been conserved through the evolutionary history of species. Therefore, the studies on the biochemistry and genetics of human memory use different animal models. In these studies, the cellular and molecular events are being correlated with the behavioral output. An important pre-condition in such studies is to be able precisely delineate the neural circuit responsible for particular behavior. For this reason invertebrate animal models such as *C. elegans*, *Drosophila melanogaster*, or marine snails *Aplysia californica* and *Hermissenda crassicornis*, having much simple neural circuits, highly reduced number of circuit elements and to large extent deciphered

genome are frequently used in molecular studies on learning and memory (Bailey and Kandel, 2008; Davis, 1996; Mayford *et al.,* 1997).

Invertebrate animal models have provided much information about the cellular and molecular mechanisms of activity-dependent neural plasticity. They are, however, unable to answer the question about localization of different cognitive functions in the mammalian and human brain. Therefore, in parallel, extensive studies are carried out on the cellular and molecular correlates of learning in mammalian brain. Hippocampus is perhaps the most studied structure in the mammalian brain. It is because of its well documented role in learning and memory and because of its simple, tri-synaptic circuitry. Although different brain structures seem to be responsible for different forms of memory, cellular and molecular processes underlying encoding, storage, and retrieval of memory may be similar throughout the central nervous system.

1.3.1 Long-term potentiation/depression (LTP/LTD) as a cellular model of memory formation

Activity-dependent changes in neural functions that modulate subsequent synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) are known as metaplasticity (Abraham and Bear, 1996; Abraham, 2008). LTP means an increase while LTD a decrease in the response of postsynaptic neuron to a single presynaptic impulse after earlier tetanic stimulation of the synapse (Lomo and Bliss, 1996; Ito, 1989).

A typical procedure followed in *in vitro* or *in vivo* experiment on hippocampal LTP include three steps:

- delivering a single stimulating pulse to either preforant path, mossy fibers or Schaffer collaterals and recording population excitatory postsynaptic potentials (EPSP) in either dentate gyrus, hippocampal CA3 field, or CA1,
- delivering a 50 to 200Hz burst of pulses to the same pre-synaptic pathway,
- recording population EPSP from dentate gyrus /hippocampal CA3/CA1 evoked by a single pulse after delay.

LTP is considered by some scientists a cellular model of learning and memory formation (Bliss and Collingridge, 1993; Lynch, 2004; Malenka and Nicoll, 1999; Squire and Kandel, 1999). It was reported that pharmacological blocking hippocampal LTP was paralled by performance deficit in hippocampus-dependent learning task (Morris *et al*., 1986). However, not all studies confirmed a direct relationship between LTP and behavioral memory (for review, see Amaral and Witter, 1989; Moser *et al.,* 1993; McEachern and Shaw, 1996). Nevertheless, experimental paradigms for LTP induction are routinely used together with animal models of trauma, Alzheimer's disease, and/or epilepsy to examine how synaptic plasticity could be changed by disease or injury.

Two types of LTP were described in hippocampus: glutamatergic NMDA receptor-dependent and NMDA-independent. NMDA-independent but cAMP-dependent occurs at mosy fibers' synapses in CA3 region, is nonassociative (similarly to sensitization) and has primarily pre-synaptic mechanism. In contrast, LTP dependent on NMDA receptors occurs at perforant pathway synapses in DG and at Schaffer collaterals in CA1 region, is characterized by

rapid induction, associativity, input specificity, and variable persistence (early-LTP and late-LTP), and has both pre- and postsynaptic mechanisms. Associativity is understood as heterosynaptic interaction of two or more inputs, over a short time scale (less than one second). It thus represents Hebbian type of neuroplasticity: the induction of LTP in DG and CA1 requires coincident activity in the postsynaptic pyramidal cells and presynaptic neuron. The input specificity of early-LTP is usually considered in relation to the compartmentalization of Ca^{2+} transients within dendritic spines and thus local Ca^{2+}-dependent phosphorylation. The input specificity of late-LTP is determined by local tags that sequester proteins manufactured as a result of a cross-talk between the synaptic membrane and the nucleus leading to alterations in gene expression, and the synthesis of new gene products that stabilize changes in synaptic responses (Frey and Morris, 1998). Early and late phase of LTP involve different molecular mechanisms. Early LTP is independent of protein synthesis and involves short-term posttranslational changes in preexisting synaptic proteins, lasting for minutes to hours, and resulting from protein kinases and proteolytic enzymes activation. Protein phosphorylation or proteolytic cleavage causes conformational (allosteric) changes in the structure of synaptic proteins on both pre- and post-synaptic membrane leading to masking/unmasking neurotransmitter receptors, receptor sensitization/desensitization, modulation of ion channel conductance, and/or enhanced neurotransmitter (NT) release (Lynch, 2004; Paratore *et al.*, 2006). Long-term changes lasting for days and sometimes life long, can be blocked via inhibitors of transcription and/or translation that indicates that they rely on modulation of gene expression and *de novo* protein synthesis (including ion channels, receptors, enzymes, membrane transporters, cytoskeleton components) and thus, lead to morphological modifications (Hawkins *et al.*, 2006; Squire and Kandel, 1999). Newly synthesized proteins in the course of memory consolidation can contribute to restructuring synaptic contacts by i.e. fiber outgrowth and new synapse formation, which will alter synaptic efficacy

beyond the duration of early-LTP/STM. Whether or not early-LTP is transformed into late-LTP depends on the history of activation of the neuron including heterosynaptic activation of aminergic as well as glutamatergic input pathways.

1.3.2 Molecular basis of learning and memory

The importance of NMDA-dependent LTP for associative learning and declarative memory arises from the unique properties of glutamatergic NMDA receptor (Citri and Malenka, 2008). NMDA receptor-channel complex is a potent glutamate and voltage dependent Ca^{2+} ionophore (Figure 4). There are two pre-requisites for activation of the NMDA-dependent signaling pathway: (1) binding glutamate to NMDA receptor and (2) concomitant partial depolarization of postsynaptic membrane. At some synapses, this depolarization may be brought about by co-activation of another (not necessarily glutamatergic) synaptic input. This way, the postsynaptic neuron is excited only in the presence of two events having close temporal relation. Because simultaneous pairing is characteristic of associative learning, the requirement of temporal contiguity is consistent with a role of NMDA-dependent LTP in declarative memory. NMDA receptor would play here a role of the cellular coincidence detector (Li and Tsien, 2009). Genetic or pharmacological interference with NMDA receptor expression and/or functions in hippocampal CA1 area was shown to affect both LTP induction and learning. Greater LTP and better performance in a spatial learning task was reported in a line of mice in which the function of NMDA receptor enhanced by overexpressing its subunit, NR2B, in the hippocampus (Tang *et al*., 1999). On the other hand, pharmacological blockade (Nakazawa *et al*., 2004) or conditional knockout of NMDA receptors in hippocampal CA1 region using loxP-cre recombinase system was reported to prevent LTP induction and impair spatial navigation in mice (Tsien *et al*., 1996b; McHugh *et al*., 1996). It

was also shown that genetically removing of the NR1 subunit in the CA1 region, which impairs NMDA receptor, makes that the place fields generated in CA1 area loose their specificity (McHugh *et al*., 1996).

Although the importance of NMDA receptors in LTP induction is well documented, another type of glutamate receptor, the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor, is postulated to play an important role in its maintenance. LTP induction leads to an increase in the number of glutamatergic AMPA receptors on the post synaptic membrane, which in turn results in increased excitability of the postsynaptic neuron (Lu *et al*., 2001; Lynch, 2004).

The LTP experiments carried out on the hippocampal slices showed that activation of NMDA receptors triggers a multiple molecular cascades that transmit synaptic signal to the cell nucleus to modulate gene expression (Flavell and Greenberg, 2008).
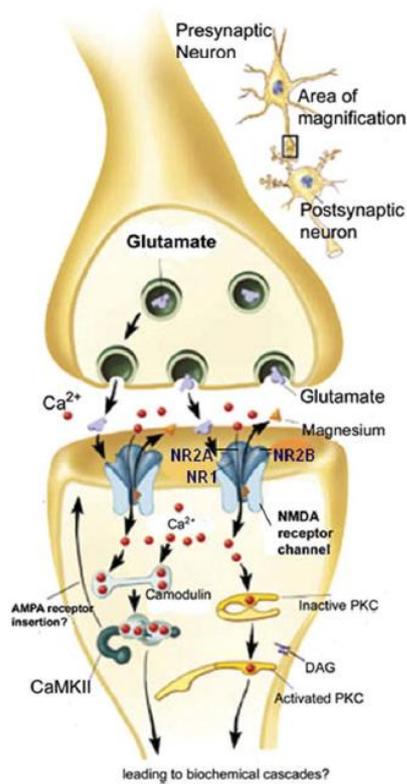
Figure 4 Molecular mechanism of synaptic plasticity. The presynaptic release of glutamate activates both AMPA and NMDA receptors. The influx of Ca^{2+} then activates biochemical cascades which finally strengthen the synapse (after Wang *et al.,* 2006).

Activation-dependent molecular changes at synapses are mediated by a rise in intracellular calcium which enters the postsynaptic neuron through NMDA ion channels. Concentration of calcium in postsynaptic region may be amplified by the activation of metabotropic glutamate receptors coupled to G-proteins via the IP3 (inositol trisphosphate) system mobilizing calcium from intracellular stores. Calcium alone or conjugated with calmodulin acts as a powerful second messenger and leads to the activation of numerous synaptic proteins via posttranslational modification and protein-protein interaction. Among those proteins are Ca^{2+}-and Ca^{2+}/calmodulin-dependent protein kinases: PKC and CaMK, controlling the function of other proteins through the phosphorylation

of Ser/Thr amino acid residues on these proteins. In the hippocampus, among many downstream signaling molecules of the NMDA receptor pathway, CaMKIIα has long been considered the key mediator in regulating the early phase of LTP and memory formation (Silva *et al.,* 1992a,b; Mayford *et al.,* 1995, Mayford *et al.,*1996, Colbran, 2004; Colbran and Brown, 2004; Lisman *et al.,* 2002; Soderling, 2000). At synapses, calcium/calmodulin binds to CaMKII after entry of Ca^{2+} through the NMDA receptors, then CaMKII translocates to post-synaptic density (PSD) via binding to the C-terminus of the NR2B subunits of NMDA receptor (see Figure 4) (Wang *et al.,* 2006). Autophosphorylation of CaMKII leads to a decrease in the dissociation of bound Ca^{2+}/CaM and extends its activation beyond the dissociation of Ca^{2+}/CaM. Thus, a transient increase in intracellular Ca^{2+} can result in prolonged α-CaMKII activation until it is dephosphorylated by protein phosphatases. The activated CaMKII at the PSD was shown to responsible for potentiating synapses, likely by enabling synaptic insertion of AMPA receptors and/or increasing their single channel conductance (Malenka and Nicoll, 1999) (see Figure 4 and Figure 5). CAMKII and PKC phosphorylates proteins interacting with long tail on GluR1, such as GRIP (glutamate receptor-interacting protein) and STG (stargazin), to deliver receptor to PDZ (post synaptic density zone) where interaction with the major postsynaptic matrix protein PSD-95 anchor receptor to the synapse In contrast to this, in the case of LTD, the removal of AMPA receptors, especially receptors containing short tail GluR2 and GluR3 subunits, takes place (Kessels and Malinow, 2009). Newly delivered GluR1-containing AMPA receptors can be phosphorylated directly by CAMK, which increases unitary conductance of the receptor and its affinity for Glu.
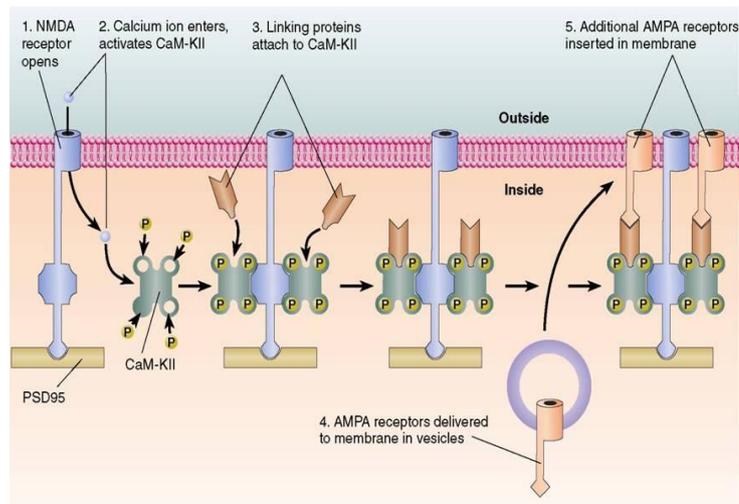
Figure 5 Insertion of AMPARs at stimulated glutamatergic synapses (after Allyn and Bacon, 2004).

Autophosphorylated persistent CaMKII form stimulates nitric oxide synthase (NOS) which leads to the raised production of the putative retrograde messenger, nitric oxide (NO) (Figure 6). NO freely diffuses across the synaptic cleft to the presynaptic cell since it is a diffusable gas and leads to a chain of molecular events which enable the presynaptic response to subsequent stimuli. In the pre-synaptic region NO activates soluble guanylate cyclase (sGC) which in turn rises the level of cGMP (an intracellular second messenger), a coactivator of protein kinase G (PKG) (see Figure 6). The presynaptic effects mediated by elevated cGMP levels and PKG activation are: decreased K+ conductance of the presynaptic membrane and reduced re-uptake of glutamate to presynaptic terminal leading to an increased neurotransmitter release and prolonged NT action and thus, increased synaptic efficacy.

PRESYNAPTIC NERVE TERMINAL

GC ← NO

cGMP

Glu   Glu   Glu

Glu

SYNAPSE   Glu

NMDA RECEPTOR

$[Ca^{2+}]$↑↑

POSTSYNAPTIC NEURON   L-arginine $\frac{CaM / NOS}{}$ NO
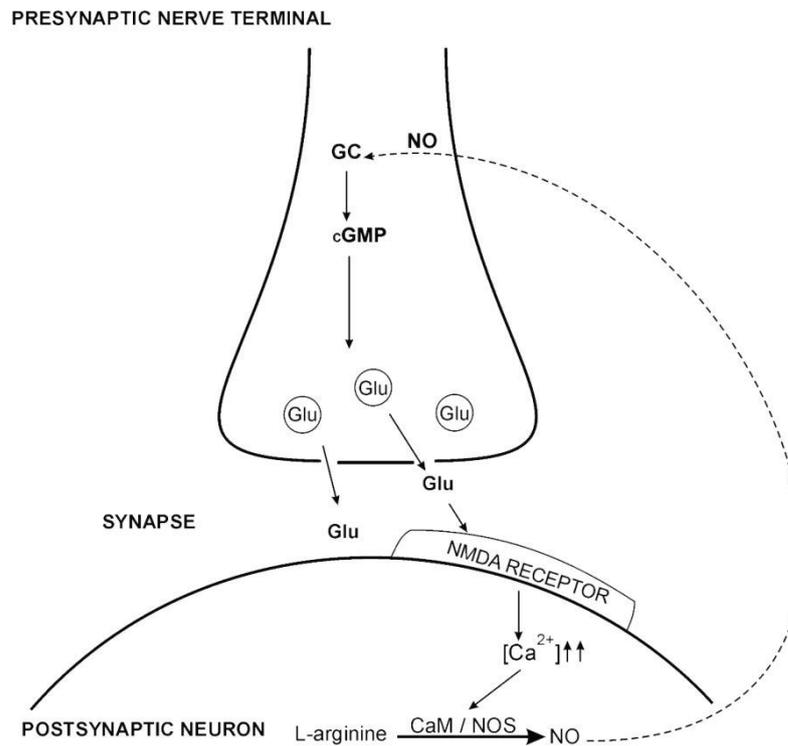
Figure 6 Retrograde synaptic signaling by NO (after Uzbay and Oglesby, 2001).

In addition to NO role as a retrograde messenger, the postsynaptic effects of this molecule were also described. In the postsynaptic cell, the components of the NO/cGMP signaling machinery are brought in close contact by the interaction with PSD-95 what allows efficient signaling without raising concentrations of this free radical throughout the cell. In the postsynaptic neuron, PKG may stimulate the release of Ca^{2+} from ryanodine receptor-gated intracellular stores, additionally increasing the Ca^{2+} concentration.

So far described synaptic changes triggered by NMDAR stimulation such as elevated Ca^{2+} levels, and activation of calcium-dependent protein kinases, are believed to support E-LTP lasting for 1-3 h.

Long-term studies run by many research groups on activity-dependent modulation of synaptic strength and structure showed that cellular and molecular processes responsible for encoding and storage of memories have been highly conserved through evolution and are very similar in invertebrate nerves system and mammalian brain (Mayford *et al*., 2012). Studies both on *Aplysia californica* and hippocampal slices demonstrated an important role of another protein kinase, the protein kinase A (PKA) during both acquisition and consolidation of memory traces (Kandel, 2001; Barco *et al*., 2006).

Activation of PKA requires activation of adenylyl cyclase (AC) and production of cAMP (negative regulator phosphodiesterase). Most of ACs are activated through neurotransmitter receptor-G protein interaction, however, some ACs isoforms (AC 1, 3, and 8 of which AC 1 and AC 8 are neuron specific) were shown to be Ca^{+2}/CaM-dependent (Riedel and Michaeu, 1999; Halls and Cooper, 2011; Wong *et al*., 1999).

PKA serves a role similar to that of CaMKII, but PKA's effects are broader. Like CaMKII, PKA phosphorylates voltage-dependent calcium channels enhancing their excitability to future stimuli. It also promotes internalization of small-conductance Ca(2+)-activated K(+) channels (SK2 channels) reducing membrane K+ conductance and thus increases neuron's excitability. These effects demonstrate PKA contribution to early phase of activity-dependent neural plasticity (i.e. E-LTP). However, both calcium stimulated AC and PKA were also shown to be critical for L-LTP and long-term memory (Wong *et al*., 1999; Kandel, 2001; Mayford *et al*., 2012). It was reported that PKA phosphorylates intracellular AMPARs stores, facilitating their insertion postsynaptically. PKA may also enhance new AMPARs delivery via activation of the mitogen-activated protein kinase/extracellular response kinase or extracellular signal-regulated kinase (MAPK/ERK) cascade**.** Cascade activation of PKA and MAPK/ERK pathways was shown to be involved in

maintaining synaptic potentiation in hippocampus beyond the initial 3 h, and thus, in LTM formation (Abel *et al.,* 1997; Chen *et al.,* 2005; Miyamoto, 2006; Roberson *et al*., 1999; Waltereit and Weller, 2003). Activated by phosphorylation MAPK translocates to the nucleus and phosphorylates the transcriptional repressor CREB2 that otherwise is inhibiting the cAMP response binding protein 1 (CREB 1), a transcriptional factor that has been shown to regulate gene expression (Kandel, 2012). With intensive synaptic stimulation, catalytic subunit of PKA is translocated to nucleus where it phosphorylates CREB1 protein. If at the same time CREB2 repressor protein is eliminated by phosphorylation by MAPK, CREB1 binds to a CRE regulatory subunit of the promoter sequence of some immediate early genes (IEGs) coding transcription factors (TFs) that induce expression of late-response structural genes (Alberini, 2009). One of these genes is encoding ubiquitin hydroxylase. Ubiquitin hydroxylase is part of ubiquitin proteosome that destroys regulatory unit of PKA what overrides the counteracting effect of protein phosphatase (PP1) and makes PKA active even after cAMP returns to normal level (persistent form of PKA) (Merrill and Strack, 2007). In a short term, PKA antagonizes the action of PP1 by phosphorylation and thus activation of constitutively expressed PP1 inhibitor-1 (I-1) which otherwise is inhibited by calcium/CaM-activated calcineurin (CN). This way active CN may inactivate CaMKII, PKA, and MAPK and thus supress long-term synaptic changes (Merrill and Strack, 2007). This example of interactions between molecules that are downstream of NMDARs shows that opposing kinases or phosphatases determine whether incoming signals enhance or suppress synaptic plasticity and therefore facilitate or supress long-term synaptic changes.

Many of IEGs encode transcription factors (TFs) such as CCAAT/enhancer-binding protein (C/EBP) or ARC (activity regulated cytoskeleton associated protein) which are responsible for activation of late-response structural genes

giving rise to the growth of the synaptic connections (ap-Elongation Factor and brain-derived neurotrophic factor (BDNF), respectively). In Aplysia, with presynaptic activation of PKA-MAPK cascade, stimulation of C/EBP activates ap-Elongation Factor promoting internalization of Aplysia cell adhesive molecules (apCAMs), defasciculation, fiber outgrowth and formation of new synaptic contacts (Alberini *et al*., 1994). On the postsynaptic side, the dendritic processes also grow to accommodate new synapses (see formation of perforated synapses). Among IEGs that have been implicated in synaptic plasticity are: c-Fos, c-Jun, Zif268, krox24, Egr-1 and earlier mentioned ARC (Alberini, 2009; Davis *et al*., 2003; Fleischmann *et al*. 2003, Plath *et al*., 2006; Kandel, 2012).

Among down-stream molecules of NMDAR activation even such fragile and short lived ones as gaseous NO were shown to contribute to long-term plastic changes in the adult brain. Already in 1999, Lu *et al*. suggested a putative role of NO in the induction of late phase of LTP. Recently, some investigators in both *in vitro* and *in vivo* experiments, demonstrated that NO via cGMP, PKG, and ERK pathway is involved in expression of some transcription factors including c-Fos and Egr-1, that are important for activity-dependent long-term neuroplasticity (Gallo and Iadecola, 2011).

As seen from the previous literature review, the most important candidate "memory molecules" are synaptic proteins and proteins implemented in cell signaling such as growth factors, neurotransmitters, and molecules belonging to the varies intracellular signal transduction pathways including enzymes and transcription factors. The major intracellular signaling pathways implemented in both short- and long-term spatial memory formation in hippocampus are NMDA-dependent pathways including activation of CaMKII, PKA, and MAPK kinases, and production of local vasodilator and at the same time messenger molecule NO.

