HIPPOCAMPAL EXPRESSION OF SELECTED CYTOCHROME P450 ISOFORMS DURING SPATIAL LEARNING IN RATS

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

BY

SENA GJOTA ERGIN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCES
IN
BIOCHEMISTRY

JANUARY 2016

Approval of the thesis:

HIPPOCAMPAL EXPRESSION OF SELECTED CYTOCHROME P450 ISOFORMS DURING SPATIAL LEARNING IN RATS

submitted by **SENA GJOTA ERGIN** in partial fulfillment of the requirements for the degree of **Master of Science in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan Adalı _____
Head of Department, **Biochemistry**

Prof. Dr. Ewa Jakubowska Doğru _____
Supervisor, **Biology Dept., METU**

Dr. Çiğdem Gökçek Saraç _____
Co-Supervisor, **Environmental En. Dept., Akdeniz University**

Examining Committee Members:

Prof. Dr. Orhan Adalı _____
Biology Dept., METU

Prof. Dr. Ewa Jakubowska Doğru _____
Biology Dept., METU

Prof. Dr. Tülin Güray _____
Biology Dept., METU

Assoc. Prof. Dr. Nursen Çoruh _____
Chemistry Dept., METU

Assoc. Prof. Dr. Michelle Adams _____
Psychology Dept., Bilkent University **Date:**
04.01.2016

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: SENA GJOTA ERGIN

Signature:

ABSTRACT

HIPPOCAMPAL EXPRESSION OF SELECTED CYTOCHROME P450 ISOFORMS DURING SPATIAL LEARNING IN RATS

Gjota Ergin, Sena

M.S., Department of Biochemistry

Supervisor: Prof. Dr. Ewa-Jakubowska Doğru

Co-Supervisor: Dr. Çiğdem Gökçek Saraç

January 2016, 93 pages

Despite very extensive studies on the molecular mechanisms of memory formation, it is still little known about the molecular correlates of individual variation in the learning skills within a random population of young normal subjects. The aim of the present study was to investigate the expression of different Cytochrome P450 (CYP) isoforms in the rat hippocampus at different stages of place learning in young Sprague-Dawley rats classified as "good" or "poor" learners. Up-to-date, it is little known about CYP expression and functions in the brain. On the other hand, these enzymes are known to be related to the metabolism of steroids, fatty acids (i.e. arachidonic acid) as well as retinoic acid, the substances important for neural functions. To address this issue, rats were trained in the partially baited 12-arm radial maze (RAM) allowing for the evaluation of both short- and long-term spatial memory. The enzyme levels were determined by Western Blot technique at early,

intermediary and late stage of the task acquisition (day 4, day 8 and after reaching a performance criterion of 83% correct responses).

Obtained results demonstrated hippocampal expression of CYP2E1, CYP2D1 and CYP7A1 but only CYP2E1 levels negatively correlated with the frequency of both, working and reference memory errors. Interestingly, CYP2E1 levels progressively increased in the course of learning. The CYP7B1- and CYP2B1/2 - immunoreactivity was not found in the examined tissue samples.

All together, these findings indicate the differential expression and differential effects of the members of CYP family in the rat hippocampus.

Keywords: Hippocampus, Spatial memory, Partially-baited 12-arm radial maze, Western Blot, Cytochrome P450, Rats

ÖZ

MEKANSAL ÖĞRENME ESNASINDA SIÇAN HİPOKAMPÜSÜNDEKİ BAZI SİTOKROM P450 İZOFORMLARININ EKSPRESYONUNUN ARAŞTIRILMASI

Gjota Ergin, Sena

Yüksek Lisans, Biyokimya Bölümü

Tez Yöneticisi: Prof. Dr. Ewa-Jakubowska Doğru

Ortak Tez Yöneticisi: Dr. Çiğdem Gökçek Saraç

Ocak 2016, 93 sayfa

Belleğin moleküler mekanizmalarını araştıran çok sayıda çalışma yapılmasına rağmen, rastgele seçilmiş genç hayvan populasyonu içinde bireysel öğrenme kapasitesindeki varyasyonun biyokimyasal temelleri hakkında oldukça az bilgi mevcuttur. Bu çalışmanın amacı, "iyi" ve "kötü" öğrenen olarak sınıflandırılan genç Sprague-Dawley sıçanların hipokampüslerinde farklı Sitokrom P450 enzimlerinin ekspresyonunun incelenmesidir. Günümüzde, Sitokrom P450'lerin beyindeki ekspresyonu hakkında çok az bilgi mevcuttur. Diğer yandan, bu enzimlerin nöral fonksiyonlar için önemli olduğu bilinen steroid, araşidonik asit ve retinoik asit metabolizmaları ile ilişkili olduğu bilinmektedir. Bu eksiği gidermek için, genç sıçanlar, hem kısa hem de uzun süreli belleği değerlendirme fırsatı veren, kolların rasgele seçilmiş yarısında pekiştirici bulundurulan (partially baited) 12 kollu radyal

labirentte eğitilmiştir. Enzimlerin ekspresyon düzeyleri Western-blot tekniği kullanılarak eğitimin erken, orta ve ileri safhalarında (4. gün, 8.gün ve %83 doğru seçim performans kriterine ulaştıktan sonra) ölçülmüştür.

Elde edilen sonuçlar CYP2E1, CYP2D1 ve CYP7A1'in hipokampüsteki ekspresyonunu gösterse bile, sadece CYP2E1 ekspresyon seviyeleri kısa süreli bellek ve uzun süreli bellek hataları ile negatif korelasyon göstermiştir. Aynı zamanda, CYP2E1 ekspresyon düzeyi öğrenmeyle birlikte gittikçe artmıştır. Diğer taraftan, CYP7B1 ve CYP2B1/2 immunoreaktivitesi test edilen doku örneklerinde gözlenememiştir.

Tüm bu bulgular, Sitokrom P450 enzim aile üyelerinin sıçan hipokampüsündeki diferansiyel ekspresyon ve diferansiyel etkilerini işaret etmektedir.

Anahtar kelimeler: Hipokampus, Mekansal bellek, Kısmi pekiştirilmiş 12-kollu radyal labirent, Western Blot, Sitokrom P450, Sıçan

To My Family,

For your endless support and love

ACKNOWLEDGEMENTS

I am deeply thankful to my supervisor Prof. Dr. Ewa Jakubowska-Doğru for her valuable guidance, patience, criticism, advice and supervision throughout this study. She had always a positive and encouraging attitude in every aspect.

I am also deeply grateful to my co-supervisor Dr. Çiğdem Gökçek Saraç for her guidance, precise commanding and advices throughout this study.

I am thankful to my examining committee: Prof. Dr. Orhan Adalı, Prof. Dr. Tülin Güray, Assoc. Prof. Dr. Nursen Çoruh and Assoc. Prof. Dr. Michelle Adams for their critics and suggestions.

I would like to thank Doç. Dr. Şevki Arslan, Dr. Tuba Çulcu and Dr. Serdar Karakurt for their technical support.

I am grateful to my lab mates Merve Akkulak, Özlem Durukan, Emre Evin, Meliha Ülger, Ezgi Balkan for their beautiful friendship, help, support and lovely atmosphere in the lab.

For the rest of my friends and all Department of Biological Sciences of METU, thanks deeply.

This thesis is dedicated to my family. It is not possible to find proper words expressing their love, trust, encouragement and endless support that brought me to these days.

I would like to thank The Scientific and Technological Research Council of Turkey (TÜBİTAK) firstly for financial support as scholarship and research support (114S807).

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

LIST OF ABBREVIATIONS

β	Beta
18-HETE	Ω-2 hydroxylated metabolite
19-HETE	19-hydroxyeicosatetraenoic acid
4DG	4 Days Group
8DG	8 Days Group
AC	Adenylate Cyclase
ACG	Acquisition Criterion Group
AhR	Aryl Hydrocarbon Receptor
APS	Ammonium per sulphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
C/EBP	CAAT Box Enhancer Binding Protein
CA	Cornu Ammonis
CA1	Cornus ammoni region 1
CA2	Cornus ammoni region 2
CA3	Cornus ammoni region 3
cAMP	Cyclic Adenosine Monophosphate
CAR	Constitutively Active Receptor
CBP	CREB Binding Protein
cDNA	Complementary DNA
CRE	cAMP Recognition Element
CREB	cAMP Response Element Binding Protein
CYP	Cytochrome P450

DA	Dopamine
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetra acetic acid
EET	epoxyeicosatrienoic acid
EGTA	Ethylene glycol tetra acetic acid
ER	Endoplasmic reticulum
ERB	Electronic Running Buffer
GABA	Gamma Aminobutiric Acid
HEPES	N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid
kDa	Kilodalton
LTD	Long term depression
LTM	Long Term Memory
LTP	Long term potentiation
MAPK	Mitogen Activated Protein Kinase
MFP	Mossy Fiber Pathway
mRNA	Messenger ribonucleic acid
MS-DB	Medial Septum-Diagonal Band
MWM	Morris Water Maze
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
NBT	Nitro blue tetrazolium
NNK	nitrosoamine 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone
PKA	Protein Kinase A
PP	Perforant Pathway

PPAR	Peroxisome Proliferator Activated Receptor
PVDF	Polyvinyl Difluoride
PXR	Pregnone X Receptor
RAM	Radial Arm Maze
RME	Reference Memory Error
SC/AC	Schaffer Collateral/Associational Commisural
SCP	Scaffer Collateral Pathway
SDB	Sample Dilution Buffer
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIRT1	Sirtuin 1
STM	Short term Memory
TBST	Tris-Buffered Saline and Tween 20
TEMED	N-N-N’-N’-tetramethylenediamine
WME	Working Memory Error

CHAPTER 1

INTRODUCTION

1.1 General information about learning and memory

1.1.1 Learning and memory definition

Answering question marks related to brain are one of the desirous goals of modern neuroscience nowadays. Thinking, perceiving, feeling, curiosity, behavior, learning and memory are phenomena occurring in the brain (Okano *et al*., 2000; Paratore *et al*., 2006). More precisely, if learning and memory is focused, it can be described as learning being a mechanism of attaining new information about the world and memory being a mechanism which retain these acquired information (Lynch, 2004). Having a very crucial role in our life's, clarifying learning process and analyzing memory forming, storing and retrieving are challenging and crowd-pulling points.

1.1.2 Learning and memory classification

On the time basis, memory is classified as either long-term memory or short-term memory. To begin with long term memory, it is divided into declarative (explicit) memory and nondeclarative (implicit) memory (Bailey *et al.* 1996; Izquerdo *et al.* 1999). Declarative memory refers to memories that are consciously recalled such as events and facts. Declarative memory is further divided into episodic memory and semantic memory. Episodic memory implies autobiographical memory of event whereas semantic memory implies general knowledge and facts. On the other hand, in contrast to declarative memory, nondeclarative memory refers to motor or sensory abilities. Categories of nondeclarative memory are procedural (related to skills and habits), priming (increase of perceptual learning skills), simple classical conditioning (association between two stimuli) and non-associative learning (habituation, dishabituation and sensitization). Therefore, examples clarifying these two categories of memory are as follows: declarative memory is responsible for reminding dates, names, faces or places, as well as autobiographic episodes, whereas nondeclarative memory stands behind riding a bicycle, butterfly swimming, declaiming the multiplication table or spelling a word (Paul *et al.* 2009; Quillfeldt *et al.* 2006; Squire *et al.* 1993). A summary of this classification is given in Figure 1.

On the other hand, short term memory also called as working memory basically stores information for a short period of time like seconds to minutes, sometimes for several hours in order to compare it with previous records, and, basing upon this, decide which behavior to express (Izquerdo *et al.* 1999; Quillfeldt *et al.* 2006).