In addition to the signalling pathways described above, hippocampal LTP can be modulated by a variety of extracellular neuromodulators. For example, the steroid hormone, estradiol, is one of the several molecules that enhances LTP by driving CREB-1 phosphorylation and subsequent dendritic spine growth. Additionally, β-adrenergic receptor agonists such as norepinephrine contribute to the protein synthesis-dependent late phase of LTP. Likewise, dopamine receptors' activation increases LTP by the cAMP/PKA signaling pathway. However, one of the most potent modulators of hippocampal circuitry seems to be acetylcholine (ACh) supplied from the medial septum known as a pacemaker region for the hippocampal theta rhythm (Stewart and Fox, 1990). The major source of cholinergic innervation to the hippocampus is provided by the medial septal nucleus (reviewed by Dutar *et al.,* 1995). The medial septal nucleus presents a direct synaptic input to both, principal neurons and interneurons (Leranth and Frotscher, 1987). Septo-hippocampal cholinergic fibres give extensive projects throughout the hippocampus to release ACh upon a diverse range of muscarinic (mAChRs) and nicotinic cholinergic receptors (nAChRs) that are differentially expressed among distinct neuron populations. Acetylcholine is a potent modulator of synaptic transmission both at glutamatergic and GABAergic synapses by both pre- and postsynaptic mechanisms through both mAChRs and nAChRs. ACh classically excites hippocampal pyramidal cells producing a sustained action potential discharge (Cole and Nicoll, 1983; Morton and Davies, 1997). Contrary to the slow sustained mAChR-mediated modulation of both pyramidal cells and interneurons, a fast and cell type-specific response is produced by the activation of nAChRs. mAChRs modulate a large amount of ionic conductances in pyramidal neurons and mAChR activation may promote long-term synaptic plasticity either directly (Auerbach and Segal, 1994) or associatively interacting with inputs of glutamatergic synapses (Huerta and Lisman, 1996). This modulation is also pathway specific like in the case of short-term modulation at the pre- and postsynaptic level: LTP is enhanced

within the DG upon mAChR activation (Burgard and Sarvey, 1990) while mAChR activation can enhance or depress LTP within CA3 pyramidal neurons (Maeda *et al*., 1993). ACh modulates cellular excitability and synaptic transmission within hippocampal circuits phasing hippocampal network activities. Shaping neuronal oscillations is particularly important because *in vivo* coherent network oscillations are considered to supply a temporal context against which the information is encoded by the precise firing of cells. In this context, hippocampal oscillations may be important in associative learning (Buzsaki, 2002) and as a reference for coding by place cells (O'Keefe and Recce, 1993).

Although not always the obtained results were consistent, most of the experimental animal studies investigating relation between ACh and memory formation brought evidence for ACh participation in mnemonic processes. In the numerous studies, it has been shown that: (1) training in various hippocampus-dependent learning tasks induces increases in hippocampal choline acetyltransferase (ChAT) levels (Park *et al.,* 1992), (2) produces increases in hippocampal extracellular ACh levels (Fadda *et al.,* 2000; Nail-Boucherie *et al*., 2000; McIntyre *et al*., 2003), and (3) alters hippocampal high-affinity choline uptake (HACU), an index of cholinergic activity (Durkin, 1994). Parallel to this, it was also found that aged rats showing cognitive deficits have decreased hippocampal ACh markers (Aubert *et al*., 1995; Baxter *et al.*, 1999b). On the other hand, lesions of the fimbria-fornix, or electrolytic or neurotoxic lesions of the medial septum, generally impair hippocampal-dependent learning and memory (Markowska *et al*., 1989; Kelsey and Vargas, 1993), although the effects of more selective lesions of cholinergic septo-hippocampal neurons produced by the immunotoxin 192 IgG-saporin were less detrimental (Kirby and Rawlins, 2003; Frick *et al*., 2004). On the contrary, intracerebral (Izquierdo *et al*., 1992; Markowska *et al*., 1995) or systemic (Hodges *et al*., 1991; Levin *et al*., 1993) administration of cholinergic agonists

as well as improving cholinergic functions by hippocampal grafts of ACh secreting cells were reported to enhance memory or reverse memory deficits (Tarricone *et al*., 1996; Cassel *et al*., 2002). It is also possible that some effects of ACh on memory may be mediated by its effects on attentional processes since attention deficits have been often reported after loss of basal forebrain cholinergic neurons, both in the MS/VDB (medial septum/vertical limb of the diagonal band) and in the NBM/SI (nucleus basalis magnocellularis/ substantia inominata) (Baxter *et al*., 1999c; McGaugh and Sarter, 1998).

Despite extended studies on the molecular basis of memory carried out in many research centers throughout the world, our present knowledge regarding molecular basis of mnemonic processes is still incomplete. To relate the region-specific protein activation (STM) or protein induction (LTM) to a learning task, different experimental approaches have been undertaken. One of them is running the studies on a group of subjects representative for the random population and carrying out the nucleic acids or protein assays for specific brain regions at selective times during and after acquisition of a learning task. The second approach is to use genetically modified animals with artificially induced mutation of the genes encoding one of the candidate "memory" proteins. Still another approach is to compare the gene expression and protein profiles between healthy young subject and subjects with learning and memory deficits either due to physiological aging or some pathological processes. In the up-to-date literature, there are, however, very few such studies investigating differences in the brain levels of proteins known to be important for learning and memory between young "good" and "poor" learners selected from the random animal population on the basis of their performance on a learning task. This approach was applied in the present study. This and similar studies provide an insight into individual variation in learning skills and memory within random animal/human populations and may also provide some new cues into how to treat memory deficits in young individuals.

The molecules examined in the present study (neuronal (n), endothelial (e) and inducible (i) NOS, CaMKIIα, PKA and ChAT), apart from their well documented role in short-term synaptic plasticity, were also shown to directly or indirectly (through the interactions with other signaling pathways) affect encoding memories for the long-term storage (Micheau and Riedel, 1999). Below there is given a detailed characteristic of the molecules considered in the present study.

1.4 A closer look at the candidate "memory molecules" examined in the present study

1.4.1 Nitric oxide synthase (NOS)

Nitric Oxide (NO) for long has been known as an agent implemented in the control of cerebral blood flow (Faraci and Brian, 1994) but at the same time it is known as an unconventional gaseous retrograde neurotransmitter (Bohme *et al.,* 1993; O'Dell *et al*., 1991; Schuman and Madison, 1991). Its production depends on the expression and activity of enzymes, NO synthases (NOSs) catalyzing the conversion of L-arginine to L-citrulline and NO (Moncada and Higgs, 1993) (Figure 7).
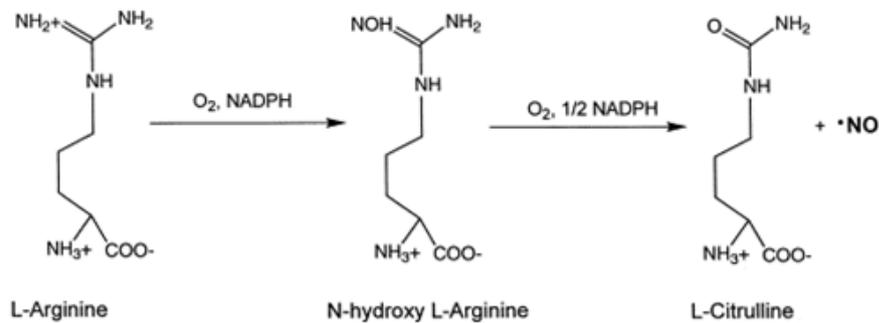
Figure 7 Oxidation of L-arginine to L-citrulline by nitric oxide synthases (after Eiserich *et al*., 1998).

Nitric Oxide (NO) is a diffusible regulatory molecule implicated in a wide range of different physiological as well as pathophysiological functions in the nervous, cardiovascular and immune systems (Lumme *et al*., 2000; Ledo *et al*., 2004; Bredt and Snyder, 1992; Hölscher, 1997; Esplugues, 2002; Prast and Philippu, 2001). In the nervous system, NO simply diffuses from the nerve terminals into adjacent cells where it forms covalent linkages to a multitude of targets one of which is an enzyme, cytosolic guanylyl cyclase (GC). GC activated by NO through binding of iron in the heme group at the active site of the enzyme, and thus altering the enzyme's conformation. High affinity binding of NO to the heme moiety leads to an up to 200-fold activation of the enzyme. Fairly recently, a dual action of NO has been postulated. First, postsynaptically produced NO can diffuse back to the pre-synaptic region and activate soluble guanylate cyclase (sGC). Activated sGC increases cGMP (an intracellular second messenger) levels which in turn can potentiate the release of glutamate and inhibit its re-uptake. As mentioned earlier, a second action of NO is the activation of GC postsynaptically (Ko and Kelly, 1999). Although primarily NO is responsible for short-lasting functional changes at synapses, it has been postulated that local production of NO during synaptic activation may also have longer-term effects on a postsynaptic neuron including its contribution to

the late-phase of long-term potentiation (L-LTP), a cellular model of memory (Lu *et al.,* 1999) and dendrite outgrowth (Inglis *et al.,* 1998), both requiring *de novo* protein synthesis.

The inactivation of NO could involve diffusion away from targets or covalent linkage to superoxide and diverse proteins. Nevertheless, being a free radical, NO is like a double edge sword. Its excess can cause cell damage and/or cell death. The resistance of glutamatergic neurons to the NO neurotoxicity may be partially related to tightly controlled subcellular location of NOS activity. NOS is inactivated by phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and/or cGMP-dependent protein kinase. By phosphorylation of NOS, enzyme translocates from membrane to soluble fractions in the cytoplasm. NO can not be generated within the cytoplasm, since phosphorylated NOS is catalytically inactive. On the contrary, nonphosphorylated catalytically active NOS is localized to the plasma membrane, from where NO is released outside the cell.

The brain levels of NO are determined by the expression and/or activity of NO synthase which is present in three isoforms: one inducible (iNOS) and two constitutively expressed: endothelial (eNOS) and neuronal (nNOS). The inducible form of NOS was found in activated macrophages, astroglia and microglia and other cells in several pathological conditions including ischemia, trauma, Parkinson's disease, multiple sclerosis, Alzheimer's disease, tumors, acquired immunodeficiency syndrome encephalitis, infection and lipopolysaccharide (LPS) treatment. The constitutively expressed endothelial form was originally found in microvessels but is also found in neurons (Dinerman *et al*., 1994; Doyle and Slater, 1997) and glial cells (Barna *et al*., 1996; Gabbott and Bacon, 1996; Iwase *et al*., 2000) while neural form was detected in different types of neurons of the cerebellum, mesencephalon, hypothalamus, striatum, cerebral cortex and hippocampus (Ihara *et al*., 2006)

31

and is also expressed in skeletal muscle. The activity of iNOS is calcium-independent and its expression increases under pathological conditions. In contrast, nNOS and eNOS are constitutive with activation dependent upon the calcium-calmodulin complex which binds to NOS following an increase in the intracellular calcium concentration (for review, see Yun *et al,*.1996; Stuehr, 1997; Weissman *et al*., 2002). In neurons, the intracellular calcium concentration is increasing as a result of synaptic activation and in particular the activation of the NMDA glutamate receptors (Stuehr, 1997). Acetylcholine acting at muscarinic receptors on endothelial cells in blood vessels activates the phosphoinositide cycle in order to generate Ca^{2+} thus stimulates NOS.

In the nervous system, by modulating NO production by either administration of NO precursors, donors, or scavengers, it was revealed that this gas neurotransmitter plays a role in synaptic plasticity and thus in learning and memory formation (Calabrese *et al.,* 2007, Prast and Philippu, 2001). Many studies demonstrated that in several brain areas including the cortex, cerebellum and hippocampus, NO is implemented in the induction of LTP and LTD considered cellular models of learning and memory (Bon and Garthwaite, 2003; Calabresi *et al.,* 1999; Haley *et al.,* 1996; Haul *et al.,* 1999; Hawkins *et al.,* 1998; Lev-Ram *et al.,*1997; Lu *et al.,* 1999; O'Dell *et al.,* 1994; Wang *et al.,* 2005; Zhuo *et al.,* 1994; Zhuo *et al.,* 1998). Parallel to this, in many studies carried out on different animal species from invertebrates to mammals, a facillitatory effect of NO was observed in several types of behavioral learning (Suswein *et al*., 2004; Paul and Ekambaram, 2011). This included olfactory learning (Kendrick *et al*., 1997; Okere and Kaba, 2000; Samama and Boehm, 1999), cerebellum-dependent motor learning (Yanagihara and Kondo, 1996), acquisition of active and passive avoidance (Antonov *et al*., 2007; Bernabeu *et al*., 1995; Huang and Lee, 1995) and hippocampus-dependent spatial learning (Pitsikas *et al*., 2005). NOS inhibition by systemic administration of nitroarginine, inhibition of soluble GC and NO-GC receptors knock-out was

reported to impair reference memory formation in the hippocampus-dependent learning tasks such as partially-baited radial arm maze, and the water maze (Zou *et al.,* 1998; Yamada *et al.,* 1995; Chapman *et al.,* 1992; Estall *et al.,* 1993).

Quantitative immunohistochemical studies showed that in the hippocampus both nNOS and eNOS are expressed in dentate gyrus but also in the stratum radiatum of CA1 region (Burette *et al*., 2002). Some studies, however, have demonstrated location of all three NOS isoforms in brain neurons (Chiang *et al*., 1994; Dinerman *et al*., 1994; Doyle and Slater, 1997; Huang *et al*., 1994; Wood *et al*., 2005) which suggests that all three NOS isoforms may be implicated in neural plasticity. However, the role of different isoforms of NO synthase in the learning process is still not clear and the data are inconsistent. In rats, an increased expression of nNOS was noted in the dentate gyrus and frontal cortex after a water-rewarded spatial alternation task (Zhang *et al*., 1998) and increased expression of both, nNOS and eNOS was observed in hippocampus and prefrontal cortex during learning of an operant conditioning task (Rapanelli *et al*., 2010). Interestingly, after place learning in the water maze, a strong induction of iNOS was also revealed by genome-scale screening at 1,6, and 24 h after training (Cavallaro *et al*., 2002). However, numerous studies examining the potential role of different NO synthases in learning and memory by either genetic or pharmacological NOS inactivation during training brought highly inconsistent results. Systemic or local (intrahippocampal) administration of NOS inhibitors such as L-nitroarginine methyl ester (L-NAME), N-omega-Nitro-L-arginine (L-NA), or 7-nitroindazole (7-NI) was reported to impair different forms of learning including conditioned eyeblink response acquisition (Chapman *et al*., 1992), the acquisition of passive avoidance task (Harooni *et al*., 2009; Mutlu *et al*., 2011) and place learning in either water maze (Chapman *et al*., 1992; Estall *et al*.,1993; Hölscher *et al*., 1995; Majlessi *et al*., 2008; Mogensen *et al*.,1995; Prendergast *et al*.,1997), or

in fully or partially baited radial arm maze (Hölscher *et al*., 1995; Yamada *et al*., 1995; Zou *et al*., 1998) and also in a shock-motivated 14-unit T-maze (Ingram *et al*., 1998). Some other works, however, indicated no effects of systemic or subtotal hippocampal inhibition of NO synthesis by pharmacological agents on learning and memory (Bannerman *et al*., 1994; Blockland *et al*., 1999; Markvartova and Vozeh, 2008; Tobin *et al*., 1995) and some reported facilitation of learning process (Du and Harvey, 1996). Few studies that used genetic inactivation of NO synthases (knockout studies) also brought discrepant results. Despite the previously reported detrimental effect of eNOS inactivation on the induction of LTP in the cortex and hippocampal CA1 area, eNOS knockout in mice was shown not to affect hippocampus-dependent spatial learning in the RAM task (Dere *et al*., 2001) and facilitate place learning in the water maze (Frisch *et al*., 2000). These discrepancies in the results obtained in the studies investigating the effect of pharmacological NOS inactivation on learning and memory might arise from the fact that applied NOS inhibitors are generally little selective and their effects depend among others on the dose and the way of administration. On the other hand, a drawback of knocking out a gene (unless it is a conditional knockout) is lack of detailed studies on how the changes produced by gene knockout during embryonic development affect the adult phenotype. It is known that a gene knockout may cause changes different than those observed when gene expression is manipulated in the adult subjects.

1.4.2 Kinases

1.4.2.1 Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα)

CaMKII is an enzyme, present in large amounts in the central nervous system and it has roles in different kind of neurobiological process such as gene expression (Yamasaki *et al*., 2008), neurotransmitter synthesis and exocytosis (Greengard *et al.,* 1993), neurotransmitter receptor and ion-channel functions (Wu *et al.,* 2001), cytoskeletal interactions and morphology (Koh *et al.,* 1999; Rongo and Kaplan, 1999; Yamasaki *et al*., 2008) and various signalling pathways (for review see Griffith, 2004; Colbran and Brown, 2004). It is known to be localised in postsynaptic densities (PSDs) of excitatory synapses as the most abundant protein (Micheaua and Riedel, 1999).

In mammals, CaMKII is encoded by four genes: α, β, γ and δ. The γ and δ forms are mostly found in peripheral tissues whereas the α and β forms are the predominant forms found in the central nervous system (Andreev, 2006). The α form is mostly expressed postnatally in forebrain structures, whereas the β, another related kinase with similar properties, is expressed more uniformly throughout the brain and during embryonic development (Burgin *et al*., 1990). The translated protein products, which are generally 50–60 kDa in size, contain a highly conserved N-terminal kinase (catalytic) domain (approximately 280 amino acids) followed by a autoinhibitory (regulatory) domain (approximately 40 amino acids) and a 150–220 amino acid C-terminal association domain (Andreev, 2006) (see Figure 8).
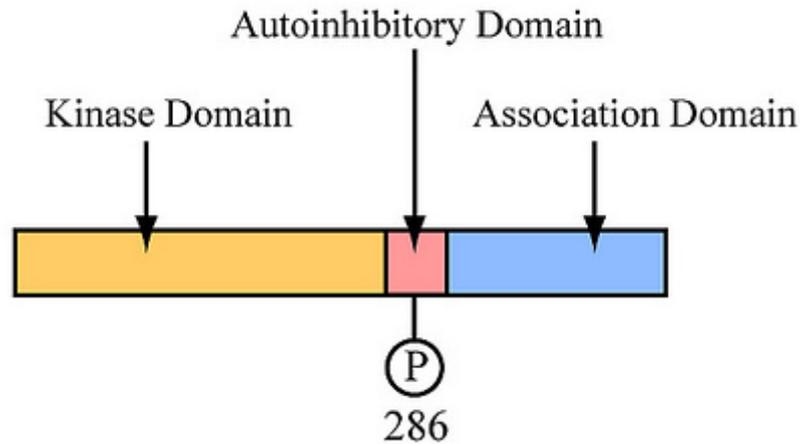
Figure 8 Schematic demonstration of domain structure of CaMKII (after Andreev, 2006).

The α isoform of CaMKII protein constitutes up to 2% of protein in the hippocampus (Micheau and Riedel, 1999). The highest concentration of CaMKII was found in the striatum pyramidale of both CA1 and CA2 by *in situ* hybridization and immunocytochemistry studies done on hippocampal slices while its expression was less common in the striatum oriens, striatum radiatum, and in all parts of the dentate gyrus (Jakobs *et al.,* 1993; Ochiishi *et al.,* 1994).

Activation of CaMKII includes steps summarized below (Micheau and Riedel, 1999; Soderling, 1993):

- Binding of calcium:calmodulin complex to the enzyme neutralizes the inhibitory role of the regulatory domain through a conformational change and frees the catalytic domain.

- The activated kinase binds Mg^{++}/ATP as a first substrate and then a rapid autophosphorylation takes place at threonine 286 (T286), a step

constituting a partially calcium-independent form of CaMKII, thus in turn provides long-term activation of CaMKII.

- Once activated, CaMKII is translocated to PSDs where it seems to be properly positioned to examine synaptic strength by sensing calcium and phosphorylating glutamate receptors' synaptic channels. Translocation of CaMKII to PSDs has also been suggested to provide a means of marking activated synapses for additional modifications that result in synapse-specific forms of plasticity (Kennedy *et al.,* 1983).

Protein phosphatases (PPs) have been shown to play a significant role in regulating CaMKII by preventing the effects of its autophosphorylation, in addition to phosphorylation of its substrates. High frequency or theta-burst electrical stimulation-induced autophosphorylation of CaMKII in hippocampal slices is only observed when protein phosphatases 1 (PP1) is inhibited following PKA-mediated activation of PP1 inhibitor-1 (Colbran, 2004).

The role of CaMKII in long term potentiation (LTP) was shown in several studies (for review see Micheau and Riedel, 1999). The first gene-targeting study of LTP and learning used mice with a null mutation for the alpha CaMKII (Silva *et al*., 1992a,b). CaMKII and its autophosphorylation at Thr286 were postulated to be essential for NMDA receptor-dependent forms of LTP in the hippocampal CA1 region (Cammarota *et al.*, 2002). It has been shown that postsynaptic injection of calcium-calmodulin induces the LTP (Ko and Kelly, 1999), whereas suppression of CaMKII either pharmacologically or genetically inhibits the formation of LTP (Fink and Meyer, 2002). Interestingly, it has been shown by previous studies that antagonists of CaMKII have no effect on previously formed LTPs in the hippocampus which indicates that CaMKII is important in early phases of LTP formation but it may have no role in the maintenance of LTP (Chen *et al*., 2001).

Increase in the function of AMPA glutamate receptors is one of the most important mechanisms that CaMKII mediate during the LTP formation (Cammarota *et al.*, 2002; Fink and Meyer, 2002). Phosphorylation of GluR1 subunits of AMPA receptor at Ser831 by CaMKII, increases the unitary conductance of the AMPA ionophore (Derkach *et al.*, 1999; Mammen *et al.*, 1997). CaMKII also facilitates synaptic insertion of new AMPA receptors into the synaptic regions (Lisman *et al.*, 2002; Fink and Meyer, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002; for review see Micheau and Riedel, 1999).