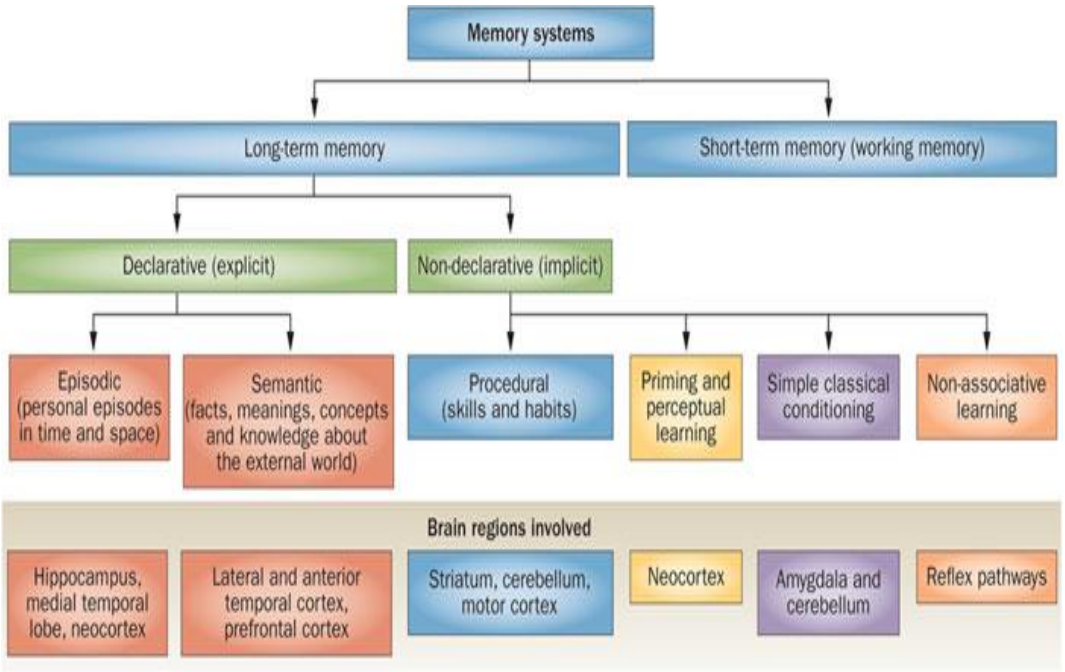


Figure 1.1: Memory system classification (Bartsch and Butler, 2013)

1.1.3 Spatial learning

Episodic declarative memory answers the questions what, where, and when. Apparently, episodic memory is different in humans and animals since the animals do not have semantic memories and spontaneous conscious recollection. Therefore, in animals, this memory is called episodic-like memory.

Spatial memory, being a form of episodic memory, gives the answer to the question "where". In the wild, spatial learning and memory help animals in finding locations providing food and safety, and therefore are crucial for survival. Tasks used to test spatial memory may provide information about its long-term and short-term aspects, namely, spatial working and spatial reference memory.

Spatial working memory may be described as a short-term storage of a spatial information in a limited amount. This information is available for a rapid access (Awh and Jonides, 2001). Despite spatial working memory, spatial reference memory implies a spatial information that is being used for many times over and over again and is acquired as a result of repeated training (Olton *et al.* 1979). These two forms can be tested by the tasks used in laboratories with animal models.

1.1.4 Prevalent experimental paradigms used in studies related to spatial learning and memory

Labyrinths, more commonly named as mazes, are experimental setups most frequently used to evaluate spatial learning and memory in rodents. During experiments, positive or negative reinforcement is used. Examples of positive reinforcements would be food, water, shelter or giving an opportunity to search a new object. Whereas examples of negative reinforcements would be electric shock, intense light, loud noise or water immersion. The maze type used depends on the aim of the test. Another crucial point is the subject. Not only the setup but also the subject should be chosen carefully. Some of the features to be evaluated and decided carefully are strain, gender, and age of the subject (Jakubovska-Doğru *et al.* 2003). Two most commonly used mazes are: radial arm maze (RAM) (Olton and Samuelson, 1976) and Morris water maze (MWM) (1979). Below (Figure 2) some maze types are presented.

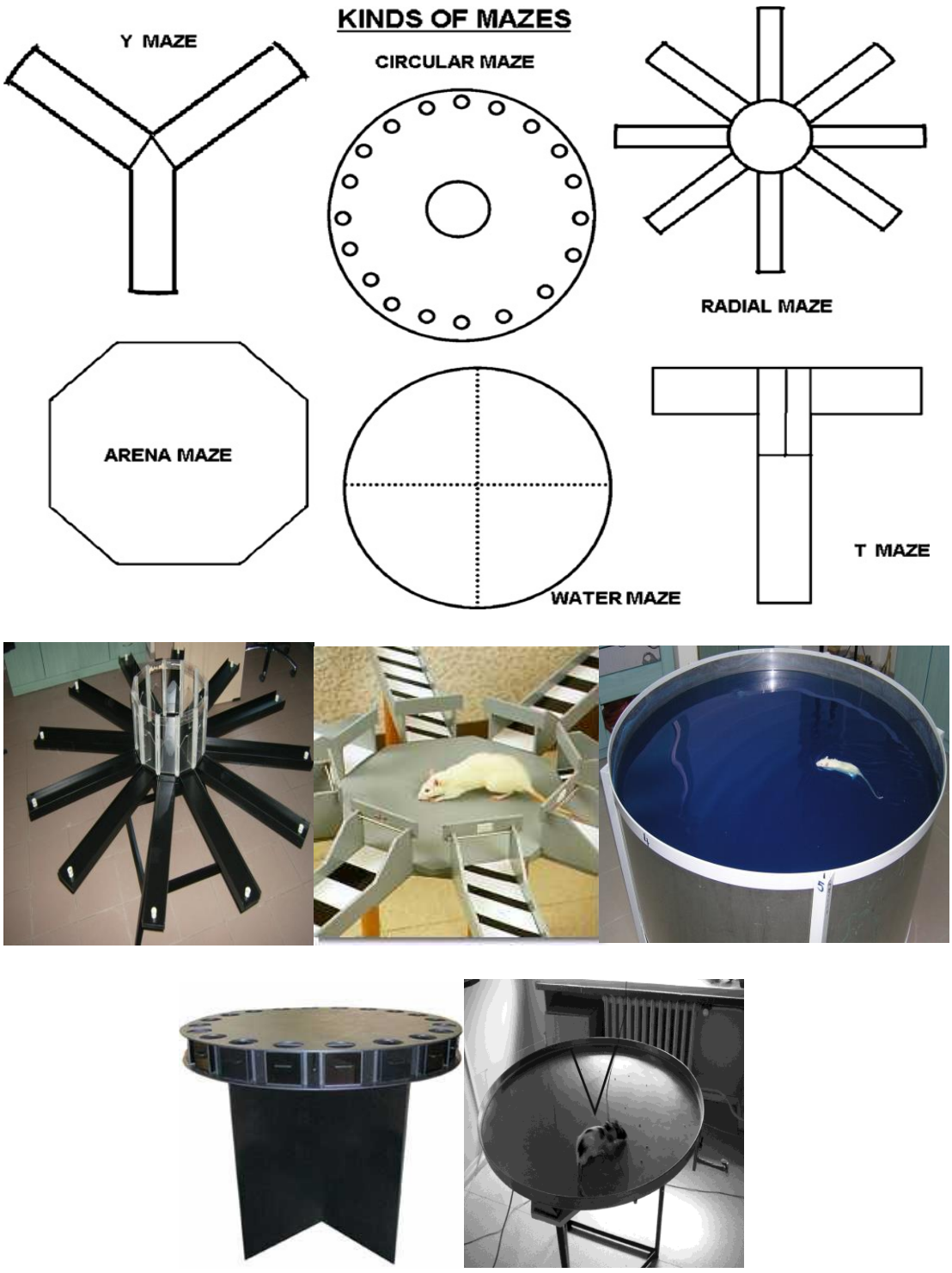


Figure 1.2: Certain types of mazes used in evaluating spatial memory (Hodges, 1996)