The up-to-date studies provided a robust knowledge about the cellular and molecular mechanisms through which CaMKII acts. However, much less is known about the behavioral effects of changes in CaMKII expression and/or activity.

Some behavioral studies show that the modulation of CaMKII expression and/or activity by genetic or pharmacological methods causes changes in animals' performances in hippocampus dependent learning and memory tasks. First-generation homozygote mice carrying the CaMKIIα mutation have been tested in lots of behavioral tasks involving spatial, nonspatial and emotional tests (Silva *et al.,* 1992a,b). In addition to their deficiency in expressing LTP, a severe deficiency in both spatial learning and retention in the water maze were observed in those mutated mice (Silva *et al.,* 1992a,b). A significantly impaired spatial learning and memory retention in the Barnes maze paralleled by an impaired LTP in the range of theta frequency were also reported in CaMKII-Asp286 mutant mice (Bach *et al.,* 1995). Besides this, a specific inhibitor of CaMKII, KN62, injected into amygdala or hippocampus of rats soon after the training trial has been shown to impair step-down inhibitory avoidance learning (Cammarota *et al.*, 1998; Wolfman *et al.*, 1994). Intrahippocampal infusion of KN62 led to full retrograde amnesia of a passive avoidance learning in rats

when given immediately, but not 120 or 240 min, after training suggesting that CaMKII activity plays an essential role at the early phases of memory formation. An increase (25-78%) in CAMKII activity was recorded in the hippocampus of rats killed immediately (0 min), but not 120 min after training. There was also observed an increase in the *in vitro* phosphorylation of CAMKIIα and β in hippocampal synaptosomal membranes (SPM) of trained rats killed immediately and 30 min after training. The learning process was accompanied by increase in AMPA GluR1 subunit phosphorylation and immunoreactivity in the hippocampus. According to Cammarota and colleagues (1998), the training in a learning task such as passive avoidance cause to quick activation of CaMKII, which, if it persists for 30 min or more, facilitates further longer-term processes. Tan and Liang (1996) reported a similar effect of the intrahippocampal injection of a CaMKII inhibitor, KN62, to rats on spatial learning in the Morris water maze. However, some discrepant results were also reported. In Mizuno *et al*. (2002) study, a significant increase in phosphorylation of PKA and CREB in the hippocampus of rats trained in radial maze was found. However, in this study, no changes were observed in the phosphorylation (activation) of CaMKII and extracellular signal regulated kinase (ERK). There was also no change in the mRNA level of the brain-derived neurotrophic factor in the hippocampus.

1.4.2.2 cAMP-dependent protein kinase (PKA)

PKA is an enzyme found in the smooth muscle tissue and in the central nervous system (CNS) including hippocampus (for review see Kandel, 2012; Nguyen and Woo, 2003; Lee, 2006). In mammals, PKA is composed of 4 regulatory (RIa, RIb, RIIA and RIIb) and 3 catalytic subunits (Cα, Cβ and Cγ) (Doskeland *et al*., 1993). The catalytic subunit of PKA is released when cAMP binds to regulatory subunit (Beebe, 1994). In brain tissue, especially Cα and Cβ catalytic subunits are present. Cα was found in both gyrus dentatus and

pyramidal cells of Ammons horn, whereas Cβ was shown to be predominantly present in gyrus dentatus (Cadd and McKnight, 1989).

Changes that occur during the signal transduction pathways leading to activation of PKA at glutamergic synapses, can be summarized as follows (Bebee, 1994, Silva and Lee, 2009):

- Simultaneous depolarization of the post-synaptic membrane caused by the impulse coming from pre-synaptic neuron and binding glutamate to NMDARs, opens NMDA calcium ionophores.

- Ca^{2+} rapidly flows into the cell in large amounts and forms Ca^{2+}/calmodulin complex (Ca^{2+}/CaM). This complex activates lots of kinases and phosphatases at the downstream pathways including CaMKII and adenylyl cyclase (AC).

- Adenylyl cyclase (AC), activated by Ca^{2+}/CaM, produces a secondary messenger called cAMP. cAMP activates PKA that is why PKA also known as cAMP-dependent protein kinase. However, activation of PKA can also occur by stimulation of some G-protein dependent receptors without any changes in intracellular Ca^{2+} levels (Kandel and Abel, 1995; Bailey *et al.,* 1996).

- Regulatory subunit isoforms of PKA bind cAMP and localize catalytic subunit isoforms near substrate proteins.

Molecular studies show that PKA like CaMKII, phosphorylates Na^+ and Ca^{2+} channels, increasing their sensitivity to the membrane voltage changes (Cantrell *et al*., 1997), suppresses membrane K^+ conductance (Yuan *et al.,* 2002; Jonathan *et al*., 2006), and causes increase in AMPA receptor-ion density, thus enhance the response of postsynaptic membrane to synaptic

stimulation (Banke *et al.*, 2000; Dell'Acqua *et al.*, 2006). As mentioned earlier in the introduction part, PKA also causes activation of mitogen-activated protein kinase (MAPK) (Roberson *et al.*, 1999; Waltereit *et al.*, 2003). The phosphorylated form of MAPK and catalytic subunit of PKA are transported to the nucleus , where through activation of transcription factor CREB-1 (known as memory enhancer) and inactivation of CREB-2 (known as memory repressor) initiate downstream events leading to long-term synaptic changes laying at the basis of long-term memory formation. At glutamergic synapses, although the initial activation of the enzyme depends on the change in intracellular Ca^{2+} levels, for transmission of the signal to be long lasting, enzymatic activity should also be maintained independent from Ca^{2+} levels. To provide Ca^{2+} independent kinase activity, there are two mechanisms: i) removal and proteolysis of regulatory subunit, and ii) conformational change in protein via auto-phosphorylation. These mechanisms ensure prolonged enzymatic activity of PKA (persistent PKA) which last even when Ca^{2+} and cAMP levels decrease (Michaeu and Riedel, 1999).

It is known that phosphorylation and dephosphorylation reactions play an important role in learning and memory formation. The balance of these reactions and production of appropriate response to outer stimulus depend on compatible functioning of protein kinases and phosphatases (Soderling and DerKach, 2000). Blitzer *et al.* (1995) showed that E- LTP in the hippocampus became cAMP-independent when protein phosphatase inhibitors were injected postsynaptically. This suggests that cAMP signaling pathway during E-LTP, is a gateway to L-LTP through the inhibition of postsynaptic protein phosphatases.

Apart from the evidence that PKA is important for E-LTP and thus short term memory formation (Blitzer *et al.*, 1995; Lisman *et al.*, 2002; Lynch, 2004), there is also an evidence for an active role of PKA during the late phase of LTP

(Frey *et al*., 1993; Malenka and Nicoll, 1999; Shinoda *et al*., 2003; Huang *et al.,* 2005; Michel *et al.*, 2008). Several studies on invertebrates (i.e. long-term facilitation in Aplysia) and vertebrates (hippocampal LTP) show that, after being transported to nucleus, active PKA provides activation of cAMP responsive element binding protein (CREB), a nuclear transcription factor that modulates transcription of genes containing cAMP responsive elements (CRE sites) in their promoters (Micheau and Riedel, 1999; Josselyn and Nguyen, 2005) and thus leads to long-term synaptic changes in stimulated cells. PKA not only triggers those changes but also it is thought to have important role in synaptic tagging, restricting the synaptic changes to activated synapses only (Young *et al*, 2006).

One of the most important studies about PKA's effect on LTP in hippocampus and other brain regions has been conducted by Abel *et al*. (1997). In this study, the expression of PKA' regulatory subunit inhibitor, R(AB), is taken under control by the promotor region of CaMKIIα in transgenic mice model. In this way, the expression of PKA inhibitor increases along with increase in CaMKIIα expression. A significant deficiency both in the late phases of LTP and in the spatial learning in the water maze was detected in transgenic mice. Despite deficits in L-LTP and long-term reference memory, no difference is observed in E-LTP and short term memory between transgenic and control animals. It was concluded that, the deficits seen in LTP and behavioral performance when PKA activity is suppressed show similarity with negative effects of anisomycin (a protein synthesis inhibitor) administration to normal mice 1 to 24 h after learning test. Also in rats trained in partially baited 8-arm radial maze a significant increase in the phosphorylation level of PKA and CREB was observed in the hippocampus (Mizuno *et al*., 2002). Interestingly, in this study, a parallel increase in both protein levels started at fourth day of training and lasted until the end of training throughout the following 4 days. Similar results have also been obtained by spatial learning in the holeboard

food-search tool (Vazguez *et al.*, 2000). All these findings indicate that those proteins plays an important role in long-term memory mechanisms in addition to they being a part of short term memory mechanisms.

1.4.3 Choline acetyltransferase (ChAT)

There are at least 6 cholinergic cell groups in the rodent brain with different projection areas (Mesulam *et al.*, 1983; Tohyama *et al.*, 1998) (Figure 9). As part of the basal forebrain, CH1 includes the medial septal nucleus (MS) and CH2 includes the vertical limb of the diagonal band of Broca (VDBB). Both have a intense projection of fibres through hippocampus (Auld *et al.*, 2002). CH3 comes up to the horizontal limb of the diagonal band of Broca (HDBB) and export fibres to both thalamic reticular region and olfactory bulb, whereas CH4, the nucleus basalis magnocellularis (NBM), connects to the thalamus besides amygdala and cerebral cortex. There are also two midbrain regions (CH5 and CH6) comprising pedunculopontine and laterodorsal tegmentum, respectively, and their projection of fibres elongate through cortical to various thalamic regions. In addition to these projection system, there are lots of cholinergic fibres typical to specific regions, such as striatum (Mesulam *et al.*, 1983; Rye *et al.*, 1987). Hippocampal cholinergic input originates from CH1 (medial septum) and CH2 (diagonal band of Broca) and is responsible for the generation of hippocampal theta rhythm (Andersen *et al.*, 1979).
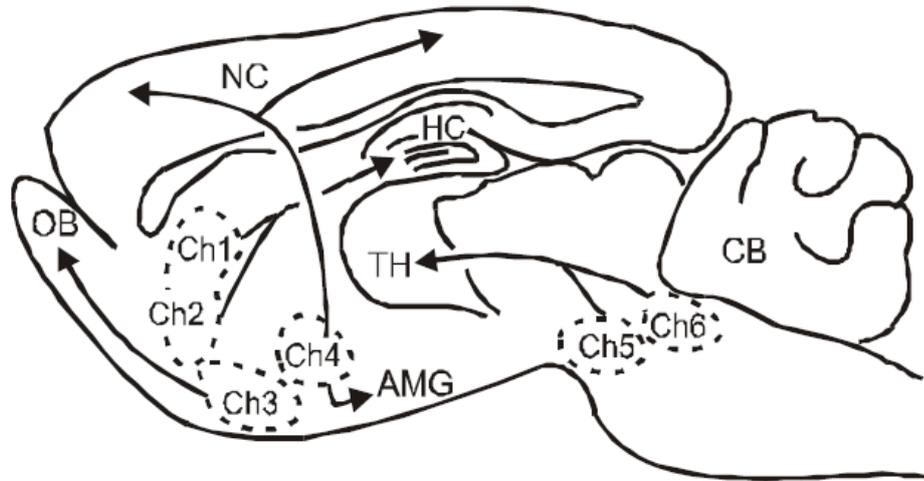
Figure 9 The cholinergic nuclei of the rat brain (Ch1-6). AMG: amygdala; CB: cerebellum; HC: hippocampus; NC: neocortex; OB: olfactory bulb; TH: thalamus (Modified after Mesulam *et al.,* 1983).

Acetylcholine (ACh), the primary transmitter of the cholinergic system, is synthesized by choline acetyltransferase (ChAT) by the choline and acetyl-CoA (Figure 10).

Figure 10 Synthesis of acetylcholine (Modified after Oda, 1999).

Acetylcholine esterase (AChE) located both at pre- and post-synaptic membrane hydrolyses and thus inactivate acetylcholine after being released from presynaptic neuron (Oda, 1999). In most species, ChAT is a 68 kDa

protein and is mostly localized in the cytoplasmic region of cholinergic nerve endings. In the central and peripheral nervous systems, ChAT is the most specific indicator for monitoring the functional state of cholinergic neurons. ChAT is encoded by a single gene that generates multiple mRNAs as a result of alternative splicing (Reis *et al.*, 2009).

The cholinergic receptors in the mammalian central nervous system can be divided into two groups: muscarinic and nicotinic receptors. In the central nervous system (CNS), muscarinic receptors are predominating (Caulfield, 1993). Five types of muscarinic receptors have been identified and classified as M_1, M_2, M_3, M_4 and M_5. Of the five subtypes of muscarinic acetylcholine receptors, the M1 subtype is the most abundant in the human cerebral cortex and hippocampus. In rat hippocampus, M1 is widely expressed in somata and dendrites of pyramidal neurons and granule cells in CA1 area and in dentate gyrus. M2 immunoreactivity is expressed mostly in nonpyramidal neurons (Levey *et al.*, 1995). Muscarinic receptors are metabotropic type of receptors linked to G proteins. In contrary, nicotinic receptors are ionotropic receptor-channel complexes. In the hippocampus, they are present mainly in the stratum oriens and in the hilus (Oda, 1999; Reis *et al.*, 2009).

In brief, the cholinergic transmission occurs in the steps below (for review see Oda, 1999) (see Figure 11):

- <u>Acetylcholine (ACh) synthesis:</u> Choline is taken by a neuron from extracellular liquid by high affinity sodium-dependent transport. Main protein in this system is the high-affinity choline uptake (HACU) transporter. Choline is combined with acetyl CoA by ChAT to form ACh.

- Storage of ACh in vesicles: Acetylcholine (ACh) is stored in vesicles by a proton pump dependent active transport mechanism. In mature vesicles, adenosine triphosphate and proteoglycan are also present.

- Release of ACh: Voltage sensitive calcium channels at pre-synaptic cell membrane are opened when action potential generated by voltage sensitive sodium channels arrive at nerve endings. Accordingly, an increase in intracellular calcium concentration is detected. Thus, synaptic vesicles fuse with cell membrane and acetylcholine is released to synaptic cleft.

- Binding of ACh to receptors: Acetylcholine (ACh) may bind to post-synaptic and/or pre-synaptic receptors. Upon binding to receptor, a biological response is generated.

- Degradation of ACh: As a result of degradation of ACh into choline and acetate in synaptic cleft by AChE, the stimulation in post-synaptic cell ceases.
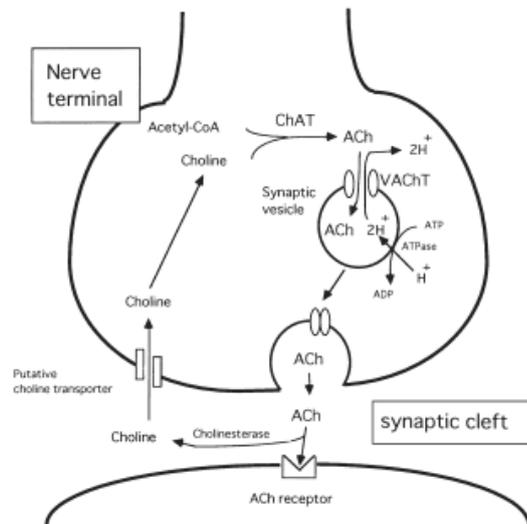
Figure 11 Schematic representation of cholinergic transmission (after Oda, 1999).

There are many studies pointing towards involvement of septo-hippocampal cholinergic system in mnemonic processes. It has been shown that damage to medial septum (MS) (the main source of hippocampal ACh) impairs both acquisition and retention of spatial memory in a variety of spatial tasks including water maze, T-maze and radial-arm maze, and results in a decrease in cholinergic markers which correlates with the task performance (Ginn and Peterson, 1992, Decker *et al.,* 1992; Dunnet, 1985; Etherington *et al.,* 1987; Fisher *et al.,* 1989; Fisher *et al.,* 1991; Kelsey and Landrey, 1988; Nilsson *et al.,* 1990; Turner *et al.,* 1992; Walsh *et al.,* 1996).

An evidence of cholinergic implementation in cognitive functions comes also from pharmacological studies. Intraseptal microinfusions of cholinergic agonist oxotremorine were reported to produce a dose-dependent improvement in choice accuracy in T-maze in old rats shifting hippocampal theta peak to a lower frequency and a higher peak power, while similar infusions of

cholinergic antagonist, scopolamine decreased choice accuracy in a dose-dependent fashion in both old and young rats producing a shift of the hippocampal theta rhythm to a higher frequency and a lower peak power (Markowska *et al.,* 1995).

Further support for the involvement of the forebrain cholinergic system in cognitive functions comes from *in vivo* microdialysis studies in which increased release of ACh in the dorsal hippocampus was noted in rats performing a spatial memory task, the radial arm maze (Stancampiano *et al*., 1999). The hippocampal ACh release in rats was also reported to correlate with animals' performance on spontaneous alternation test (McIntyrre *et al.,* 2002). Interestingly, in this study ACh release in amygdala correlated negatively with the performance level in amygdala-dependent learning task, the conditional place preference. The increase in the hippocampal ACh release was shown to be greater at more advanced stages of training on the hippocampal-dependent learning task, the spontaneous alternation test (Fadda *et al*., 1996). Results obtained in some other studies, however, indicate that the ACh release in hippocampus is increased during early acquisition phase and low during consolidation of memory traces (see Deiana *et al*., 2011).

There is also number of reports indicating that ChAT has role in pathological and age-dependent memory deficits. Both Alzheimer's disease and senile dementia of the Alzheimer's type are progressive neurodegenerative diseases that overwhelmingly involve memory and cognitive disorders. In these diseases, the cholinergic system is constantly and profoundly affected among others. A significant reduction of acetylcholine and ChAT concentrations in the cerebral neocortex and hippocampus are observed. Also, in the nucleus basalis of Meynert (Ch4), a significant reduction in the number of cholinergic neurons is observed (Oda, 1999).

In senescence accelerated mice, (SAM prone/8 (P8), a poor performance in water maze correlated with low ChAT expression in cerebral cortex, hippocampus as compared to normal aging strain, SAM resistant/1 (R1) mice (Wang *et al.,* 2009). Similar results were presented in the study by Luine and Hearns (1990), where aged rats with impaired performance on the radial arm maze were reported to have decreased ChAT activity in the vertical diagonal band nucleus (vDB), the dentate gyrus and striatum as compared to young rats. A discrepant result was reported in Colombo and Gallagher (1998) study with young (6 months-old) and aged (24 months-old) rats were compared for their performance in place-learning tasks: the water maze and the radial arm maze, and for ChAT activity and ChAT immunoreactivity in the forebrain. In aged rats impaired in spatial learning lower ChAT immunoreactivity was found in nucleus basalis magnoselularis (NBM) and striatum compared to young and aged rats with preserved cognitive function. However, no correlation between ChAT activity in the septum, hippocampus, or cortex and cognitive performance has been shown by these studies. Despite some discrepancies in the literature, most of the studies reported a decrease in the activity of ChAT in cholinergic system in aged subjects with memory deficits (Deina, 2011).

To restore cholinergic function in aged brains, exogenous ChAT supplementation can be an alternative therapy (Fu *et al*., 2005). ChAT can pass the blood-brain barrier and enter the neurons when delivered with so called protein transduction domain (PTD). In age mice with learning deficits, improvement of memory was shown after PTD-ChAT administration (Fu *et al.*, 2005).

According to the data in the literature, the involvement of PKA pathway in cholinergic transmission, and some of the memory deficits that induced by PKA II inhibition may be related to altered cholinergic transmission (Sharifzadeh *et al*., 2005). By using the effects of intrahippocampal infusion of

H-89, which is a selective PKA II inhibitor, into the brain regions, the action of protein kinase A II (PKA II) in spatial memory retention in rats and its regulation of cholinergic gene expression were reported. In a Morris water maze task, the changes in escape latency, the swimming speed, and the traveled distance of rats were recorded. Animals were subjected to 3 days training; each day consisted of two blocks, and each block included four trials. After the last trial on the third day of training, stereotaxic surgery was employed for the infusions, and after 48 h of surgery the animals were tested. The significant changes in escape latency and traveled distance but not swimming speed were obtained by the result of bilateral intrahippocampal infusion of H-89 (2.5 or 5 microM) into the CA1 region. The response was quite dose dependent, and the maximum effect was achieved with 5 microM H-89. After behavioral testing, several infused animals were transcardially perfused, their brains were removed and brain tissue sections were subjected to immunohistochemical staining analysis using anti-ChAT antibodies. These analyses revealed that the density of ChAT-containing cholinergic nerve terminals in the dorsal hippocampus was reduced by the intrahippocampal infusion of 5 microM H-89. The 5 microM H-89 infusions also lead to a reduction in the number of ChAT-containing neurons in the medial septum. When all these results are considered, the involvement of PKA II in regulation of cholinergic gene expression and its important role in spatial memory retention in rats can be inferred.

1.5 Aim of the study

The aim of the present study was to correlate the individual variation in short- and long-term spatial memory occurring within the random population of young rats, with the hippocampal levels of the above mentioned candidate "memory molecules" including three isoforms of nitric oxide synthase: neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS);

protein kinase A (PKA), calcium-calmodulin dependent protein kinase II alpha (CaMKIIα), their phosphorylated forms (pPKA, pCaMKIIα) and choline acetyltransferase (ChAT). Rats were classified as "good" and "poor" learners on the basis of their performance in the partially baited 12-arm radial maze. Western blot technique was used to determine the tissue levels of proteins. Taking into consideration the differences between rodent strains in learning skills (Dere *et al*., 2001) and NOS expression (Blackshaw *et al*., 2003; Hölscher, 2002), the study investigating the relation between animals' cognitive status and hippocampal levels of NOS isoforms was carried out on two strains of laboratory rats: the inbred Wistar (W) and outcrossed Wistar/Sprague Dawley (W/S) rats. The studies examining hippocampal levels of remaining enzymes were run on pigmented Long-Evans rats.

CHAPTER 2

MATERIALS AND METHODS

2.1 Subjects

At the first stage of the experiments, 3 months old inbred male albino Wistar (W) (n=30) and outcrossed male Wistar/Spraque Dawley rats (W/S) (n=35) obtained from GATA Animal Facility, Ankara, Turkey were used (Figure 12). At the second stage of the experiments, 3 months old inbred male pigmented Long-Evans rats (n=32) obtained from Charles River Laboratory, Germany, were used as an experimental subject.

Albino rat

Pigmented rat

Figure 12 Picture from albino and pigmented rat strains (http://www.quora.com/How-do-scientists-get-animals-for-experiments)

Since in the present studies rats were subjected to the learning tests with food as a reinforcement, to increase animals' motivation, one week prior to, the experiments, rats were subjected to a food restricted diet with fixed daily food portion of 3 standard pellets per rat given at a fixed time. Before rats were taken into experiments, their *ad libitum* body weight was reduced by 15%. (Jakubowska-Dogru *et al*., 2003). Rats body weight was recorded daily and kept at a stable level across the experiments. Once a day, subjects were taken into experiments in the same order and approximately at the same time of the day. The animal care procedures and all manipulations of experiments were pursued in accordance with the METU Ethic Committee Regulations (Protocol No: 2009/01).

2.2 Activity measurements

Since locomotor hyper- or hypoactivity may affect the animals' performance in the learning task, in W and outcrossed W/S rats' experiments during the shaping training in radial arm maze (RAM) task, five rats that did not show any locomotor activity were discarded from further experiments. In this way the group numbers decreased to W (n=27) and outcrossed W/S (n=33).

In experiments with Long-Evans rats, animals' locomotor activity was assessed in the activity boxes (MAY 9908 model Activity Monitoring System: Commat Ltd, Turkey). This system consisted of a plexiglas box in 42x42x30 cm dimensions, with an infrared light source. In each activity box, there were 15 photocell emitters and sensors on each side, placed 4.5 cm above the ground and 2.5 cm away from each other. Another 15 photocell emitters were placed at 11.5 cm height. Rats' vertical and horizontal movements were sensed by photocells and recorded by a computer based "special motion recognition software" with 0.1 sec sensitivity. To assess ambulatory activity several measures were taken including total travelled distance, total movement time,

average velocity of the motion and total number of motions within the test period (15 min). The rearing movements were recorded by the vertical sensors at the bands of the box. The total number of vertical movements, total time and average time of a single motion were recorded. All those recorded data was stored as "activity score" with the help of the software. None of the Long-Evans rats were discarded from further experiments.

2.3 Twelve-arm Radial Maze Apparatus

The apparatus used in the experiment was a twelve arm radial maze (RAM), made of plywood, grey in color and 80 cm above from floor of a room including a variety of distinct distal cues. The central platform of the maze was 40 cm in diameter and arms were 60 cm in length and 9 cm wide. Each arm was framed by 15 cm high sidewalls made of clear Plexiglas which prevents rats from directly crossing from one arm to the other, but allows them to see the visual cues belonging to the room. A guillotine door was placed at the entrance to each arm. The doors of the apparatus could be controlled individually or in concert. At the end of each arm, there was a food well (2 cm wide and 2 cm deep). To unify the food odor traces throughout the maze, in all food wells, 1 cm from the bottom, a perforated partition was inserted beneath which food pellets (two 2g chocolate flavored rice puffs) were placed. The same food pellets were used as a bait during the training. The dim homogenous light was used to illuminate the maze. The animal behaviour was monitored by a video camera system (EthoVision 3.1 System by Noldus Information Technology, Holland) fixed at the ceiling.

2.4 Behavioral procedure

The behavioral procedure was adopted from Jakubowska-Dogru *et al.* (2003). Before the experiments, all rats were daily handled individually for 5 minutes for six consecutive days. At the beginning of the experiments, for five days, rats were subjected to habituation and shaping training. During this training, rats were allowed to explore the maze for 10 min each day, and eat all the food pellets initially scattered throughout the maze. The number of pellets was reduced on each successive day and they were placed closer to the ends of the arms. At the end of the shaping training, food was positioned only in the food wells of six semi-randomly selected arm (Figure 13).
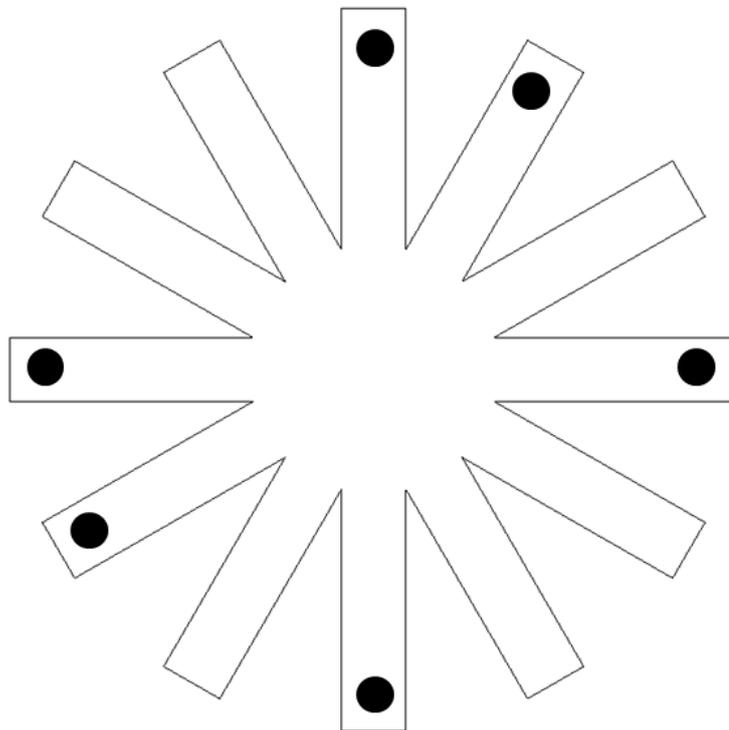
Figure 13 The scheme of 12-arm radial maze with baited arms marked by black dots.

A partially baited radial arm maze such as used in the present study allows simultaneous estimation of reference memory errors (RMEs) and working memory errors (WMEs). One trial was given daily. On the daily trials, rats were placed on the central platform each time facing different directions. The guillotine doors were raised to allow the first choice by entering one of the arms. Each time, after making a choice, rats returned to the central platform and the guillotine doors were shut for the following 5 sec before allowing animals to make another choice. Rats were allowed to make choices among the arms until they finished the trial either by eating all food pellets, or making 12 choices, or 10 min had passed, whichever came first. The entry to the arm was counted only when the rat crossed the midpoint of the alley with its two forepaws. All rats were trained to the criterion of three consecutive daily trials with maximum 3 incorrect arm entries out of the total 18 choices (the first 6 choices per trial were considered).

The following measures were recorded in the course of the experiments:

a) Number of choices to the acquisition criterion,
b) Total number of entries to unbaited arms (reference memory errors, RMEs),
c) Total number of re-entries to either baited or unbaited arms (working memory errors, WMEs).

On the basis of their performance in partially baited 12-arm radial maze, the individual rats were classified as "good" (total number of choices to the acquisition criterion ≤ group mean - 3SEM) and "poor" (total number of choices to the acquisition criterion ≥ group mean + 3SEM) learners.

2.5 Tissue sample preparation

After all rats have reached the acquisition criterion, a single retraining session was given to the rats and 15 min later, animal were sacrificed by decapitation. The brains were rapidly removed and placed on dry ice, hippocampi dissected and immediately frozen in liquid nitrogen and then stored at -80ºC until the biochemical assay were performed.

Left and right hippocampi of "good" and "poor" learners from rats were homogenized by ultrasonication in the presence of protease inhibitors. Tissue samples were transferred to a centrifuge tube containing 800 µl of Triton lysis buffer (1% Triton X-100, 20 mM Tris-HCL pH 7.4, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10% glycerol, 20 mM HEPES and protease inhibitor cocktail (Roche)). Then, samples were subjected to sonication using a sonic dismembrator in an ice-bath with a microtip probe set to a power output of 40 W for three cycles of 5 sec each (15 sec interval between sonications). Afterwards they were centrifuged at 17000 xg at speeds of 13500 rpm for 30 min at 4°C, then supernatant was removed and stored at -80°C until use.

2.6 Determination of protein concentration

Protein concentrations were determined in the cleared supernatant of "good" and "poor" learners from W, outcrossed W/S and Long-Evans rats by both Lowry *et al*. (1951) method and Bicinchoninic acid (BCA) method of Smith *et al.* (1985) using crystalline bovine serum albumin (BSA) as a standard. Since these two methods gave consistent results, only one of them was chosen for protein determination of samples and given in the result part.

2.6.1 Lowry method

Principle of the Lowry method is the reactivity of the peptide nitrogen with the copper ions under alkaline conditions and following this reaction, with copper-catalyzed oxidation of aromatic acids reduction of the Folin-Ciocalteu phosphomolybdic and phosphotungstic acid to heteropolymolybdenum blue. Tyrosine and tryptophan are reduced by that reagent. The Lowry method is sensitive to determination of low protein concentrations ranging from 0.005-0.10 mg of protein per ml.

The reagents for Lowry method are listed below:

Reagent I: 2% (w/v) $CuSO_4.5H_2O$, 100 ml (Copper source).

Reagent II: 2% (w/v) Na-K tartarate, 100 ml ($NaKC_4H_4O_6$), store at +4 ^{0}C. (Prevent precipitation of Cu^{++} ions in alkaline medium).

Reagent A: 2% (w/v) Na_2CO_3 (buffering the environment) in 0,1 N NaOH 1L (It is used to make the medium alkaline).

Stock solutions are prepared as follows:

- ✓ 2 grams of $CuSO_4.5H_2O$ is dissolved and final volume completed to 100 ml with dH_2O,
- ✓ 2 grams of Na-K tartarate is dissolved and final volume completed to 100 ml with dH_2O,
- ✓ 4 grams of NaOH is dissolved in a little amount of dH_2O and 20 grams of Na_2CO_3 are dissolved with dH_2O then these two suspensions are mixed and final volume is completed to 1 L.

To prepare the Lowry Alkaline Cupper Reagent, Reagent I, Reagent II and Reagent A are mixed in the given order with the ratio of 1:1:100. Folin-Phenol Reagent is prepared by diluting 2 N stock solution to 1 N with distilled water. Prepared Folin-Phenol reagent should be stored in dark bottle since its light sensitive.

Before the preparation of the reaction tubes, initial dilution is done for sample fraction (50 times dilution). Besides initial dilutions, dilution within tube is carried out by taking 0.1 to 0.5 ml of initially diluted samples into reaction tubes and completing the final volume to 0.5 ml by addition of distilled water. Then, 2.5 ml of Lowry Alkaline Copper Reagent is added to each tube. All tubes are mixed and incubated at room temperature for 10 min for copper reaction in alkaline medium. Then, 0.25 ml of diluted Folin reagent (1 N) is added to the each tube and tubes are mixed by vortex within 8 sec. The tubes are left for incubation at room temperature for 30 min.

The intensity of resulting color is measured at 660 nm using spectrophotometer. Standard tubes with five different protein concentrations (20, 50, 100, 150, 200 μg BSA in 0.5 ml final volume) are prepared from crystalline BSA and same steps used for sample tubes are carried for standard tubes, too. A standard curve is drawn using intensity readings of standard and slope of this curve is used for determination of protein concentrations in samples.

Protein concentration is calculated by the following formula:

$$\text{Protein Concentration} \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{\text{OD}_{660\text{nm}}}{\text{Slope of Standards}} \times \text{Dilution}_{\text{in tubes}} \times \text{Dilution}_{\text{initial}} \qquad (2.1)$$

2.6.2 Bicinchoninic acid (BCA) method

The BCA method (also known as the BCA assay or Smith assay) is a biochemical assay for determining the total amount of protein in a solution. The total protein concentration is determined calorimetrically by change in color from green to purple in proportion to protein amount in sample solution. BCA replaces the Folin reagent in the Lowry method by forming complexes between Copper ions (Cu^{2+}) and peptide bonds to produce purple colored end product. The most prominent features of BCA method are that the reagent can remain fairly stable under alkaline conditions and the reagent allows application in one step through adding into the copper solution. Like Lowry method, a molybdenum/tungsten blue product is also produced.

In this method, sample fractions and standard protein solutions are diluted 1:200 in 1.5 ml eppendorf tubes. Standard solutions are prepared to contain 0.02 mg/ml, 0.05mg/ml, 0.075mg/ml and 0.1mg/ml of BSA (Bovine Serum Albumin). Each sample and standard solutions are transferred to 96-well tissue culture plates as duplicates, each well having 100 μl of solution. Then, 100 μl of BCA solution is added to each well and mixed in shaker. To begin reaction and get color change, plates are incubated at 60^0C for 15 minutes. The intensity of resulting color change is measured with the help of ELISA plate reader at 570 nm.

Protein concentration is calculated by the following formula:

$$\text{Protein Concentration} \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{\text{OD}_{570\text{nm}}}{\text{Slope of Standards}} \times \text{Dilution}_{\text{in tubes}} \times \text{Dilution}_{\text{initial}} \qquad (2.2)$$

BCA solution is composed of combination of three reagents given in below:

Reagent A: 8 g $Na_2CO_3H_2O$ (sodium carbonate monohydrate) and 1.6 g Sodium tartarate (Rochella salt) are dissolved in distilled water and the pH of the solution is titrated to 11.25 with sodium bicarbonate. The volume is completed to 100 ml to prepare Reagent A.

Reagent B: Prepared by dissolving 4 g of BCA in 100 ml dH_2O.

Reagent C: 0.4 g of Cu SO_45H_2O is dissolved in 10 ml dH_2O.

After preparation, reagents should be stored at +4 ^{O}C, but prior to use they should be at room temperature.

BCA solution should be prepared as 25 (A): 25 (B): 1 (C) (v/v/v) just before using. Reagent A and C should be mixed at first and then reagent B should be added to prevent precipitation. It should be prepared in dark bottle or bottle should be covered with aluminum folio since the solution is light sensitive.

2.7 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Sample proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS using the method of Laemmli (1970). Mini-PROTEAN Tetra cell 165-8033 equipment (Bio-Rad Laboratories, Richmond, CA, USA) was used for gel preparation and electrophoresis. The separating (6% and 7.5%) and stacking gel (4%) solutions were prepared following a procedure below in a given order (Table 1).

Table 1 Components of SDS-PAGE separating and stacking gels

	Separating Gel (0.375 M Tris, pH 8.8)		Stacking Gel (0.125 M Tris, pH 6.8)
Monomer concentration	**6%**	**7.5%**	**4%**
Gel solution (ml)	6	7.5	1.3
Distilled water (ml)	16.035	14.53	6.1
Separating gel buffer (ml)	7.5	7.5	-
Stacking gel buffer (ml)	-	-	2.5
10% SDS (ml)	0.3	0.3	0.1
10% APS (ml)	0.15	0.15	0.05
TEMED (ml)	0.015	0.015	0.01
Total volume (ml)	**30**	**30**	**10**

The gel sandwich made between two glass plates was used to prepare polyacrylamide slab gels. First, separating gel solution was added to the gel sandwich till the desired height of the solution was obtained between glass plates. Top of the polymerizing gel was covered with a layer of isobutanol to obtain smooth gel surface. Then, the gel was incubated for polymerization for about 30 min at room temperature. After polymerization, the alcohol layer was poured totally. The stacking gel polymerization solution was prepared and added to the gel sandwich till the sandwich was filled completely. Following stacking gel addition, a 1.0 mm Teflon comb containing 15 wells was inserted into the layer of polymerizing stacking gel solution. The attention was given not to get any bubbles at the edges of the comb during placing the comb. Polymerization was completed in about 30 min at room temperature. The teflon comb was carefully removed without damaging the wells. Then, wells were filled with electrode running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) with a fine needle syringe for removal of any formed air bubbles or unpolymerized chain particles. Finally, the gel running module was filled with a sufficient volume of electrode running buffer.

Protein samples were diluted with 4x sample dilution buffer containing 0.25 M Tris-HCL, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol and 0.01% bromophenol blue in the ratio of 1:3 (3 part sample and 1 part buffer) and samples were boiled in water bath for 1.5 min. Then, protein samples and 3 µl of commercially available protein molecular weight marker (Fermentas, SM0671) (Figure 14) were applied to different wells by the Hamilton syringe. The loaded amount of protein samples were given in Table 2.
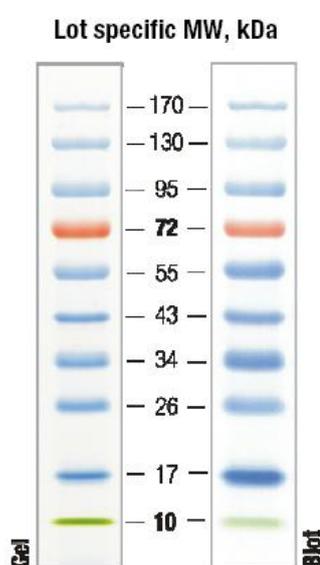
Figure 14 Image of pre-stained protein ladder (http://www.fermentas.com)

Table 2 The amount of each protein samples loaded into wells

Protein name	Loaded amount
nNOS	20 µl (2µg/µl protein)
eNOS	20 µl (2µg/µl protein)
iNOS	20 µl (2µg/µl protein)
CaMKIIα	10 µl (0.5µg/µl protein)
pCaMKIIα	15 µl (0.5µg/µl protein)
PKA	7.5 µl (0.5µg/µl protein)
pPKA	10 µl (0.5µg/µl protein)
ChAT	15 µl (0.5µg/µl protein)

After application of the samples, gel running module was placed to main buffer tank containing sufficient amount of electrode running buffer. The electrophoresis unit was connected to the power supply to run electrophoresis at 20 mA and 100 V in stacking gel and 40 mA 160 V in separating gel.

2.8 Western blotting

Techniques commonly used to detect differential protein expression in the samples of brain tissue are: gene expression profiling by microarray analysis (Mirnics *et al*., 2001), quantifying mRNA levels by Northern blot analysis (Bagasra *et al*., 1995), RNase protection assay (Wei and Mojsov, 1995), and recently more in use Real-Time Quantitative RT-PCR method (Berti *et al*., 2002), or directly quantifying protein levels by Western blot analysis (Krupinski *et al*., 1996). On the other hand, short-lasting functional changes, the attributes of working memory, may be assessed by measuring levels of enzymatic activity (Ellman *et al*., 1961). The interpretation of the results obtained in gene expression profiling studies is very difficult since usually an applied learning task induces changes in expression of numerous genes of which some are up- and some other are down-regulated. The changes induced by learning should be dissected from the changes induced by physical activity. The observed changes depend on the brain region and the time window during or after the training, in which biochemical assays are conducted. The identified genes may be pleiotropic and/or interact functionally to effect memory formation. In addition, each of them may act in a complex networks not in a linear sequence. Therefore in the present study, to evaluate hippocampal levels of proteins of interest Western Blot analysis was applied.

After electrophoresis was completed, gels were removed from set-up for western blotting. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 min with constant shaking to adjust the

final size of gel and remove the buffer salts and SDS coming from SDS-PAGE step. Nitrocellulose membrane was prepared by cutting 1 cm larger than the dimension of the gel and two pieces of filter paper (Whatman #1) were cut in dimensions little bit larger than the membrane.

Nitrocellulose membrane, two filter papers and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as seen in Figure 15. A test tube was used to remove any air bubbles between the layers by gently rolling over the sandwich. This was the very critical step since any air bubbles formed between layers block the transfer of proteins. Then, the sandwich was put into the Mini Trans-Blot module 165-8033 (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with cold transfer buffer. Voltage and current were set to 90 V and 400 mA, respectively. Transfer process was carried for 90 min with Bio-Rad PowerPac basic power supply (Bio-Rad Laboratories, Richmond, CA, USA).
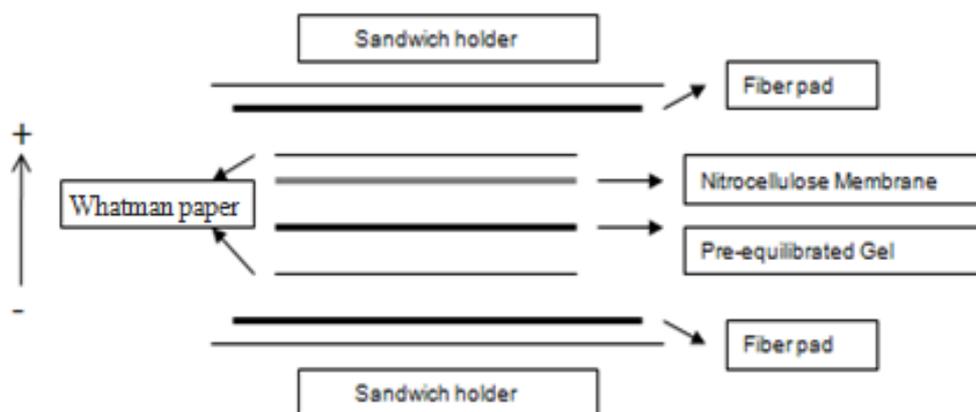
Figure 15 Preparation of western blot sandwich.

At the end of this process, the membrane carrying the transferred protein on it, i.e. "blot" was obtained and removed from the module. Then, membrane was transferred to a plastic dish as protein side facing upwards and washed with TBS-T (Tris Buffered Saline plus Tween 20: 20 mM Tris-HCL, pH 7.4, 0.5 M NaCl and 0.05 % Tween 20) for 10 min. This washing step removes the salts and buffers from transfer medium. Then, the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBS-T) for 60 min so that empty spaces between transferred proteins were filled. This filling inhibits the non-specific binding of antibodies to the membrane. After that, the blot was incubated with primary antibody for 2 hours. The list of primary antibodies for each protein was given in Table 3. Following this step, the blot was washed 3 times with 50 ml TBS-T for 5 min each time. The washing steps were necessary for removal of excess of antibody from the membrane. The blot was then incubated with secondary antibody that conjugated to marker enzyme-alkaline phosphatase for 1 h (see Table 3). The blot was then washed 3 times with 50 ml TBS-T for 5 min each to remove excess antibody. The complete removal of TBS-T between each washing steps were extremely important since non-specifically bound regions can give reaction with substrate solution.