1.1.4.1 Radial arm maze

In 1976, Olton and Samuelson built the first radial-arm maze in order to evaluate spatial memory in small rodents such as rats and mice. A maze may have eight or more arms with the bait (food pellets) placed in the food wells at the ends of the arms. This is a classical design of the Olton's radial maze serving tests on short-term (working) spatial memory. In a single experimental session, re-entries to already visited arms constitute the working memory errors. The newer version of the RAM is a partially baited version where only half semi-randomly selected arms are baited (Packard, 1989, Whishaw and Brooks, 1999; Jakubowska-Doğru *et al.* 2003). Such maze may be used for testing both, working and reference spatial memory with entries to never baited arms counted as reference memory errors. The food deprivation applied to the animals shortly before and during the experiments which increases their motivation to search for food pellets when in the maze. The optimum, energy-saving strategy in the partially baited RAM is that subject retrieves all food pellets by visiting only once each baited arm, and omitting non-baited arms. Depending on the experimental design, the arm choice may be based on different strategies: (1) so called guidance strategy when baited arms are marked by distinct intramaze cues, (2) building a cognitive map of the environment according to the relative locations of distant, usually visual-spatial cues referred to as allothetic or allocentric cues, or (3) spatial navigation guided by so called idiothetic or egocentric cues generated by self-movement (proprioceptive and vestibular cues used i.e. in the darkness).

Each of these strategies depends on different memory system in the brain: the guidance strategy on dorsal striatum and amygdala, allothetic strategy on medial temporal lobe including hippocampus, and idiothetic strategy on cerebellum.

1.1.5 Hippocampus and Its Circuitry

Name “hippocampus” comes from the Greek hippos “horse” and kampos “sea monster” meaning seahorse (Figure 1.3).

Figure 1.3: Hippocampus removed from brain compared to seahorse (Neuroscientifically Challenged, n.d)

Hippocampus is a structure located in medial portion of the temporal lobe and belongs to allocortex phylogenetically older than neocortex. It is characterized by having only three up to four layers, in contrast to the six layers of the neocortex. It is a part of the limbic system originally referred to as an emotional brain. In humans hippocampus has a size about of a child’s thumb (Squire and Kandel, 1999). The hippocampus is formed by two interlocking sheets of cortex forming its two major regions: dentate gyrus (DG) and Cornu Ammonis (CA). In cross-section it has a strict laminar organization with principal cells, granular in dentate gyrus (DG) and

pyramidal in Cornu Ammonis (CA). CA is divided into subregions known as CA1, CA2, and CA3. Neural connections also have laminar distribution and are as a rule uni-directional. It receives input nearly from all neocortical regions also from many subcortical regions directly or indirectly, via the entorhinal cortex (Kubik *et al.* 2007).

DG is composed of three layers. First one is the granule layer including densely packed cell bodies of the granule cells. Second one contains apical dendrites of the granule cells and their afferents meshed in order to form molecular layer. The last third layer is named the polymorph layer and contains initial segments of the granule-cell axons as they pool together in order to form the mossy fiber bundle largely targeting pyramidal cells in the CA3 field. The hippocampus proper (CA region) is composed of four fundamental layers: stratum radiatum, stratum pyramidale, stratum oriens and straitum lacunosum/moleculare. At the bottom there is alveus, consisting of axons of the pyramidal cells which are directed towards the fimbria or the subiculum. The stratum oriens contains basal dendrites of the pyramidal cells, the stratum pyramidale is dominated by the cell bodies of these cells, while the stratum radiatum and the stratum lacunosum/moleculare contain segments of apical dendritic trees of pyramidal cells. (Figure 1.4) (O'Keefe and Nadel, 1978).

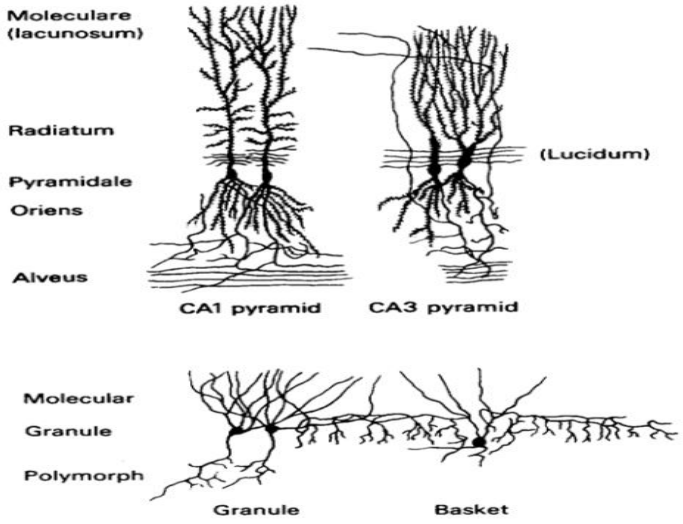


Figure 1.4: Internal layering of the hippocampus (O'Keefe and Nadel, 1978)

There are three principal pathways by which information flow occurs in the hippocampus: Perforant pathway (PP), mossy fiber pathway (MF) and Schaffer Collateral pathway (SC). The major afferent input coming to the hippocampus is through perforant pathway from the enthorhinal cortex being a gateway of many other cortical areas. Axons from its II/IV layers project to the granule cells in the dentate gyrus (DG) and to the pyramidal cells of the CA3 region, on the other hand axons from the III/V layers project to the pyramidal cells of the CA1 and the subiculum. Apart from the afferent input, another extrinsic input originating from medial septum-diagonal band (MS-DB), constitutes of cholinergic afferents (Yoshida and Oka, 1995).

In mossy fiber pathway, mossy fibers are the axons of dentate gyrus granule cells projecting to CA3 pyramidal cells. Whereas in Schaffer Collateral/Associational Commisural (SC/AC) pathway, Schaffer collaterals and commisural fibers are branching axons of CA3 pyramidal neurons projecting to the ipsi-lateral and contra-lateral hippocampal CA1 regions, respectively.