Table 3 List of primary and secondary antibodies used for each protein, with their brands and the optimum dilution rates

Protein name	Primary Ab	Dilution	Brand	Secondary Ab	Dilution	Brand	β-actin/Primary Ab	Dilution	Brand	β-actin/Secondary Ab	Dilution	Brand
nNOS	Monoclonal mouse anti-nNOS	1/1000	Invitrogen 37-2800	Goat Anti-Rat IgG-ALP Conj.	1/2000	Santa Cruz 3824	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
eNOS	Monoclonal mouse anti-eNOS	1/250	Santa Cruz 136977	Goat Anti-Rat IgG-ALP Conj.	1/1000	Santa Cruz 3824	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
iNOS	Monoclonal mouse anti-iNOS	1/1000	Santa Cruz 7271	Goat Anti-Rat IgG-ALP Conj.	1/2000	Santa Cruz 3824	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
CaMKIIα	Monoclonal mouse anti-CaMKIIα	1/1000	Santa Cruz 32288	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
pCaMKIIα	Monoclonal mouse anti-pCaMKIIα	1/500	Santa Cruz 32289	Goat Anti-Mouse IgG-ALP Conj.	1/1000	Santa Cruz 2008	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
PKA	Purified mouse anti-PKA RIIβ	1/5000	BD Bioscience 610626	Goat Anti-Mouse IgG-ALP Conj.	1/10000	Santa Cruz 2008	Mouse monoclonal IgG₁ β-actin (C4)	1/250	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/5000	Santa Cruz 2008
pPKA	Purified mouse anti-PKA RIIβ pS114	1/1000	BD Bioscience 612550	Goat Anti-Mouse IgG-ALP Conj.	1/2000	Santa Cruz 2008	Mouse monoclonal IgG₁ β-actin (C4)	1/500	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/2000	Santa Cruz 2008
ChAT	Monoclonal mouse anti-ChAT	1/1000	ABCAM 78023	Goat Anti-Mouse IgG-ALP Conj.	1/2000	Santa Cruz 2008	Mouse monoclonal IgG₁ β-actin (C4)	1/500	Santa Cruz 47778	Goat Anti-Mouse IgG-ALP Conj.	1/2000	Santa Cruz 2008

Blots were then incubated with substrate solution that described by Ey and Ashman (1986) for visualization of specifically bound antibodies (Table 4). Blots were then incubated with a monoclonal antibody directed against β-actin (see Table 3), which was used as a control to normalize protein expression levels. The final images were photographed using a computer-based gel imaging instrument (Vilber Lourmat) with Infinity-Capt version 12.9 software. Immunoreactive protein bands were then quantified by densitometric scanning method using an Image J software package program developed by NIH.

Table 4 Preparation of substrate solutions for immunodetection

Solution A	Solution B
2.67 ml of 1.5 M Tris-HCL, pH 8.8	2 mg/ml phenazine methosulfate in distilled water
4 ml of 1M NaCl	**Solution C**
0.82 ml of 100 mM $MgCl_2$	5.44 mg BCIP/0.136 ml N,N-dimethyl formamide
0.04 ml of 100 mM $ZnCl_2$	
0.096 ml Diethanolamine	**Finally,** NBT/BCIP substrate solution was prepared by mixing **solution A** with **solution C** and 0.268 ml of **solution B**.
12.2 mg NBT	
Distilled water to 40 ml	
pH of the solution was adjusted to 9.55 with saturated Tris before completing to final volume	

2.9 Statistical analysis

The obtained data from both behavioral tests and biochemical assays were assessed with the help of SPSS v.18 (Chicago, IL) statistical package program. One-Way ANOVA, Pearson's correlation and Mann-Whitney tests were used for evaluation of data. Data were expressed as mean ± standard error of mean (SEM).

CHAPTER 3

RESULTS

3.1 Results of behavioral tests

3.1.1 Partially baited 12-arm radial maze results of Wistar (W) and outcrossed Wistar-Spraque (W/S) rats

The criterion of radial-arm maze training was to make maximum 3 incorrect arm entries out of the total 18 choices (first 6 choices per each trial) within 3 consecutive daily trials. During training, total number of choices to the acquisition criterion, total number of both working memory errors (WMEs) and reference memory errors (RMEs) were recorded and compared between W (n=27) and outcrossed W/S (n=33) rat groups (Figure 16).
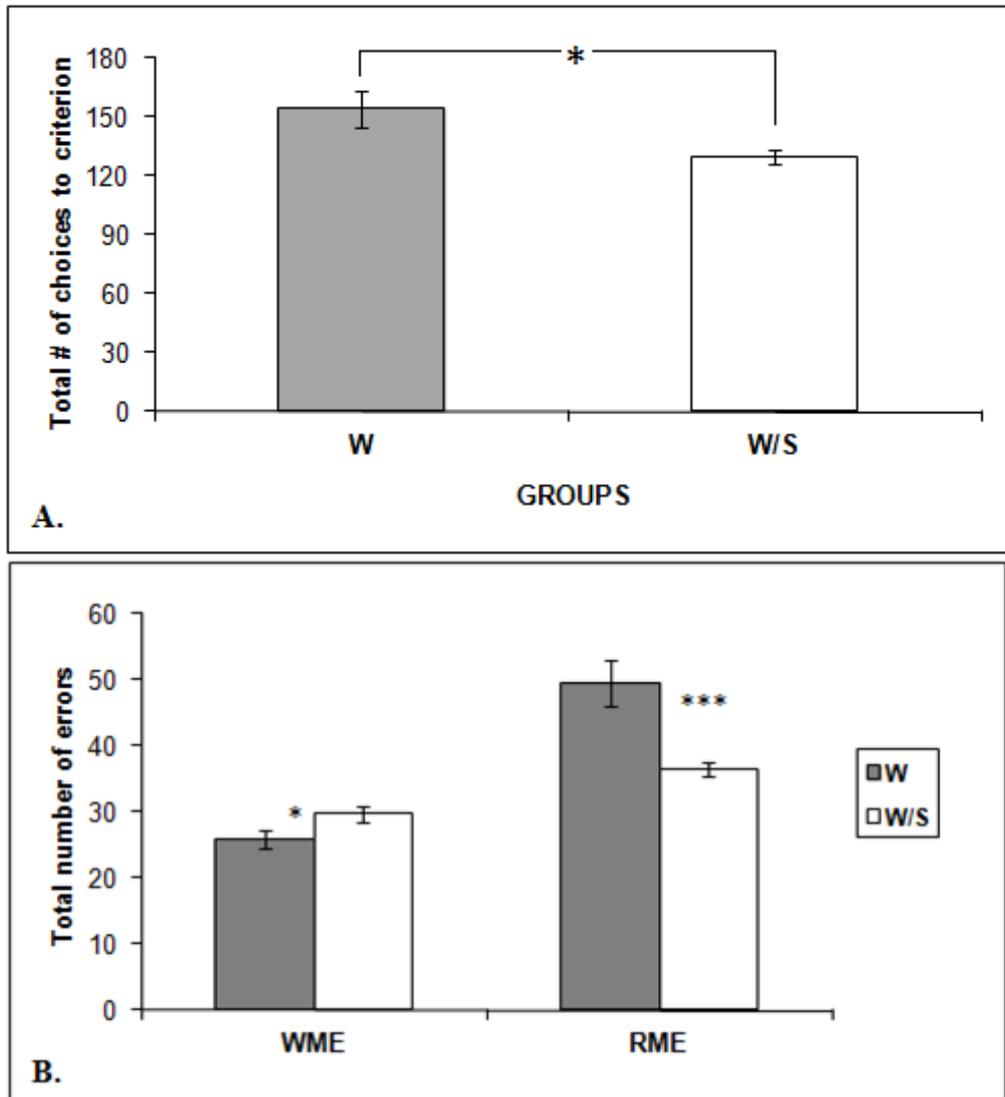
Figure 16 Mean (± SEM) number of choices to the arbitrary acquisition criterion (A.), and mean (± SEM) numbers of working and reference memory errors in W and outcrossed W/S rat groups (B.). Error bars denote ± SEM. Asterisks denote the level of significance: *p<0.05, ***p<0.001.

One-Way ANOVA revealed a significant difference between W and outcrossed W/S rats in the total number of choices to criterion ($F_{(1;59)}=6.919$, $p \leq 0.01$) with W rats executing significantly more reference ($F_{(1;59)}=14.59$, $p \leq 0.001$) and

significantly less working memory errors ($F_{(1;59)}$=4.41, p $\leq$0.04) as compared to outcrossed W/S rats. In both rat groups, the mean total number of RMEs (49.48 ± 3.53 and 36.5 ± 1.02, respectively) was significantly (p$\leq$0.001) higher than the mean total number of WMEs (25.92 ± 1.40 and 29.72 ± 1.16, respectively).

According to their performance in the partially baited radial-arm maze task, 9 W (33%) and 8 W/S (24%) rats were classified as "good" learners (total number of choices to the acquisition criterion $\leq$ group mean - 3SEM) and 8 W (30%) and 5 W/S (15%) rats were classified as "poor" learners (total number of choices to the acquisition criterion $\geq$ group mean + 3SEM) with the remaining rats classified as "intermediate" learners (Figure 17).

Figure 17 Distribution of "good", "poor" and remaining "intermediate" learners in W (A.) and outcrossed W/S (B.) rat groups.

Figure 18 presents the mean numbers of total choices to criterion (A.) and mean numbers of working and reference memory errors (B.) estimated for "good" and "poor" learners from W and outcrossed W/S rat groups. The original data of those measures was given in Table 5.
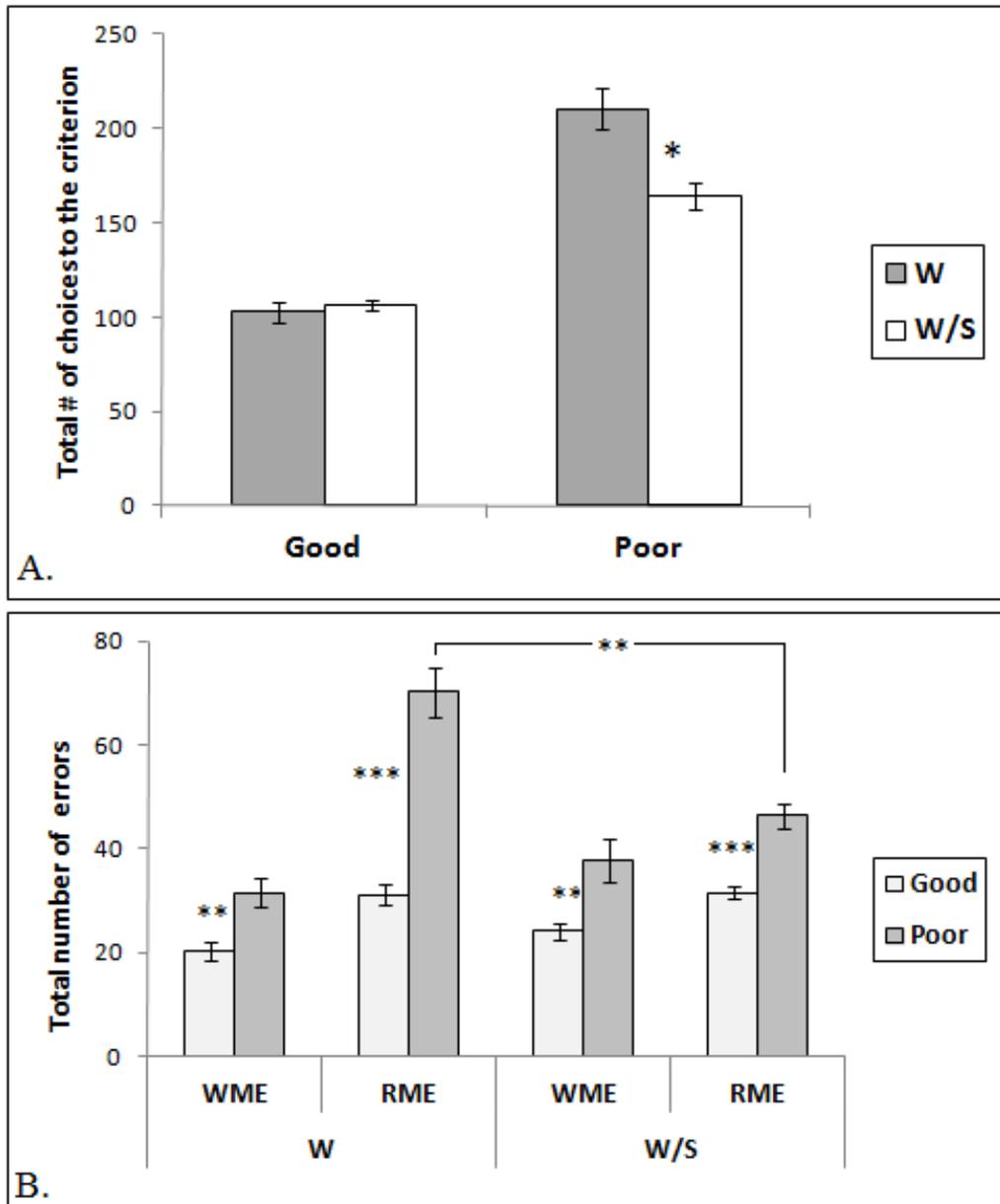
Figure 18 Mean number (± SEM) of total choices to criterion (A.) and mean number (± SEM) of working and reference memory errors (B.) emitted by "good" and "poor" learners from W and outcrossed W/S rat groups, respectively. Error bars denote ± SEM. Asterisks denote the level of significance: *p<0.05, **p<0.01, and ***p<0.001.

Table 5 The data of total # of choices, number of both WMEs and RMEs of "good" and "poor" learners from W and outcrossed W/S rats

RAT-ID	TOTAL # OF CHOICES	NUMBER OF WME	NUMBER OF RME
W-GOOD			
W1-8	97	14	31
W1-9	126	21	37
W2-1	73	10	21
W2-6	108	24	35
W2-7	113	25	38
W2-9	99	26	27
W2-13	111	21	34
W2-14	115	24	33
W2-15	85	17	24
W-POOR			
W1-3	281	38	101
W1-5	194	22	67
W1-11	191	32	66
W1-16	226	30	73
W2-4	211	47	63
W2-10	193	32	59
W2-11	196	27	65
W2-12	191	24	67
W/S-GOOD			
W/S-9	102	18	27
W/S-19	116	26	31
W/S-21	116	27	29
W/S-5	96	23	31
W/S-12	104	20	33
W/S-14	107	30	31
W/S-15	107	22	35
W/S-17	105	27	36
W/S-POOR			
W/S-11	163	33	49
W/S-12	176	52	53
W/S-14	183	37	46
W/S-3	157	28	45
W/S-8	143	39	39

One-Way ANOVA revealed a significant difference between "poor" learners from W and outcrossed W/S rat groups in the mean number of total choices to criterion ($F_{(1;12)}$=9.225, p≤0.011). No such difference was found between W and outcrossed W/S "good" learners.

In all animal groups, RMEs significantly outnumbered WMEs. Within both W and outcrossed W/S rat groups, "poor" learners compared to "good" learners, showed significantly higher number of both WMEs ($F_{(1;16)}$=11.684, p≤0.004; $F_{(1;12)}$=14.461, p≤0.003, respectively) and RMEs ($F_{(1;16)}$=65.435, p≤0.001; $F_{(1;12)}$=43.700, p≤0.001, respectively). Highly significant difference was observed between W and outcrossed W/S "poor" learners in the number of RMEs ($F_{(1;12)}$=14.58, p≤0.003), with "poor" learners from the outcrossed W/S rat group making much less RMEs than the "poor" learners from the W rat group. Conversely, there was no significant difference between W and outcrossed W/S "poor" learners in the number of WMEs. There was also no significant difference in the count of either RMEs or WMEs between "good" learners from W and outcrossed W/S groups.

3.1.2 Activity measurement results of Long-Evans rats

Mean numbers of activity scores obtained from activity measurements were compared between "good" and "poor" learners from Long-Evans rats (Table 6). The mean number of activity score in "good" learners was 2597.21 while in "poor" learners it was 2454.9, which was significantly different between learning groups ($F_{(1,23)}$=7.836, p≤0.001). This result suggested that active rats were also successful in radial-maze learning task.

Table 6 Activity scores of "good" and "poor" learners from Long-Evans rats

ACTIVITY SCORES			
RAT-ID	GOOD	POOR	
LE-4	1249	LE-3	2213
LE-5	2703	LE-6	1896
LE-8	1934	LE-7	2009
LE-10	1736	LE-9	2745
LE-21	3723	LE-13	2835
LE-23	2953	LE-14	2121
LE-24	2373	LE-15	2790
LE-25	3491	LE-33	3067
LE-26	3747	LE-38	1953
LE-27	2696	LE-40	2920
LE-28	2173		
LE-29	2421		
LE-30	2207		
LE-32	2955		

3.1.3 Partially baited 12-arm radial maze results of Long-Evans rats

On the basis of their performance in partially baited 12-arm radial maze, Long-Evans rats (n=32) were classified as "good" (n=14) (44%) (total number of choices to the acquisition criterion ≤ group mean - 3SEM), "poor" (n=10) (31%) (total number of choices to the acquisition criterion ≥ group mean + 3SEM) with the remaining rats classified as "intermediate" learners (Figure 19).
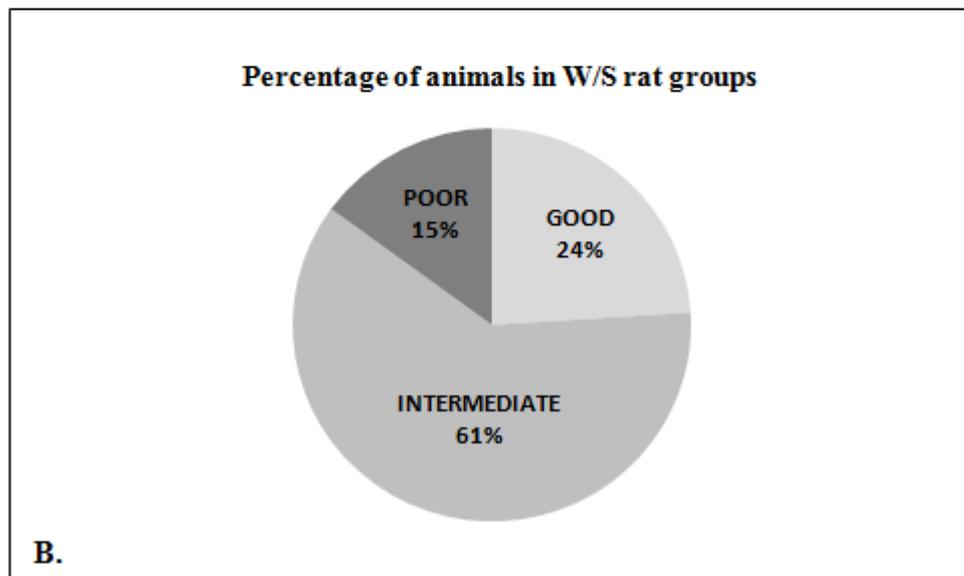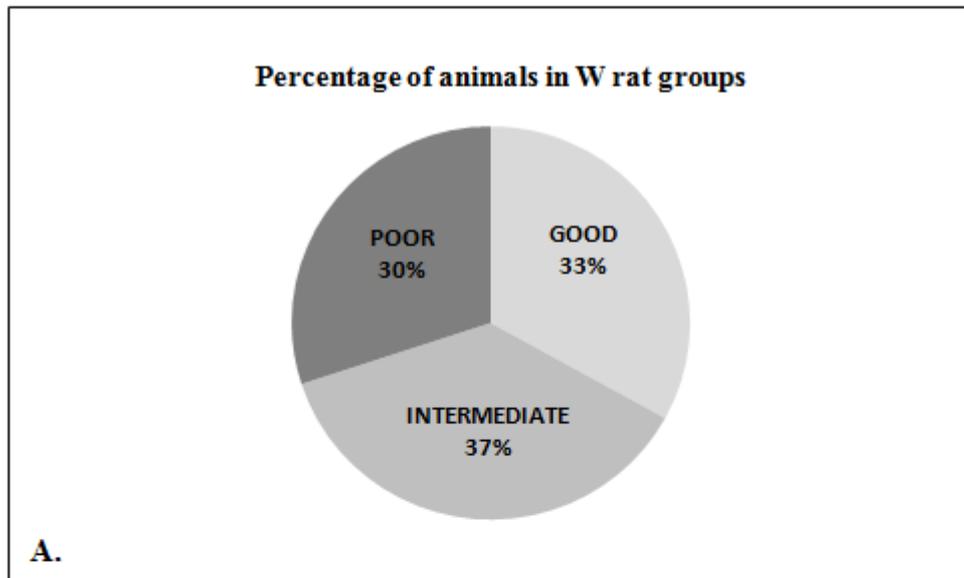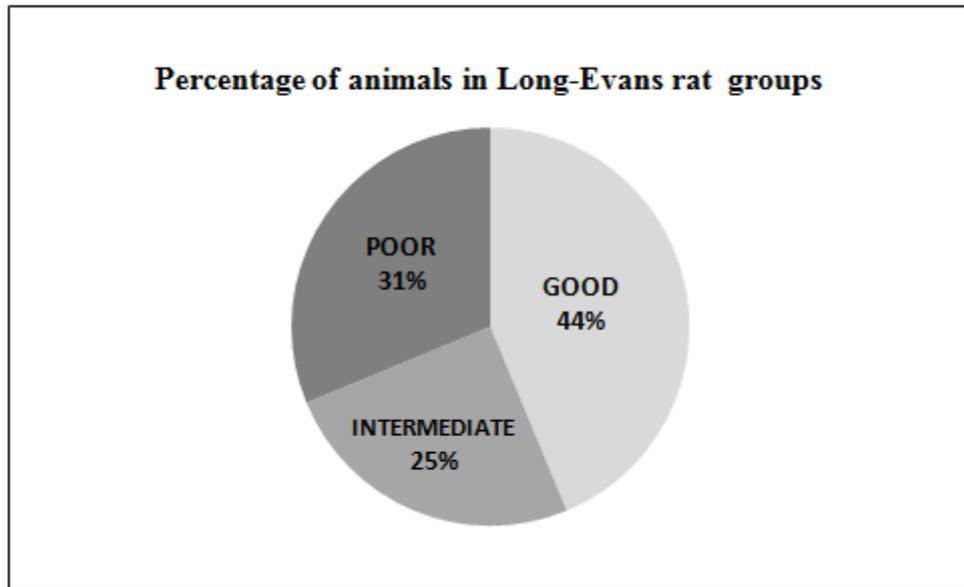
Figure 19 Distribution of "good", "poor" and remaining "intermediate" learners in Long-Evans rat groups.