Hippocampal efferent projections go back to the septum and from CA1 region out of the hippocampus (Figure 5) (O'Keefe and Nadel, 1978; Suzuki and Amaral, 2004).

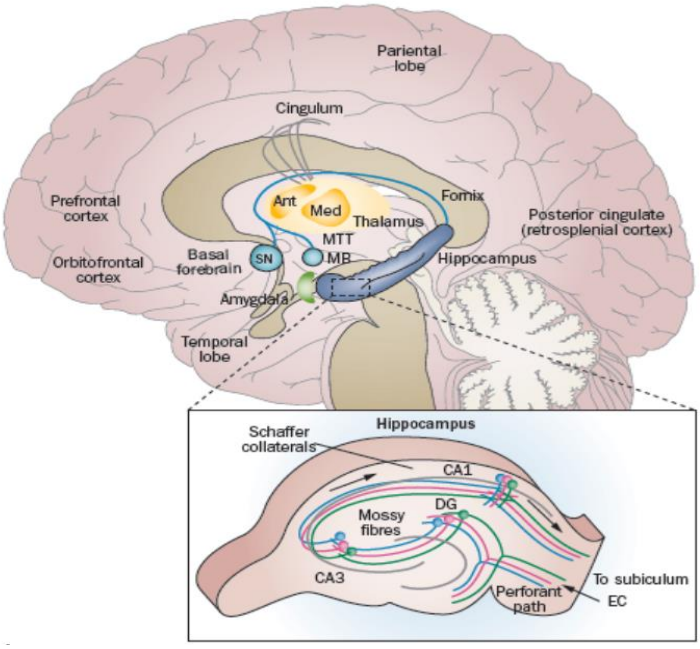


Figure 1.5: Hippocampus location and its circuitry (O'Keefe and Nadel, 1978**)**

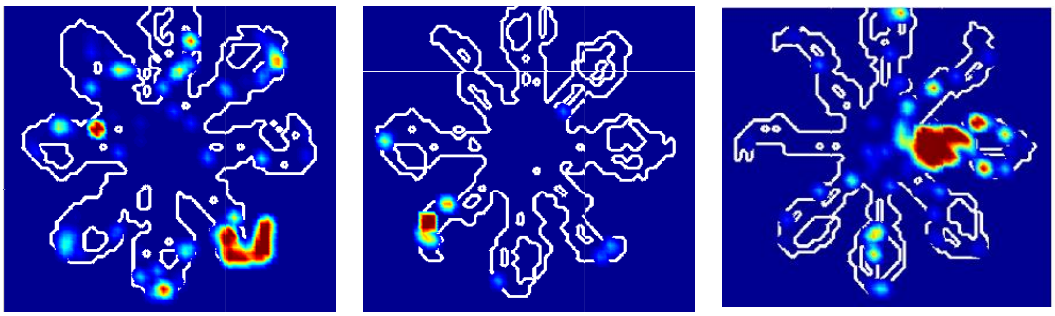
Importance of the hippocampus for memory formation was first documented by clinical case of patient H.M. This case was reported in 1957 when the patient was knocked down by a bicycle having a head injury which resulted in epilepsy. He was having seizures and by the age of 27, he was highly incapacitated. He was then subjected to a bilateral removal of the anterior two thirds of his hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae in order to cure the epilepsy. This surgery left him with a devastated memory which never recovered. When he was asked to remember a number, only after a minute or two later after directing his attention to another task, he was not able to remember the number (Squire and Kandel, 1999; Lynch, 2004).

Cellular model of memory formation being declared as activity-dependent long-term potentiation (LTP) of synaptic transmission, was first proposed by Hebb (1949) and, in 1968, experimentally demonstrated in the hippocampus by Tim Bliss and Per Andersen (Lømo, 2003).

Electrophysiological and behavioral experiments confirmed that hippocampus forms internal representations of the spatial environment, named as "cognitive maps" (O'Keefe and Nadel, 1978; Squire and Kandel, 1999). It was observed that pyramidal cells of the mouse hippocampus (talking about a million pyramidal cells), and related cortices encode features of the environment contributing in this way to the formation of a reference frame used in spatial orientation and navigation. O'Keefe and colleagues discovered in the hippocampus individual cells called "place cells" that fire only when an animal is in the particular location (O'Keefe and Dostrovsky, 1971). Relatively recently Edvard Moser and his collaborators discovered entorhinal grid cells serving as an inner global positioning system (GPS)

of the body monitoring self-position in the environment for purposes of navigation (Fyhn *et. al.* 2007). According to the recent observations, the grid cells are like a graph paper and place cells are like the markings on the graph paper that form the territory map.

A.



B.

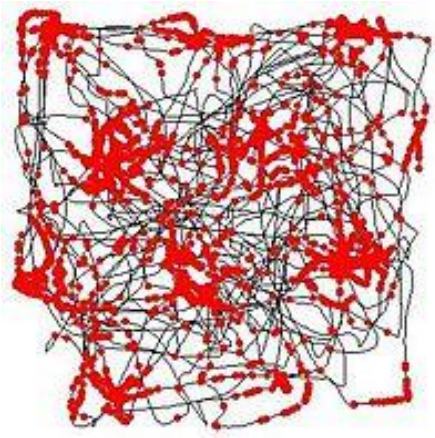


Figure 1.6: A. Place cells. B. Grid cells (Fyhn *et. al.* 2007).

1.1.6 Molecular basis of short-term and long-term memory formation

Short term memory (STM) is a form of memory lasting from minutes to hours and involving covalent modifications of pre-existing proteins usually by phosphorylation making these modifications more effective whereas long term memory (LTM) lasts for days or years sometimes lifelong and involves CREB-mediated gene expression and synthesis of new mRNAs and proteins (Hawkins *et al.* 2006).