The total number of choices to the acquisition criterion of 3 consecutive daily trials with maximum 3 incorrect arm entries out of the total 18 choices (first 6 choices per each trial) and total number of errors were compared between "good" and "poor" learners of Long-Evans rats (Figure 20). The original data of those measures was given in Table 7.

Table 7 The data of total # of choices, number of errors, number of both WMEs and RMEs of "good" and "poor" learners from Long-Evans rats

RAT-ID	TOTAL # OF CHOICES	NUMBER OF ERRORS	NUMBER OF WME	NUMBER OF RME
GOOD				
LE-4	139	60	13	47
LE-5	133	63	25	38
LE-8	146	80	32	48
LE-10	132	66	25	41
LE-21	113	73	34	39
LE-23	132	64	23	41
LE-24	135	52	16	36
LE-25	107	74	24	50
LE-26	142	61	15	46
LE-27	127	68	27	41
LE-28	129	63	19	44
LE-29	134	65	26	39
LE-30	135	51	23	28
LE-32	98	53	20	33
POOR				
LE-3	279	152	58	94
LE-6	304	163	73	90
LE-7	268	133	48	85
LE-9	301	169	68	101
LE-13	301	174	82	92
LE-14	314	167	81	86
LE-15	305	170	72	98
LE-33	273	137	64	73
LE-38	276	121	45	76
LE-40	298	163	82	81
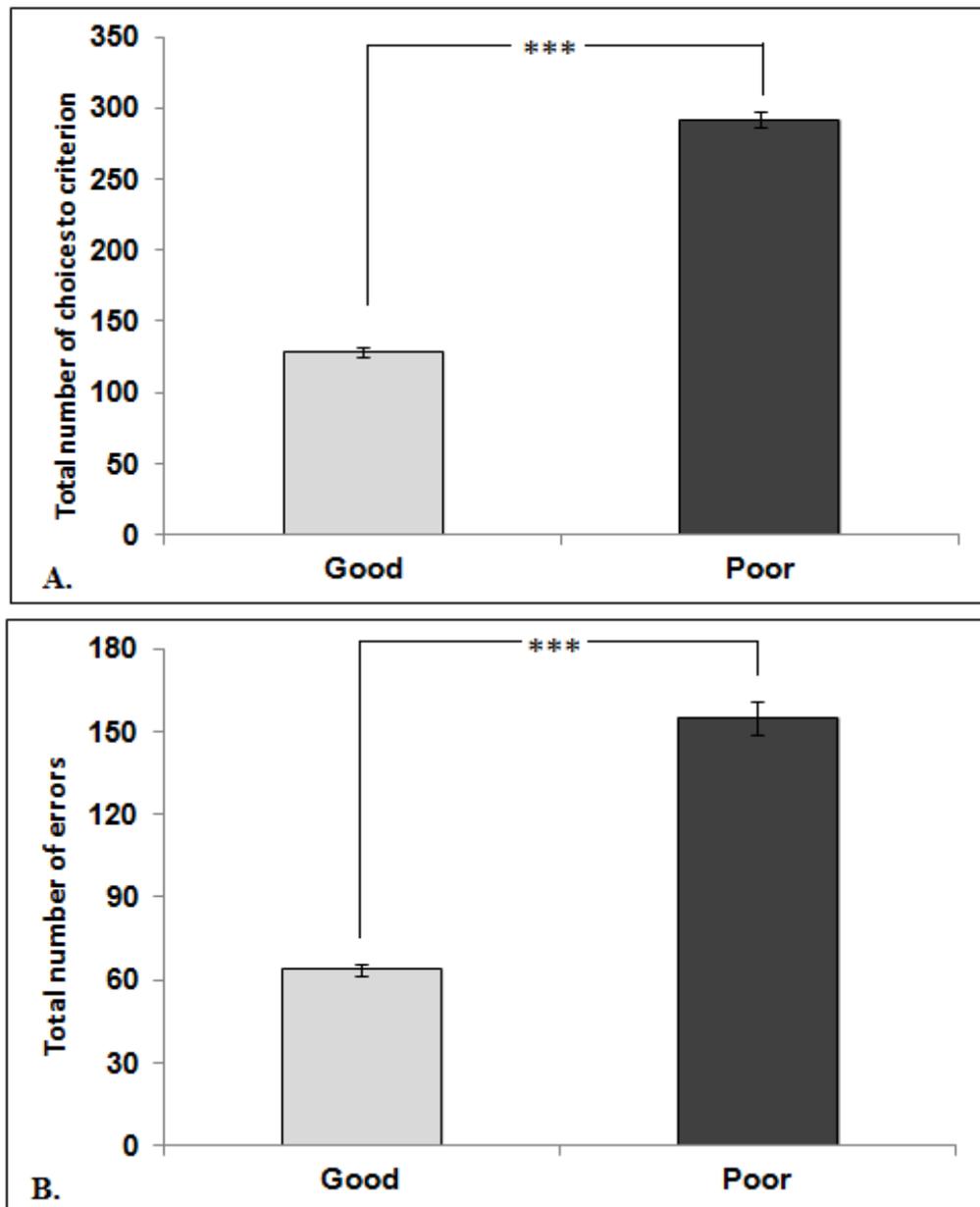
Figure 20 Mean number (± SEM) of total choices to criterion (A.) and mean number (± SEM) of errors (B.) emitted by "good" and "poor" learners. Error bars denote ± SEM. Asterisk denote the level of significance: ***$p<0.001$.

As seen from Figure 20, "good" learners reached the arbitrary performance criterion significantly almost 2.5 times faster than "poor" learners with

significantly making less errors ($F_{(1;23)}=719.503$, $p\leq0.001$; $F_{(1;23)}=270.046$, $p\leq0.001$, respectively).

The mean total number of both working (WME) and reference memory errors (RME) of Long-Evans rats were compared between "good" and "poor" learners and given in Figure 21. The original data of those measures was given in Table 7.
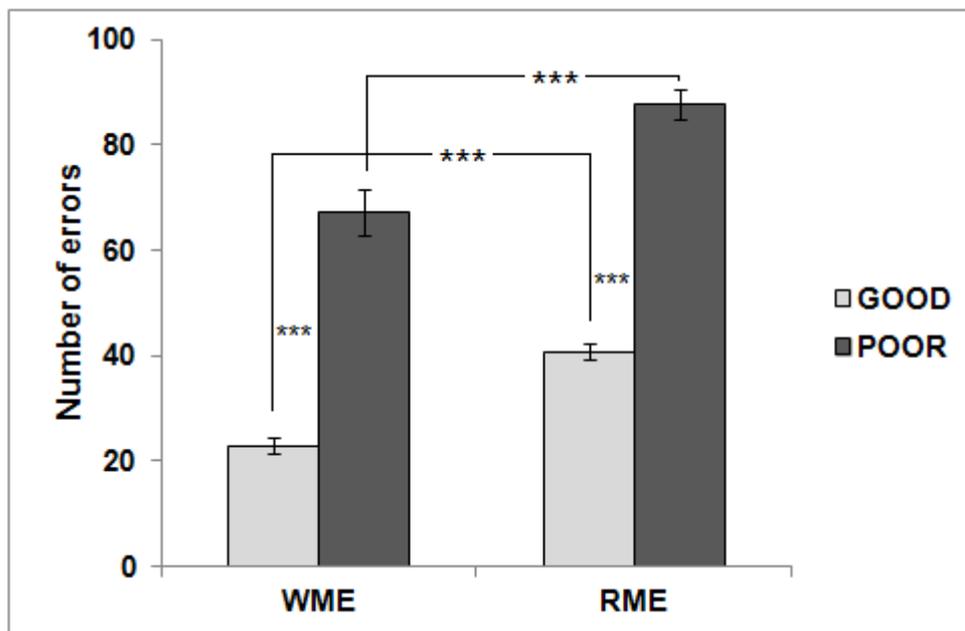
Figure 21 Mean number (± SEM) of working and reference memory errors emitted by "good" and "poor" learners. Error bars denote ± SEM. Asterisk denote the level of significance: ***p<0.001.

As seen from Figure 21, "poor" learners emitted significantly more both RMEs ($F_{(1;23)}$=228.892, p≤0.001) and WMEs ($F_{(1;23)}$=119.017, p≤0.001) than "good" learners, and RMEs were significantly higher than WMEs within both "good" ($F_{(1;27)}$=60.707, p≤0.001) and "poor" learners ($F_{(1;19)}$=15.476, p≤0.001).

3.2 Results of biochemical assays

3.2.1 Results of Western blotting assay for NOS isoforms

The Figure 22 presents a BSA standard calibration curve obtained from Lowry (1951) protein determination method while Table 8 shows the protein concentrations (mg/ml) of samples from the left and right hippocampi of "good" and "poor" learners of W and outcrossed W/S rats. Western blotting assay was carried out for left and right hippocampi of "good" and "poor" learners from W and outcrossed W/S rats, separately. Therefore, for each group, the number of samples was twice the number of subjects: W "good'" n=18; W "poor" n=16; W/S "good" n=16; W/S "poor" n=10. Since the band quantification results obtained from the left and right hippocampi of "good" and "poor" learners were statistically insignificant (Mann-Whitney test, p≥0.1), for further analyzes the average values were used.
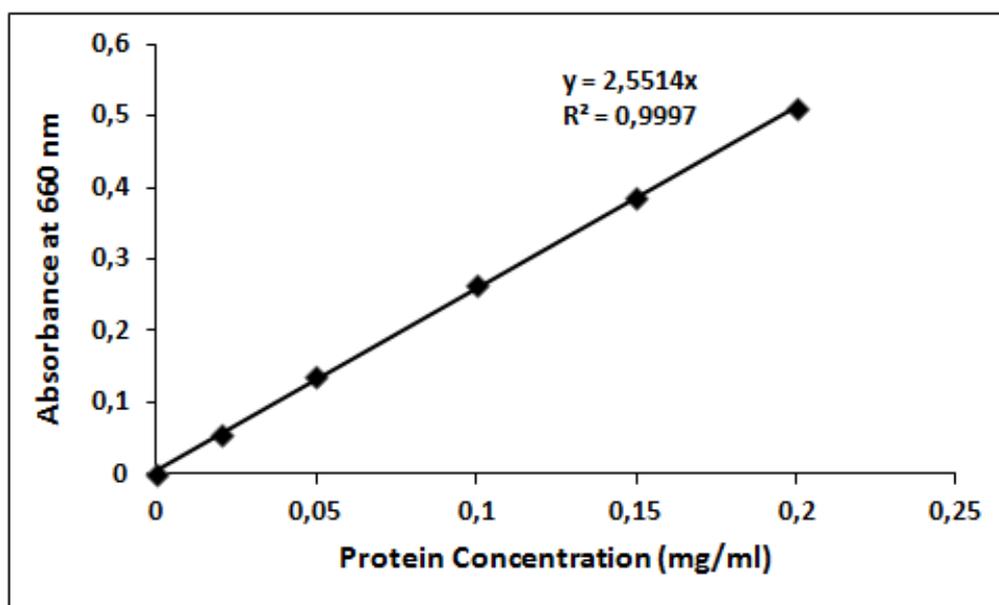
Figure 22 Standard calibration curve for Lowry protein determination method

Table 8 Protein concentrations (mg/ml) of left and right hippocampi of "good" and "poor" learners from W and outcrossed W/S rats

W-GOOD		W-POOR		W/S-GOOD		W/S-POOR	
RAT-ID	Protein Concentration (mg/ml)	RAT-ID	Protein Concentration (mg/ml)	RAT-ID	Protein Concentration (mg/ml)	RAT-ID	Protein Concentration (mg/ml)
W1-8-L	8,252	W1-3-L	8,465	W/S9-L	11,368	W/S11-L	10,658
W1-8-R	9,184	W1-3-R	9,979	W/S9-R	11,609	W/S11-R	10,509
W1-9-L	9,490	W1-5-L	10,120	W/S19-L	10,790	W/S12-L	11,423
W1-9-R	9,600	W1-5-R	10,220	W/S19-R	11,190	W/S12-R	12,141
W2-1-L	11,495	W1-11-L	12,420	W/S21-L	10,700	W/S14-L	9,533
W2-1-R	12,001	W1-11-R	10,760	W/S21-R	10,662	W/S14-R	10,861
W2-6-L	12,390	W1-16-L	10,848	W/S5-L	11,747	W/S3-L	10,887
W2-6-R	13,490	W1-16-R	11,129	W/S5-R	9,939	W/S3-R	11,396
W2-7-L	12,830	W2-4-L	13,800	W/S12-L	12,752	W/S8-L	12,700
W2-7-R	12,150	W2-4-R	12,260	W/S12-R	11,167	W/S8-R	12,320
W2-9-L	9,552	W2-10-L	10,980	W/S14-L	9,227		
W2-9-R	9,763	W2-10-R	11,810	W/S14-R	10,470		
W2-13-L	8,298	W2-11-L	12,290	W/S15-L	11,314		
W2-13-R	9,131	W2-11-R	12,720	W/S15-R	10,922		
W2-14-L	11,310	W2-12-L	11,880	W/S17-L	10,624		
W2-14-R	12,160	W2-12-R	12,020	W/S17-R	10,854		
W2-15-L	11,087						
W2-15-R	9,295						

82

In the Western blot analysis of nNOS, eNOS, and iNOS, immunoreactive proteins were detected as bands of 170 kDa, 133 kDa, and 130 kDa, respectively (Figure 23). Relative NOS protein expressions were determined by comparing band intensities with that of β-Actin, which was detected at the position corresponding to a molecular weight of 42 kDa. and was used as the internal sample loading control (Figure 24, 25 and 26).

Figure 23 Immunoreactive protein bands representing nNOS (the upper lane, 170kDa), eNOS (the middle lane, 133 kDa), and iNOS (the bottom lane, 130kDa) from left (L) and right (R) hippocampi of "good" (G) and "poor" (P) learners in W (A) and outcrossed W/S (B) rat groups. The β-Actin band (42 kDa) was used as an internal control for each gel.

Quantitative immunoblot analysis of nNOS, eNOS, and iNOS levels in the hippocampus of W and outcrossed W/S rats done by One-Way ANOVA revealed significantly lower expression of all three NOS isoforms in W rats as compared to outcrossed W/S rats with the greatest difference in nNOS level ($F_{(1;59)}=346.852$, $p \leq 0.001$; $F_{(1;59)}=5.610$, $p \leq 0.021$ and $F_{(1;59)}=26.204$, $p \leq 0.001$, for nNOS, eNOS and iNOS, respectively) (Figure 24).
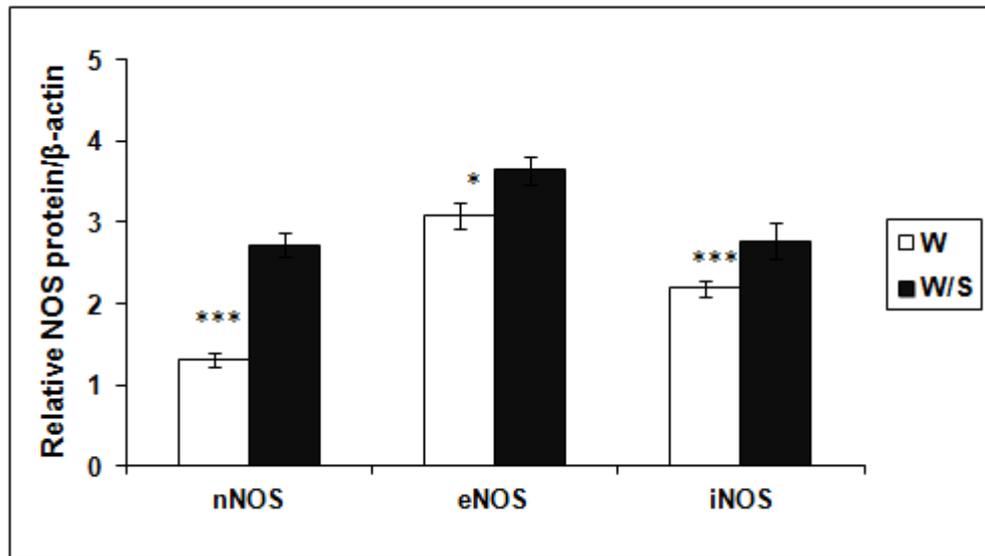
Figure 24 Comparison of the levels of nNOS, eNOS, and iNOS in the hippocampus of W and outcrossed W/S rats. Band quantification is expressed as the mean ± SEM of the relative intensity with respect to that of β-Actin, used as the internal control. Error bars denote ± SEM. Asterisks denote the level of significance: *p<0.05 and ***p<0.001.

The Figure 25 illustrates the differences in the levels of three NOS isoforms between "good" and "poor" learners in W and outcrossed W/S group, independently. One-Way ANOVA applied to these data in both groups yielded a significantly higher expression of hippocampal nNOS ($F_{(1;33)}=7.129$, $p \leq 0.012$ and $F_{(1;25)}=6.487$, $p \leq 0.018$ for W and outcrossed W/S groups, respectively) and

significantly lower expression of hippocampal iNOS ($F_{(1;33)}$=6.896, p≤0.013 and $F_{(1;25)}$=30.671, p≤0.001, respectively) in "good" learners as compared to "poor" learners. "Good" learners of both groups, also exhibited lower hippocampal levels of eNOS, however, this difference was yielded statistically significant only in W group of rats ($F_{(1;33)}$=56.094, p≤0.001).
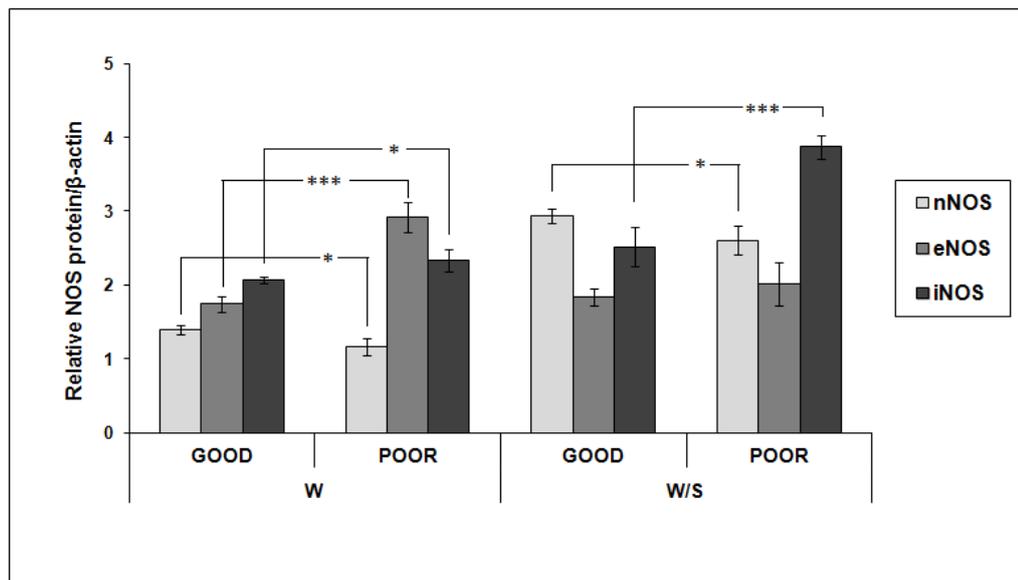
Figure 25 Comparison of the levels of nNOS, eNOS, and iNOS in the hippocampus of "good" and "poor" learners from W and outcrossed W/S rat groups, independently. Band quantification is expressed as the mean ± SEM of the relative intensity with respect to that of β-Actin, used as the internal control. Error bars denote ± SEM. Asterisks denote the level of significance: *p<0.05 and ***p<0.001.

Pearson's correlation analysis performed on the data from W rat group confirmed a significant positive correlation between the total number of choices to criterion and both eNOS (r=0.853[**], p≤0.001) and iNOS expression (r=0.487[**], p≤0.003). In W group, the negative correlation between the nNOS level and the number of choices to reach the performance criterion was only

marginally significant (r=-0.274, p=0.116). Similar analysis applied to the data from outcrossed W/S rat group revealed a significant negative correlation between the total number of choices to criterion and nNOS expression (r=-0.530[**], p≤0.005) and a positive correlation between the total number of choices to criterion and iNOS expression (r=0.609[**], p≤0.001).

Comparison of NOS isoforms levels in the hippocampus of "good" and "poor" learners from W and outcrossed W/S rat strains were presented in Figure 26 (A.) and (B.).
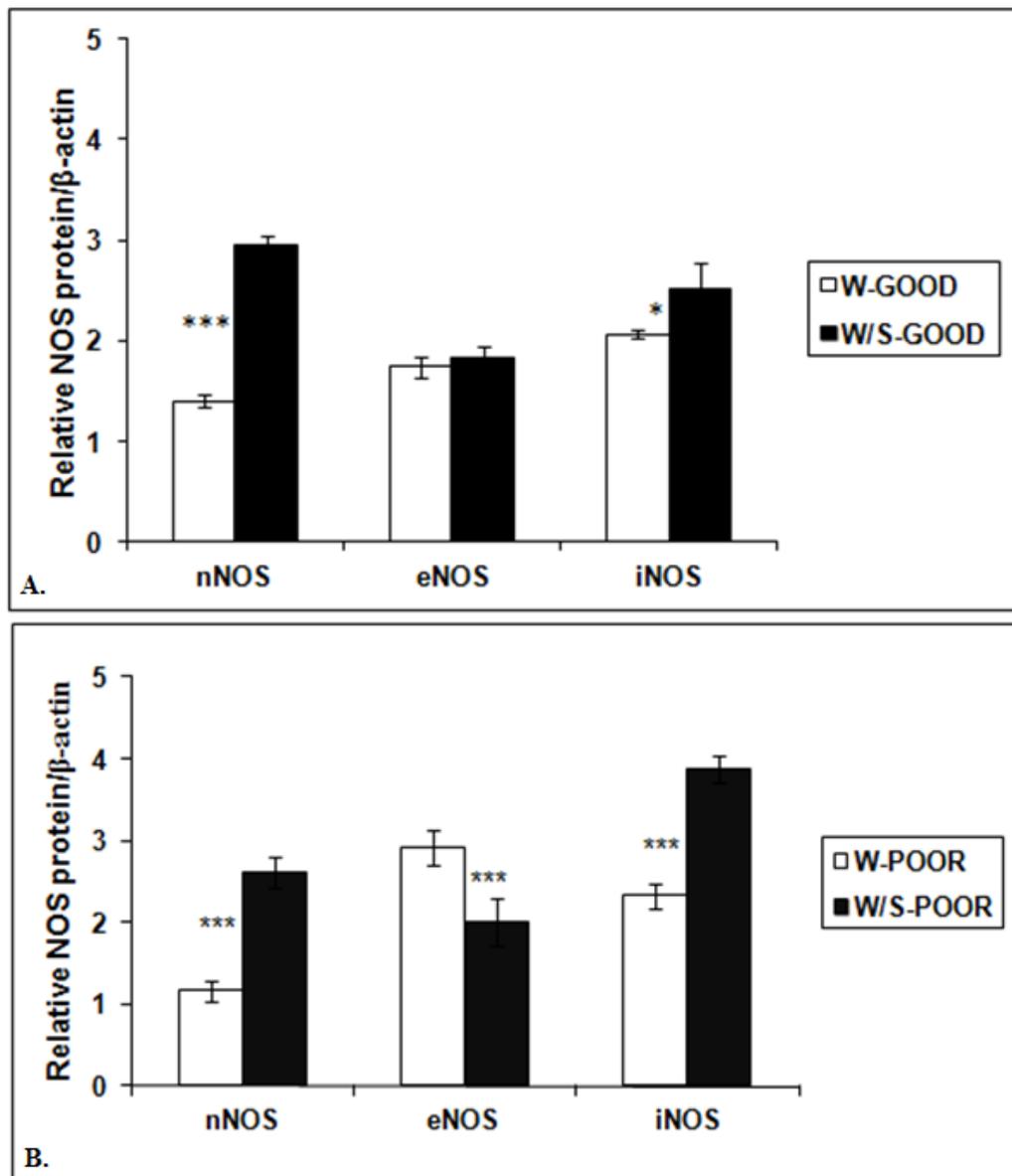
Figure 26 Comparison of the levels of nNOS, eNOS and iNOS in the hippocampus of "good" (A.) and "poor" (B.) learners from W and outcrossed W/S rat strains. Error bars denote ± SEM. Asterisks denote the level of significance: *p<0.05 and ***p<0.001.

The comparison between "good" learners from W and outcrossed W/S groups showed significantly higher level of nNOS ($F_{(1;33)}$=404.295, p≤0.001) and iNOS ($F_{(1;33)}$=6.865, p≤0.013) in outcrossed W/S "good" learners as compared to W "good" learners (Figure 26A.).

The comparison of "poor" learners from W and outcrossed W/S groups showed significantly lower hippocampal nNOS ($F_{(1;25)}$=102.761, p≤0.001) and iNOS ($F_{(1;25)}$=99.430, p≤0.001) expression and significantly higher eNOS level ($F_{(1;25)}$=14.254, p≤0.001 in W "poor" learners as compared to outcrossed W/S "poor" learners (Figure 26B.).

3.2.2 Results of Western blotting assay for CaMKIIα, PKA, their phosphorylated forms and ChAT

The Figure 27 presents a BSA standard calibration curve obtained from BCA protein determination method while Table 9 shows the protein concentrations (mg/ml) measured from the left and right hippocampi of "good" and "poor" learners from Long-Evans rats.
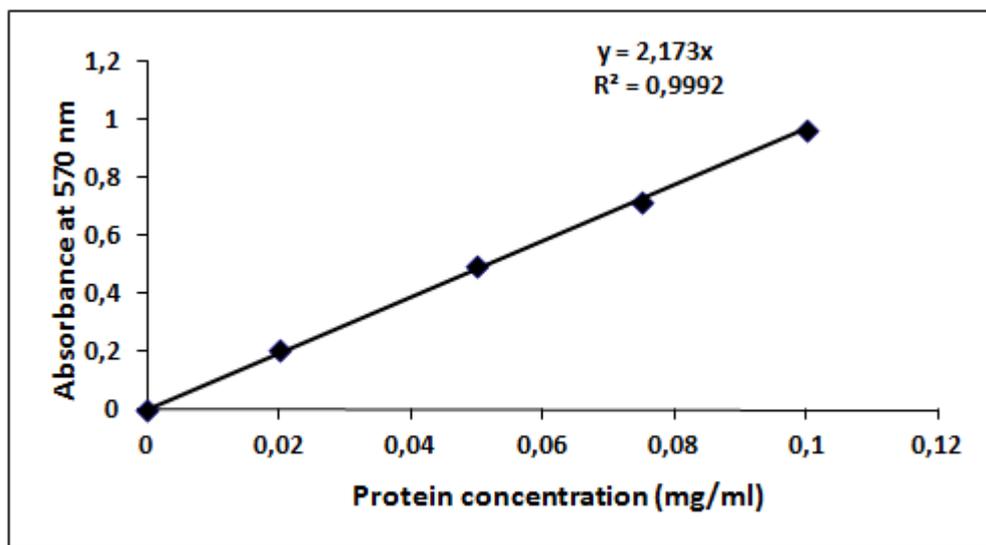
Figure 27 Standard calibration curve for BCA protein determination method

Table 9 Protein concentrations (mg/ml) of left and right hippocampi of "good" and "poor" learners from Long-Evans rats

RAT-ID	PROTEIN CONCENTRATION (mg/ml)		
GOOD		**POOR**	
LE-4-L	11,990	**LE-3-L**	7,900
LE-4-R	12,350	**LE-3-R**	7,190
LE-5-L	7,070	**LE-6-L**	8,540
LE-5-R	8,400	**LE-6-R**	8,870
LE-8-L	8,530	**LE-7-L**	10,490
LE-8-R	8,330	**LE-7-R**	10,440
LE-10-L	9,510	**LE-9-L**	8,200
LE-10-R	9,050	**LE-9-R**	8,940
LE-21-L	11,311	**LE-13-L**	9,789
LE-21-R	12,201	**LE-13-R**	9,416
LE-23-L	9,910	**LE-14-L**	8,974
LE-23-R	9,850	**LE-14-R**	7,227
LE-24-L	9,080	**LE-15-L**	8,034
LE-24-R	9,700	**LE-15-R**	9,573
LE-25-L	8,280	**LE-33-L**	8,264
LE-25-R	8,310	**LE-33-R**	8,066
LE-26-L	8,490	**LE-38-L**	8,136
LE-26-R	8,690	**LE-38-R**	7,946
LE-27-L	8,490	**LE-40-L**	8,723
LE-27-R	9,710	**LE-40-R**	9,840
LE-28-L	9,360		
LE-28-R	8,430		
LE-29-L	8,770		
LE-29-R	8,560		
LE-30-L	9,070		
LE-30-R	10,700		
LE-32-L	9,472		
LE-32-R	9,607		

Western blotting assay was carried out for left and right hippocampi of "good" and "poor" learners from Long-Evans rats, separately. Therefore, for each group, the number of samples was twice the number of subjects: "good'" n=28; "poor" n=20. Since the band quantification results obtained from the left and right hippocampi of "good" and "poor" learners were statistically insignificant (Mann-Whitney test, $p \geq 0.1$), for further analyzes the average values were used. In the Western blot analysis of CaMKIIα and pCaMKIIα, immunoreactive proteins were detected as bands of 50 kDa, PKA and pPKA were detected as 53 kDa, and ChAT was detected as 70 kDa (Figure 28). Relative protein expressions were determined by comparing band intensities with that of β-Actin, which was detected at the position corresponding to a molecular weight of 42 kDa. and was used as the internal sample loading control (Figure 29 and 30).

The Figure 28 illustrates the immunoreactive protein bands of enzymes obtained from the left (L) and the right (R) hippocampi of Long-Evans rats classified as "good" and "poor" learners.
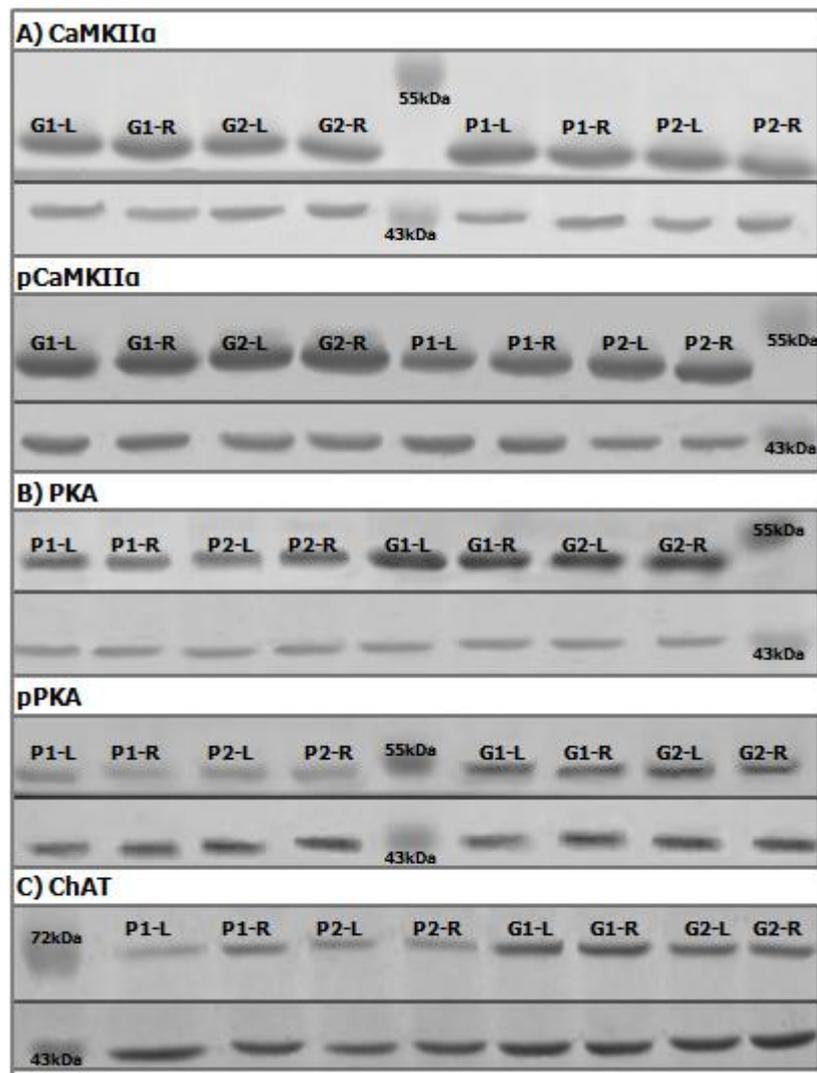
Figure 28 Immunoreactive protein bands representing A) CaMKIIα (50kDa) and pCaMKIIα (50kDa); B) PKA (53 kDa) and pPKA (53kDa); and C) ChAT (70kDa) from left (L) and right (R) hippocampi of "good" (G) and "poor" (P) learners in Long-Evans rats. The β-actin band (42kDa) was used as an internal control for each gel.
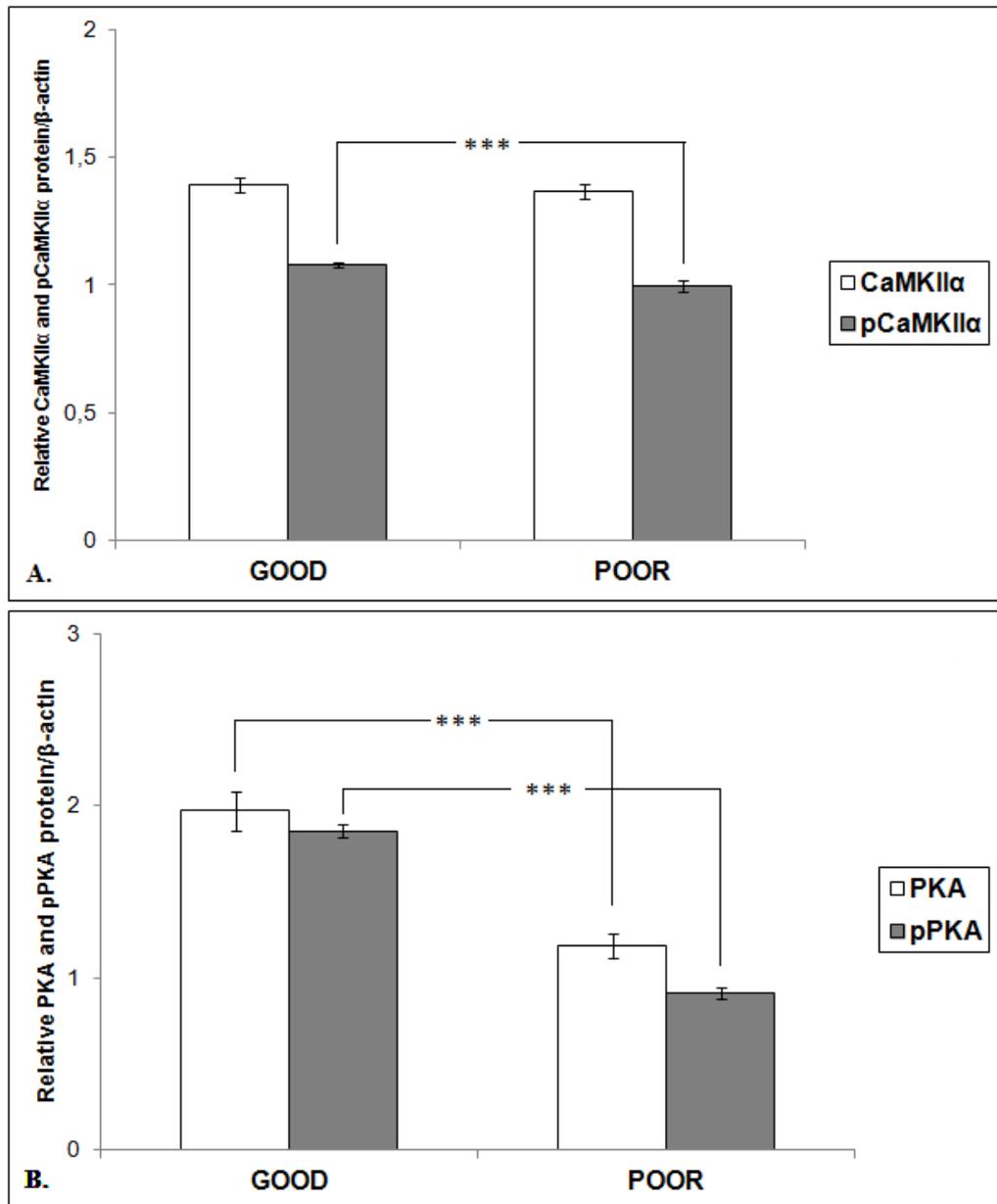
Figure 29 Comparison of the levels of A) CaMKIIα and pCaMKIIα; B) PKA and pPKA, in the hippocampus of "good" and "poor" learners from Long-Evans rats. Band quantifications are expressed as the mean ± SEM of the relative intensity with respect to that of β-actin, used as the internal control. Error bars denote ± SEM. Asterisks denote the level of significance: ***p<0.001.

As seen from Figure 29A. the difference in the total hippocampal levels of CaMKIIα between "good" and "poor" learner groups was insignificant. However, the hippocampal level of the phosphorylated form of CaMKIIα, reflecting activation of this enzyme, was significantly higher in "good" as compared to "poor" learners ($F_{(1;47)}$=16.890, $p \leq 0.001$). On the other hand, both the hippocampal levels of the total PKA and its phosphorylated form (pPKA), the latter indicating enzyme activation, were significantly higher in "good" as compared to "poor" learners ($F_{(1;47)}$=27.342, $p \leq 0.001$ and $F_{(1;47)}$=318.979, $p \leq 0.001$, respectively) (Figure 29B.). In addition, "good" learner group had significantly higher level of the total hippocampal ChAT compared to "poor" learner group ($F_{(1;47)}$=174.957, $p \leq 0.001$) (see Figure 30).
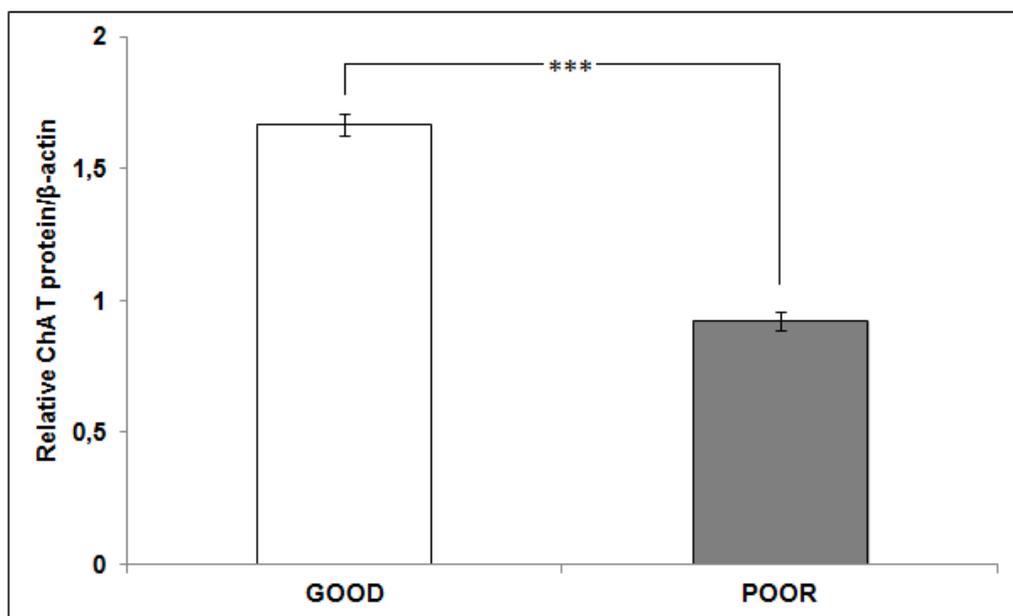
Figure 30 Comparison of the levels of ChAT in the hippocampus of "good" and "poor" learners from Long-Evans rats. Band quantifications are expressed as the mean ± SEM of the relative intensity with respect to that of β-actin, used as the internal control. Error bars denote ± SEM. Asterisks denote the level of significance: ***$p < 0.001$.

Pearson's correlation analysis performed on the data from Long-Evans rats confirmed a significant negative correlation between the total number of choices to criterion and pCaMKIIα (r=-0.523[**], p≤0.001); PKA (r=-0.590[**], p≤0.001); pPKA (r=-0.929[**], p≤0.001); ChAT (r=-0.878[**], p≤0.001) expression and the lack of significant correlation between the total number of choices to criterion and CaMKIIα (r=0.093, p≤0.528) expression.

CHAPTER 4

DISCUSSION

4.1. Correlation between hippocampal levels of neuronal, endothelial and inducible NOS and spatial learning skills in rats

Studies on physiological, molecular, and genetic aspects of synaptic plasticity are undoubtedly very important for better understanding the role of different molecules from the intracellular signal transduction cascades in learning and memory formation. However, studies designed to elucidate (unravel) the potential role of synaptic plasticity in the regulation of whole-animal behavior by learning and memory are of equal importance (Mayford *et al.*, 2012). Therefore, in the present work, an attempt was taken to correlate the hippocampal expression of some major enzymes from the signal transduction pathways and the behavioral output: the animals' performance in a hippocampus-depended spatial learning task. The learning task used to assess animal's cognitive skills was partially baited 12-arm radial maze which allows evaluation of both short-and long-term memory formation.

In the present study, a significant difference in hippocampus-dependent spatial learning was shown between an inbred line of W rats and the outcrossed W/S rats. A genetically more homogenous group of laboratory line of W rats had a higher percent of "poor" learners and manifested a slower rate of acquisition of

a spatial memory task due to the significantly higher number of reference memory errors (RMEs) compared to the genetically less homogenous outcrossed W/S rats. Between-group comparison of learning scores done for "good" and "poor" learners independently, revealed a lack of a significant difference in the rate of learning between W and outcrossed W/S "good" learners. However, the number of RMEs was significantly higher and thus, the rate of reaching performance criterion significantly slower in the inbred line of W "poor" learners compared to outcrossed W/S "poor" learners.