As mentioned above, short-term memory involves modifications in pre-existing molecules at activated synapses. In the invertebrate model of, Aplysia *californica* extensively studied by the Eric Kandel's group (Goelet *et al.,* 1986, Kandel, 1991, 2001; Hawkins *et al*., 2006), it was shown that synaptic stimulation activates intracellular signal transduction pathways leading to an increase in the levels of so called second messenger molecules such as cAMP regulating activity of different protein kinases. Kinase activation results in the phosphorylation of synaptic proteins such as membranous ion channels and receptors, followed by the transient conformational changes in their structure which either enhance or damp their functions. Transient changes in ion channels' conductance and increase in the sensitivity of postsynaptic receptors to neurotransmitters cause transient augmentation in synaptic efficacy. In Aplysia model, an increase in the intracellular cAMP concentration leads to a short-term behavioral sensitization (strengthening of gill withdrawal reflex in response to the touch of siphon/mantel area) that lasts for minutes and is correlated to an increase in synaptic strength of the sensory-to-motor

neuron connection referred to as short-term facilitation. It results from serotonin release by the modulatory interneuron at its axo-axonic synapse with the sensory neuron, transient depression of the presynaptic potassium channels at the sensory-motor junction, broadening of presynaptic potential in the sensory neuron terminal, and increased neurotransmitter release. (See Figure 1.7).

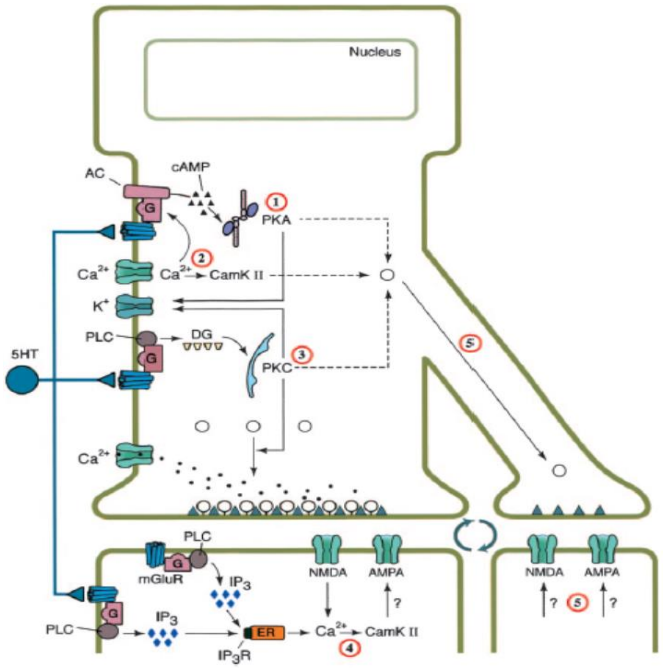


Figure 1.7: Short-term memory cascades (Hawkins *et al*., 2006).

Consolidation of synaptic modification and thus conversion of short-term memory into a long-term memory, is based on synthesis of new mRNAs and proteins. This means that long-term memory needs gene expression. Repeated serotonin arousal causes increase in cAMP levels. This leads to activation of the cAMP-dependent PKA by dissociation of its catalytic subunits from the regulatory subunit. In a short-term response to synaptic activation, the catalytic subunit of PKA phosphorylates

different substrates such as potassium channels or proteins involved in neurotransmitter exocytosis producing transient increase in synaptic function. However, for a more persistent change in synaptic efficacy, the catalytic subunit recruits mitogen activated protein kinase (MAPK) and both kinases translocate to the nucleus. Within the nucleus, the transcription factor, cAMP response element binding protein (CREB-1), is activated by phosphorylation binding to the cAMP recognition element (CRE) located within the promoter or enhancer regions on the DNA. CREB-binding protein (CBP), the transcription coactivator, is recruited to the related promoter region by phosphorylated CREB-1 in order to turn on gene transcription.

Recruiting RNA polymerase II to the promoter or functioning as acetyltransferase (adding acetyl groups to substrate-specific lysine residues) are crucial functions of CBP. One of the fundamental substrates of CBP are histone proteins (components of nucleosomes blocking chromatin), which when acetylated cause structural and functional changes in the nucleosome. These changes are chromatin transformation and facilitation of DNA transcription. Furthermore, activation of MAPK leads to inactivation of transcription factor CREB-2 which is a transcriptional repressor. Expression of genes related to learning and memory is induced by CREB-1 activation and CREB-2 suppression. Among early response genes induced by CREB-1 is ubiquitin carboxy terminal hydroxylase which enables ubiquitin mediated protein degradation, Degradation of PKA regulatory subunit is needed for persistent PKA activation. Another early response gene induced by CREB-1 is CAAT box enhancer binding protein (C/EBP) a transcription factor which interacts with the CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine) box motif, present in several gene promoters controlling the late gene cascades synthesizing proteins necessary for the growth of the new synaptic connections (see Figure 1.8) (Hawkins *et al*., 2006; Kandel *et al*., 1991).

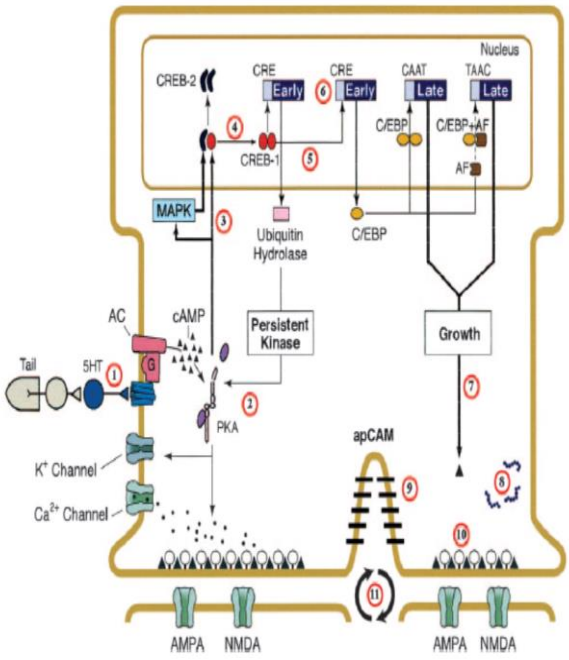


Figure 1.8: Long-term memory cascades (Hawkins *et al*., 2006).

1.2 General information about Cytochrome P450 (CYP)

Cytochromes P450 are heme-containing monooxygenases long believed to be present only in liver due to their drug metabolizing function but later on, they have been found in many other tissues including lungs, gut and brain (Hedlung *et al*. 2001; Miksys and Tyndale, 2002; Azenbacher and Azenbacherova, 2003). CYPs are phase I enzymes being involved in oxidative activation or deactivation reactions.

They are implemented in the metabolism of xenobiotics (i.e. drugs) as well as endogenous substrates such as thromboxane, prostacyclin, bile acids, calcitriol and steroid hormones. Nomenclature of CYP is based on amino acid similarities: more

than 40 % of sequence identity in families and 55 % or more of sequence identity in subfamilies. The family is expressed by a number, subfamily by a letter and enzyme itself by a number again (i.e. CYP2E1) (Hedlung *et al*. 2001; Miksys and Tyndale, 2002).

Table 1.1: CYP examples expressed in brain grouped according to their acting substrates (Ferguson and Tyndale, 2011)

Exogenous substrates					Endogenous substrates		
Clinical drugs			Neurotoxins	Drugs of abuse	Fatty acids	Steroids	Neurotransmitters
Antidepressants	Antipsychotics	Other					
CYP1A	CYP1A	CYP1A	CYP1A	CYP2B	CYP2J	CYP1A	CYP2B
CYP2B	CYP2D	CYP2B	CYP1B	CYP2D	CYP2U	CYP1B	CYP2D
CYP2C	CYP3A	CYP2C	CYP2D	CYP2E1	CYP4A	CYP2B	
CYP2D		CYP2D	CYP2E1			CYP2C	
CYP3A		CYP2E1	CYP3A			CYP2D	
		CYP3A					

Cytochromes P450 are membrane-bound enzymes located either in endoplasmic reticulum (ER) or in the inner mitochondrial membrane. Cytochromes P450 located in ER (microsomal CYPs) need additionally a flavoprotein cytochrome P450 reductase transferring electrons from NADPH to CYP whereas CYPs located in mitochondria need additionally adrenodoxin reductase, a flavoprotein transferring electrons from NADPH to small iron-sulfur protein, and adrenodoxin reducing CYP (Hedlung *et al*. 2001). These enzymes are part of a cycle starting with the binding of a substrate to Fe^{3+} (ferric) form of CYP enzyme (Fig.1.9). A typical reaction catalyzed by CYP is $NADPH + H^+ + O_2 + RH \Longrightarrow NADP^+ + H_2O + R\text{-}OH$ where NADPH is an electron donor.

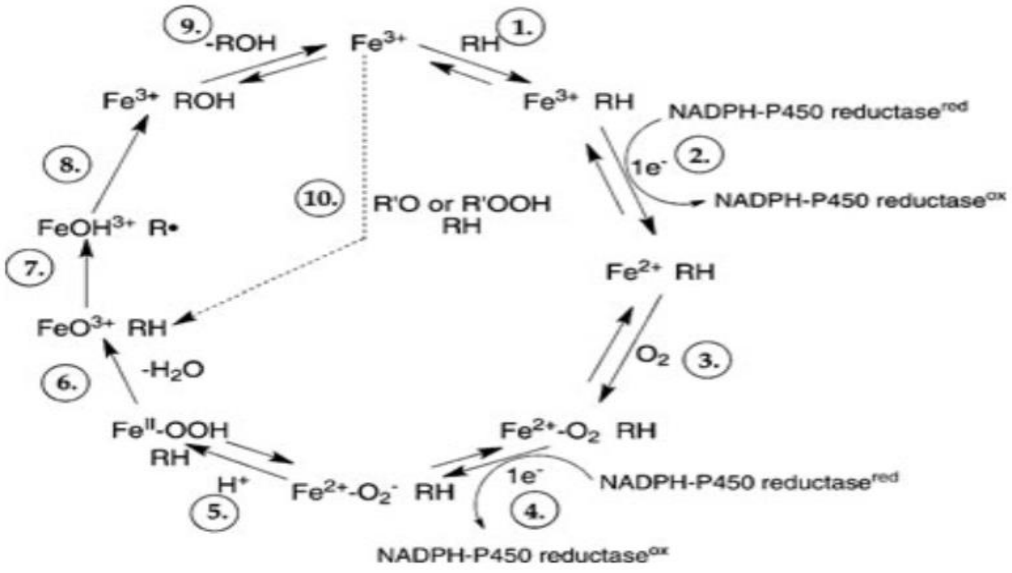


Figure 1.9: Catalytic cycle steps of CYP reactions (Guengerich, 2001).

Total CYP amount in the brain is very low compared to liver, approximately 0.5-2% of that of liver. In the brain of human as well as monkey, dog, mouse, and rat different CYP species have been detected. The expression levels of CYP in the brain differ among brain regions therefore it can be said that its distribution is heterogeneous (Ferguson and Tyndale, 2011). There are four receptor-dependent mechanisms of the induction of CYP gene expression by the xenobiotics. Transcriptionally activated CYPs belong to CYP1-4 families. One of the involved receptors is aryl hydrocarbon receptor (AhR) which has a dimerization partner both regulating CYP1A and CYP1B transcription. Second receptor is constitutively active receptor (CAR), a key factor conferring phenobarbital-inducible gene expression of CYP2B genes. Third one is pregnane X receptor (PXR) which leads to the induction of CYP3A gene expression by binding some compounds i.e. dexamethasone. The last one is peroxisome proliferator activated receptor (PPAR)

mediating induction of CYP4A gene by interacting with some acidic chemicals or endogenous compounds such as fatty acids. CYP4A enzymes catalyze biologically crucial hydroxylation or epoxygenation of fatty acids (Hedlung *et al*. 2001). Cytochromes seem to be derived from a single ancestral gene ~1.36 billion years ago and are highly conserved among the species. However, CYP nomenclature may differ from species to species (see Table 1.2) (Martignoni *et al.* 2006).

Table 1.2: CYP families in human, rat, mouse, dog and monkey (Martignoni *et al.* 2006)

Family	Subfamily	Human	Mouse	Rat	Dog	Monkey
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1	1B1	1B1
CYP2	A	2A6, 2A7, 2A13	2A4, 2A5, 2A12, 2A22	2A1, 2A2, 2A3	2A13, 2A25	2A23, 2A24
	B	2B6, 2B7	2B9, 2B10	2B1, 2B2, 2B3	2B11	2B17
	C	2C8, 2C9, 2C18, 2C19	2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55	2C6, 2C7*, 2C11*, 2C12*, 2C13*, 2C22, 2C23	2C21, 2C41	2C20, 2C43
	D	2D6, 2D7, 2D8	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D15	2D17‡, 2D19‡, 2D29‡, 2D30‡
	E	2E1	2E1	2E1	2E1	2E1
CYP3	A	3A4, 3A5, 3A7, 3A43	3A11, 3A13, 3A16, 3A25, 3A41, 3A44	3A1/3A23, 3A2*, 3A9*, 3A18*, 3A62	3A12, 3A26	3A8

*Gender difference.
‡Strain specific.

In the present study, the examined CYP isoforms were: CYP2E1, CYP2D1, CYP7A1, CYP2B1/2, and CYP7B1.