Interestingly, the levels of all three NOS isoforms were significantly higher in the hippocampus of outcrossed W/S rats compared to inbred W rats with the most prominent difference in the constitutive expression of nNOS. These results demonstrate that learning skills and the expression of NOS isoforms vary markedly in different strains. Strain-dependent variation in NOS expression was also previously reported by other authors (Blackshaw *et al.,* 2003; Dere *et al.,* 2001). Assuming that depending on its concentration NO may act as a excitatory or inhibitory modulator of learning-related neuroplasticity (Du and Harvey, 1996; Huang and Lee, 1995), strain-dependent variation in NOS expression may partially account for the inconsistent results regarding the role of different NOS isoforms in learning and memory formation obtained in the experiments wherein NO synthesis was either inhibited or elevated by pharmacological agents. In our experiments too, strain-dependent differences in NOS expression make the interpretation of the obtained results more difficult. Certainly, the lower overall levels of all three NOS isoforms in W rat group cannot be held responsible for the deficient learning in these rats compared to the outcrossed W/S rats. Our attempt to correlate the expression of NOS isoforms with individual learning skills of animals representing random rat populations based on detailed analysis of behavioral and western blot data in the subclasses of "good" and "poor" learners provided an evidence for the existence of a direct relationship between

nNOS level and the rate of place learning in the RAM task. This notion is supported by significantly higher nNOS levels in "good" learners" as compared to "poor" learners observed in both W and outcrossed W/S rat groups and a positive correlation between nNOS expression and individual animal performance assessed for each group independently by Pearson's correlation test. These results are consistent with the previous findings demonstrating deficient learning in rats trained in spatial memory tasks, a water maze and an 8-arm radial maze, after administration of a selective inhibitor of nNOS, 7-nitro indazole (7-NI), which was shown to reduce neuronal NO synthase by 85% without affecting blood pressure (Holscher *et al.,* 1995). Administration of 7-NI was also reported to impair memory retention as assessed by the probe trial of a morris water maze (MWM) task in mice (Mutlu *et al*., 2011). Also, bilateral injections of L-NAME, a preferential nNOS inhibitor, to the hippocampal CA1 region were reported to result in dose-dependent impairment of place learning in the water maze in Wistar rats. This impairment was reversed by co-administration of L-arginine, the NO precursor, suggesting involvement of nNOS in the water maze place learning (Majlessi *et al*., 2008). Morris water maze (MWM) is a typical reference memory task and deficient performance in this learning task observed after NOS inactivation indicates the role of NO in long-term reference memory formation. Conversely, a partially baited 12-arm radial maze task applied in the present study includes components of both, reference and working memory. The data collected in this study shows that although in both W and outcrossed W/S rat groups, "poor" learners emitted significantly more working and reference memory errors, the frequency of RMEs was significantly higher than that of WMEs. Also when comparing W and outcrossed W/S laboratory rat lines, a highly significant difference was noted only in RME counts. Similar results were reported by Zou and colleagues (Zou *et al*., 1998) who observed impaired performance in partially baited 8-arm RAM in rats administered NOS inhibitors L-NAME and 7-NI with greater impact on reference than on working memory errors**.** Taken

together, these observations confirm that local production of NO during synaptic activation in addition to short-lasting functional changes at synapses also contributes to long-term remodeling of neural connections responsible for establishing long-term memories (Inglis *et al.,* 1998).

Despite the higher overall levels of all three NOS isoforms in outcrossed W/S rats compared to W rat line, in W "poor" learners, eNOS level was significantly higher than that in outcrossed W/S "poor" learners. eNOS was also significantly higher in W "poor" learners compared with W "good" learners. Pearson's correlation analysis confirmed an inverse relationship between hippocampal level of eNOS and the learning rate in inbred W rats but not in outcrossed W/S rats. The negative correlation between hippocampal level of the constitutively expressed eNOS and the spatial learning capability observed in the present study in the laboratory strain of W rats is consistent with some previous reports by other authors who demonstrated accelerated place learning, better retention of the acquired place preference, and improved reversal learning in the water maze in the eNOS **-/-**mice (Frisch *et al*., 2000). However, the latter result could also be due to the impact of eNOS knockout during embryonic development on some other aspects of behavior relevant to the learning process such as activity or anxiety. NOS-like activity was found in regions involved in activity and anxiety, such as basal ganglia and amygdala (Dinerman *et al.,* 1994; Dun and Förstermann, 1994; West and Grace, 2000).

In contrast to these results, the *in vitro* experiments on LTP in mice hippocampus demonstrated the most profound attenuation in CA1 LTP in animals with double nNOS-/eNOS- mutation which suggested that both NOS isoforms play a role in long- term synaptic potentiation (a cellular model of memory formation) and may compensate for each others effects in mice with single mutation (Son *et al.,* 1996). However, a direct relationship between LTP and memory was questioned by some authors on the basis of anatomical and

functional data (for review, see Amaral and Witter, 1989; Moser *et al.,* 1993; McEachern and Shaw, 1996).

The positive correlation found in the present study between the spatial learning capacity and the hippocampal nNOS levels and the negative correlation between the learning scores and the levels of hippocampal eNOS in W rats may be related to apparently different neurophysiological roles of these two NOS isoforms. It has been demonstrated that genetic inactivation of eNOS but not of nNOS led to a strong reduction in NMDA-induced GABA release in several brain regions, including the hippocampus, whereas NMDA-induced glutamate release was reduced only by the inactivation of the neuronal isoform (Kano *et al*., 1998). According to these results, elevated eNOS could facilitate the inhibitory while elevated nNOS excitatory neural actions. However, it should be noted that NO is at the same time a potent vasodilator and eNOS located in brain microvessels and astrocytes is implemented in the regulation of cerebral circulation and thus supply of O_2 and glucose to the active brain regions (for review see Faraci, 2006). Impaired eNOS-dependent regulation of cerebral blood flow is considered one of the cardiovascular risk factors contributing to stroke and vascular cognitive impairment during aging. Our results suggest that in some rat strains including W rats, the role of eNOS in neurosignaling may be greater than its role in the regulation of cerebral blood flow.

In both, W and outcrossed W/S rat lines the overall level of hippocampal eNOS was significantly higher than that of n and iNOS (see Figure 24). Interestingly, in both rat groups the level of iNOS expression was relatively high (higher than or equal to that of nNOS, see Figure 24). This result is consistent with the strong induction of hippocampal iNOS in rats trained in place learning task previously reported by other authors (Cavallaro *et al.,* 2002). In addition, in this study, a significant negative correlation was found between rate of learning

and hippocampal iNOS levels in both rat lines. Similarly, in aged Long Evans rats trained in the Morris water maze decreased nNOS and increased iNOS levels were found in the hippocampus and the cortex of cognitively impaired animals (Law *et al.,* 2002). All this suggests that iNOS in addition to its well-known role in brain defense mechanisms and its deleterious function in neurological disorders may also play some role in learning and memory formation.

Taken together, our results showed that there are strain-depended differences in the overall levels of neuronal, endothelial and inducible NOS which may partially explain the discrepancies in the literature regarding the role of different NOS isoforms in NO-dependent neuroplasticity. In both rat lines, rate of learning positively correlated with hippocampal levels of nNOS and negatively correlated with iNOS levels. Hippocampal eNOS levels correlated negatively with animals' performance but only in the W rats and not in the outcrossed W/S rats. These results suggest that not only nNOS and eNOS but also iNOS may impact adult neural plasticity laying the basis of learning process and memory formation in young subjects although mechanistic explanation of iNOS role requires further elucidation. The opposite effects of elevated nNOS and eNOS expression on learning observed in W rat line is in accordance with different localization of these two enzymes in the hippocampal neurons and indicates the different roles of these two NOS isoforms in neuro-signaling. In the present study, the individual and strain-dependent differences in animals' performance in the partially-baited RAM reflected differences in reference rather than working memory. Thus, the obtained results are consistent with a notion that NO synthases responsible for local NO production during synaptic activation in addition to short-lasting functional changes contribute also to the long-term neuroplasticity.

4.2. Correlation between hippocampal levels of CaMKIIα, pCaMKIIα, PKA, pPKA and ChAT and spatial learning skills in rats

Undoubtly, physiological and molecular studies on synaptic plasticity have a major contribution to our understanding the cellular and molecular mechanisms of activity-dependent neural plasticity. However, it is also important to examine how the cellular and/or molecular changes correlate with behavioral output. Therefore, in the present study, we focused on the correlation between expression levels and activity of some of the candidate "memory molecules" and the animals' learning skills assessed by their performance in a hippocampus-dependent learning task.

It has been well documented that phosphorylation/dephosphorylation reactions are critical for encoding, retaining and modifying information in neural circuits (Cammarota *et al*., 2002; Chen *et al*., 2005; Colbran, 2004; Hu *et al*., 2002; Sanhueza *et al*., 2007; Micheau and Riedel, 1999). This is why in the present study, focus was put on two major kinases: CaMKII and PKA. Additionally, the hippocampal expression of ChAT, a major modulatory neurotransmitter in the hippocampus, was also evaluated.

Experiments carried out on Long-Evans rats did not reveal a significant difference in the hippocampal levels of the total CaMKIIα between "good" and "poor" learners, however, the level of the pCaMKIIα, an indicator of CaMKII activation, was significantly higher in "good" learners as compared to "poor" learners. Conversely to that, hippocampal levels of both PKA and pPKA, were significantly higher in "good" than in "poor" learners. Similarly, the hippocampal level of ChAT was significantly higher in "good" learners.

The increased activation of hippocampal CaMKII in animals showing significantly better performance in a spatial learning task found in the present study, confirms the involvement of CaMKII in behavioral memory. An important role of a CaMKII, the main protein of the postsynaptic density and a sensor of the Ca(2+) signals generated by activation of NMDA receptors, in learning and memory formation was shown in several previous studies (i.e. Cammarota *et al*., 2002, Mayford *et al.,* 1996; Micheau and Riedel, 1999). A deficiency in expressing LTP paralleled by a severe deficiency in both spatial learning and memory retention in the water maze (Silva *et al.*, 1992 a,b) and in the Barnes maze (Bach *et al.,* 1995) were observed in alpha-calcium-calmodulin kinase II mutant mice. Also in pharmacological studies carried out on rats in the Morris water maze, pretraining injections of a specific inhibitor of CAMK II (KN62) retarded task acquisition in comparison to the vehicle-injected controls, while no impairment was observed in the nonspatial task (Tan and Liang, 1996). An increase in the activation of hippocampal CaMKII within first 30 min after one-trial passive avoidance training was also reported by some authors (Cammarota *et al*., 1998). However, there are also some discrepant results. For instance, Mizuno and co-workers (2002) did not record an increase in the hippocampal pCaMKII levels in rats trained in a partially baited 8-arm RAM as compared to control animals which were also exposed to the maze but not trained.

Our results do not allow for the evaluation of a potential, learning-dependent increase in the hippocampal pCaMKII activity since all the animals were trained in a learning task. The results of the present study, however, significantly correlate animals' learning capability with the levels of CaMKII activation in the hippocampus. Similar findings to ours were presented by Solomonia *et al.* (2005) in a study carried out on chickens. In the latter study, the level of pCaMKII in ventral hyperstriatum and mid-medial mesopallium regions (regions important for visual imprinting) positively correlated with

chicks learning a visual imprinting task. Present results also show that the level of protein activation not always is proportional to the level of protein expression indicating that the level of the protein enzymatic activity is more reliable molecular marker of memory than protein expression *per se*.

Many of the previous studies indicated special importance of CaMKII for early phases of both cellular (LTP) and behavioral memory formation. Chen *et al*. (2001) reported that administration of CaMKII inhibitors have no effect on earlier established LTP which indicates that CaMKII is important LTP induction but not so much for its maintenance. It was also shown that intrahippocampal infusion of a specific inhibitor of CAMK II (KN62) prevented acquisition of one-trial passive avoidance task in rats when given immediately, caused partial memory impairment when given 30 min after training, and showed no effect 120 min or more elapsed. Parallel to this, an increase (25-78%) in CAMKII activity was recorded in the hippocampus of rats killed immediately, but not 120 min, after training (Cammarota *et al*., 1998; Wolfman *et al*., 1994). These results suggest that training in a learning task leads to a fast activation of CaMKII, which, if persistent for 30 min or more, enables further long-term processes (Izquierdo *et al.,* 1995). Persistent activation of CaMKII after synaptic activation during LTP induction or after training in a learning task just like in the present study, is considered by some authors a gateway to long-term memory formation and fits to a postulated CaMKII role of a molecular switch between short- and long-term memories (Lisman *et al.,* 2002).

Positive correlation demonstrated in this study between the animals' cognitive capacity and hippocampal CaMKII activation suggests that CaMKII activity in the hippocampus can be used as index of episodic memory in young healthy subjects.

In the present study, in contrast to CaMKIIα, the hippocampal levels of both, constitutively expressed PKA and its phosphorylated form were significantly higher in "good" as compared to "poor" learners trained in a spatial learning task. This result is consistent with previous findings by other authors showing importance of PKA activity in LTP (Abel *et al.,* 1997**,** Bernabeu *et al*., 1997, for review see Nguyen and Woo, 2003) as well as behavioral learning and memory in both invertebrates (Kandel, 2001; McGuire *et al.,* 2005) and vertebrates (for review see Abel and Nguyen, 2008). Initial increase in the activity of hippocampal PKA was observed in the course of spatial learning in the partially baited 8-arm radial maze at fourth day of training (Mizuno *et al*. 2002) and in the holeboard food-search task already at first day of training (Vazguez *et al.*, 2000).

Positive correlation between animals' performance and the hippocampal levels of both, basal and phosphorylated PKA may be accounted for by a well documented involvement of PKA in both, short-term memory requiring functional changes in protein structure including PKA autophosphorylation, as well as long-term memory requiring *de novo* protein synthesis including upregulation of the enzyme protein itself. On the other hand, the positive correlation between the hippocampal level of phosphorylated but not basal CaMKII can be related to the predominating role of CaMKII in early, short-term, activity-dependent synaptic changes underlying short-term memory with little impact on late, long-term synaptic modifications underlying long-term memory. In the present study, basal hippocampal levels of all examined enzymes as well as the levels of phosphorylated forms of CaMKII and PKA were estimated in well trained animals, all having reached an arbitrary performance criterion. Their long-term (reference) memory of the location of food wells was well established. After reaching criterion performance, animals were sacrificed 15 min after a re-training trial which, however, required operation of short-term, working memory. The re-training trial might have

induced the phosphorylation of hippocampal CaMKII with basal level of this kinase remaining unchanged and equal in both learning groups.

In the current study, in "good" learners, the higher hippocampal levels of PKA were accompanied by relatively high levels of hippocampal ChAT. A plentitude of lesion, electrophysiological, pharmacological, biochemical, and behavioral studies demonstrated importance of the cholinergic septo-hippocampal system for functional integrity of the hippocampus and hippocampus-dependent forms of contextual and episodic memory (for review see Deiana *et al.,* 2011; Robinson *et al.,* 2011) although disparate results were also presented (for review see Micheau and Marighetto, 2011; Parent and Baxter, 2004). ACh through its basal forebrain systems: nucleus basalis-prefrontal cortex, and septum-hippocampus, was shown to impact both short-term, working memory and long-term, reference memory, with prefrontal cortex more implemented in the short- while hippocampus in the long-term neural plasticity. Recently, however, new neuropsychological data more and more support the view that the medial temporal lobe structures including hippocampus are also critical to short-term memory in associative learning (Kumaran, 2008). Since in the learning task applied in the present study, both types of memory (working and reference) were required for the optimal animal performance, a positive correlation between hippocampal ChAT levels and animals' learning scores was expected.

The lower basal level of ChAT found in the present study in "poor" learners as compared to "good" learners is consistent with low cholinergic markers and low ChAT activity in the forebrain of aged rats manifesting memory deficits (Dunbar *et al.,* 1993; Luine and Hearns, 1990; Markowska *et al.,* 1998). In the study conducted by Wang *et al*. (2009), a strong relationship was found between the poor water maze performance in aging accelerated P8 mice and the low levels of ChAT expression in the hippocampus and cerebral cortex of

these animals. A positive correlation found in the present study between hippocampal ChAT expression and animals' performance in the spatial memory task is also consistent with the results of our previous study, wherein enhancement of hippocampal cholinergic functions by chronic intraventricular NGF administration produced significant memory improvement in young but memory-deficient rats (Jakubowska-Dogru *et al*., 2005).

An extensive crosstalk among different signal transduction pathways has been reported in neurons (for review see Shobe, 2002). Among other effects, it has been shown in *in vivo* experiments that in the septo-hippocampal system, ChAT expression is regulated by PKAII. Sharifzadeh *et al*. (2005) showed that bilateral injection of PKAII inhibitor, H-89, into hippocampus of aged rats causes a significant decrease in cholinergic markers along the septo-hippocampal pathway. In the light of these findings, parallel trends observed in the present study, in the expression of PKA and ChAT in the rat hippocampus gain additional meaning.

CHAPTER 5

CONCLUSION

To our knowledge, this is the first study investigating the correlation between hippocampal activity and/or levels of selected enzymes known as "memory molecules" and individual variation in learning skills in random populations of young rats belonging to different genetic lines: inbred Wistar (W), the outcrossed Wistar/Spraque Dawley (W/S), and pigmented Long-Evans rats.

In the present study, hippocampal expression and activation of two major protein kinases, CaMKII and PKA, as well as the expression of three NOS isoforms and ChAT, the enzymes responsible for the synthesis of two important modulatory neurotransmitters: NO and ACh, were examined in young, healthy rats classified as "good" and "poor" learners on the basis of their performance in the hippocampus-dependent learning task: partially baited 12-arm radial maze requiring both short- and long-term memory. Taken together, presented results indicated a great individual and strain-dependent variation in the learning skills. The individual and strain-dependent differences in animals' performance in the partially-baited radial arm maze reflected between-subject differences in reference rather than working memory. Strain-dependent differences were also observed in the overall levels of NOS isoforms. A positive correlation was revealed between animals' learning capability and the hippocampal levels of nNOS, constitutively expressed PKA and its phosphorylated form pPKA, phophorylated form of CaMKII but not the basal CaMKII, and the hippocampal level of ChAT. A negative correlation was

found between animals' performance in the learning task and hippocampal level of iNOS. Similarly, a negative correlation was noted between animals' performance and eNOS levels but only in the inbred laboratory line of W rats but not in outcrossed W/S rats. Results of NOS studies suggest that all 3 NOS isoforms are implemented in the learning process playing, however, different roles in neural signaling. The results of studies on kinases' expression and activity point towards CaMKII involvement primarily in the short-term while PKA in both short-and long-term synaptic plasticity.

This study provided an insight into individual variation in the learning skills and memory within a random young animal populations pointing towards a potential molecular basis of this variation. The present study, as most of other studies on the molecular basis of learning and memory was correlative and thus, the obtained results do not permit to establish a direct causal relation between molecular estimates and a very complex processes which is behavioral learning and memory. However, the results of this and similar studies may be indicative of molecular targets when designing memory-enhancing treatments against memory deficits which are not related to pathological processes.

Future Perspective: In the present study, the levels of hippocampal enzymes were estimated shortly after memory re-consolidation during a retraining trial. It will be of future interest to examine the hippocampal activity and/or expression of selected "memory molecules" in young "good" and "poor" learners during distinct phases of memory processing including early phase of task acquisition, memory consolidation and memory retrieval after delay.

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MEETING PRESENTATIONS

Saraç G.Ç., Adalı O. and Doğru J.E. 2012. Investigation of the possible role of CaMKIIα, PKA and their phosphorylated forms in spatial learning capacity of Long-Evans rats. 8th FENS Forum of European Neuroscience, Barcelona, Spain. July 14-18. (Poster presentation accepted).

Saraç G.Ç., Keçecioğlu E., Adalı O. and Doğru J.E. 2011. Study of the relation between the animals' learning capacity and the hippocampal levels of CaMKIIα and pCaMKIIα in random population of Long Evans rats. 10. Ulusal Sinirbilimleri Kongresi, İstanbul, Turkey. (Poster Presentation).

Karakurt S., Sever M., **Saraç G.Ç**., Doğru J.E., Adalı O. 2010. In vitro effects of plant phenolic compounds on liver glutathione S-transferase activity. Turk J Bioch. 35 (1), 78, XXII. National Biochemical meeting, Eskisehir, Turkey.

Saraç G.Ç., Karakurt S., Adalı O. and Doğru J.E. 2010. Study of the relation between the spatial learning capacity and the hippocampal expression of

different NOS isoforms in the random populations of young-adult laboratory rats. 9. Ulusal Sinirbilimleri Kongresi, Turkish Journal of Neurology, 16(1):165, 13-17 April, İstanbul, Turkey. (Poster Presentation).

Saraç G.Ç., Karakurt S., Adalı O. and Doğru J.E. 2010. Investigation of the potential role of nitric oxide synthase (NOS) isoforms in spatial learning in rat hippocampus. 7th FENS Forum of European Neuroscience, Amsterdam, Holland. July 3-7. (Poster Presentation).

Saraç G.Ç., Karakurt S., Koçak Ö., Adalı O. and Doğru J.E. 2009. Correlation between spatial learning and the expression of different Nitric Oxide Synthase (NOS) isoforms in rat hippocampus. Society for Neuroscience (SfN) Neuroscience 2009, Chicago IL, USA. October 17-21. (Poster Presentation).

SCIENTIFIC PUBLICATIONS

Saraç G.Ç., Karakurt S., Adalı O. and Doğru J.E. 2012. Correlation between hippocampal levels of neural, epithelial and inducible NOS and spatial learning skills in rats. Manuscript submitted to journal of "Behavioral Brain Research".

SCHOOL ATTENDANCE

First Kemali-IBRO Mediterranean School of Neuroscience, "The synapse from beach to bedside: Synaptic transmission, plasticity, synaptopathies", Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy. September 21-30, 2009.

SCHOLARSHIP

Research scholor for one year in 2010 at the University of California, Davis (UCDavis) Department of Pharmacology, Davis, California. Supported by Middle East Technical University, Ankara Turkey.

Full grant to attendance "First Kemali-IBRO Mediterranean School of Neuroscience, "The synapse from beach to bedside: Synaptic transmission, plasticity, synaptopathies", Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy. September 21-30, 2009.

CERTIFICATE

Certificate of Animal Use, University of California, Davis (UCDavis) Department of Pharmacology, Davis, California, 2010.

Certificate of Animal Use in Experimental Research, Gulhane Military Medical Faculty, Ankara, Turkey, November, 2008.