1.2.1 CYP2E1

CYP2E1 as many other CYP enzymes is strongly conserved among species. Human CYP2E1 and rat CYP2E1 have a similarity of 80%. CYP2E1 has different metabolic roles. One of them is ethanol detoxification which helps in prevention of tissue ethanol concentrations to reach excessive levels. It also plays a role in metabolism of fatty acids and participate in converting ketones to glucose under starvation or low-carbohydrate diet (Martignoni *et al.* 2006). CYP2E1 gene is located on 10. Chromosome and its molecular weight is 52 kDa. It is composed of 493 amino acids. CYP2E1 is mainly located in endoplasmic reticulum, Golgi apparatus and plasma membrane (Seliskar, 2007; Martignoni *et al.* 2006). See Figure 1.9 for the structure of CYP2E1.

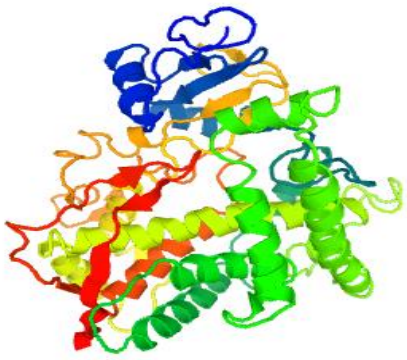


Figure 1.10: 3D structure of rat CYP2E1 (Protein Modal Portal, 2015).

CYP2E1 activity is inducible by ethanol, acetone isoniazid, pyridine and pyrazole in both rodents and non-rodents. The regulation of CYP2E1 involves many different mechanisms including transcriptional, post-transcriptional, translational and post-translational mechanisms.

CYP2E1 plays an important role in ketones and fatty acids metabolism especially in diabetes or obesity (Lieber, 2004). In addition it was reported to metabolize certain neuroactive substrates, including exogenous compounds such as anesthetics, organic solvents and muscle relaxants and endogenous substrates such as arachidonic acid (Tindberg and Ingelman-Sundberg, 1996). A drawback of CYP2E1 mediated metabolism is generation of oxygen radicals. When this overrides the cellular detoxification capacity, it results in oxidative stress with its different pathologic consequences. The action of CYP2E1 is similar in both humans and rodents, therefore rodents can be chosen conveniently as a model organisms in studies on this cytochrome P450 (Novak and Woodcroft, 2000; Zuber *et al.* 2002).

1.2.2 CYP2D1

CYP2D isoforms are reported to be involved in the monooxygenation of different drugs including antidepressants and β-blockers, (Hiroi *et al.* 2002). There are six CYP2D isoforms (CYP2D1, -2D2, -2D3, -2D4, -2D5 and -2D18) that have been identified by genomic analysis in rats (Nelson *et al.* 1996). The human and rat CYP2D isoforms have a high sequence similarity (> 70%) (See Table 1.3) (Funae *et al.* 2003; Venhorst *et al.* 2003). The six isoforms of CYP2D6 are expressed in different tissues such as liver, kidney and brain (Komori, 1993). The mRNA of

CYP2D isoforms shows a specific tissue distribution depending on their catalytic properties and the roles played by them in different tissues (Hiroi *et al.* 1998, 2002). Among the six isoforms, CYP2D1 is the rat orthologue of human CYP2D6. See Figure 1.10 for the crystal structure of CYP2D1.

Table 1.3: Sequence similarities (%) among human and rat CYP2D isoforms (Funae *et al.* 2003).

	CYP2D1	CYP2D2	CYP2D3	CYP2D4	CYP2D5	CYP2D18	CYP2D6
CYP2D1		73	79	72	95	72	71
CYP2D2	45		78	73	73	73	71
CYP2D3	59	59		75	79	75	72
CYP2D4	50	41	45		73	99	77
CYP2D5	82	41	55	50		73	71
CYP2D18	50	41	45	100	50		77
CYP2D6	59	41	55	59	50	59	

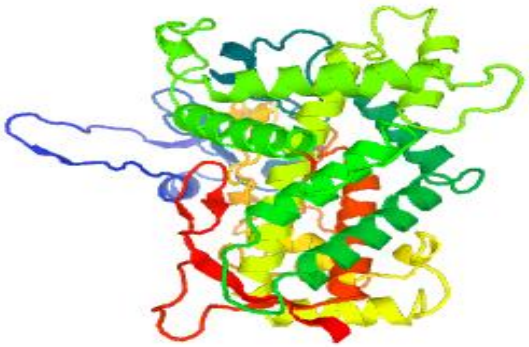


Figure 1.11: 3D structure of CYP2D1 (Protein Modal Portal, 2015).

In the brain, CYP2D isoforms, participate in the metabolism of neurotoxins and psychotropic drugs such as tricyclic antidepressants, selective serotonin reuptake inhibitors, amphetamines and neuroleptics. However, its pharmacological and physiological importance is not yet completely clarified (Funae *et al.* 2003). It is thought that CYP2D6 may have a modulatory effect on dopamine metabolism although it does not take part in dopamine synthesis (Niznik *et al*. 1990). Involvement of CYP2D6 in dopaminergic pathway may be observed in Alzheimer's disease (Chen *et al.* 1995), Parkinson's disease (Kurth and Kurth, 1993) or under nicotine addiction (Boustead *et al.* 1997).These varies effects of CYP2D6 may be related to its genetic polymorphism. All these should to be still clarified in future.

1.2.3 CYP7A1

Cholesterol 7α-hydroxylase (CYP7A1) (see Figure 1.12 for its 3D structure), catalyzes the first and the major rate-limiting step of the classical pathway for the bile acid biosynthesis (see Figures 1.13).

This is important for the balanced cholesterol degradation in the cells. CYP7A1 is considered to be liver-specific (Norlin and Wikwall, 2007; Nebert *et al.* 2013). In the nervous system, most of the cholesterol (about 70% in rodents) is found in the myelin sheaths that surrounds axons, whereas the smaller fraction (about 30%) is found in the plasma membranes of neurons and glial cells (Davidson, 1965). The bile acids not only in hepatocytes but also in the brain highly regulate the gene expression and the activity of the CYP7A1 (Quinn and DeMorrow, 2012). It is observed that, deficiency of CYP7A1 in mice led to 75% reduction of bile acid biosynthesis and no other enzyme compensated this (Nebert *et al.* 2013).

Figure 1.12: 3D structure of CYP7A1 (Protein Modal Portal, 2015).

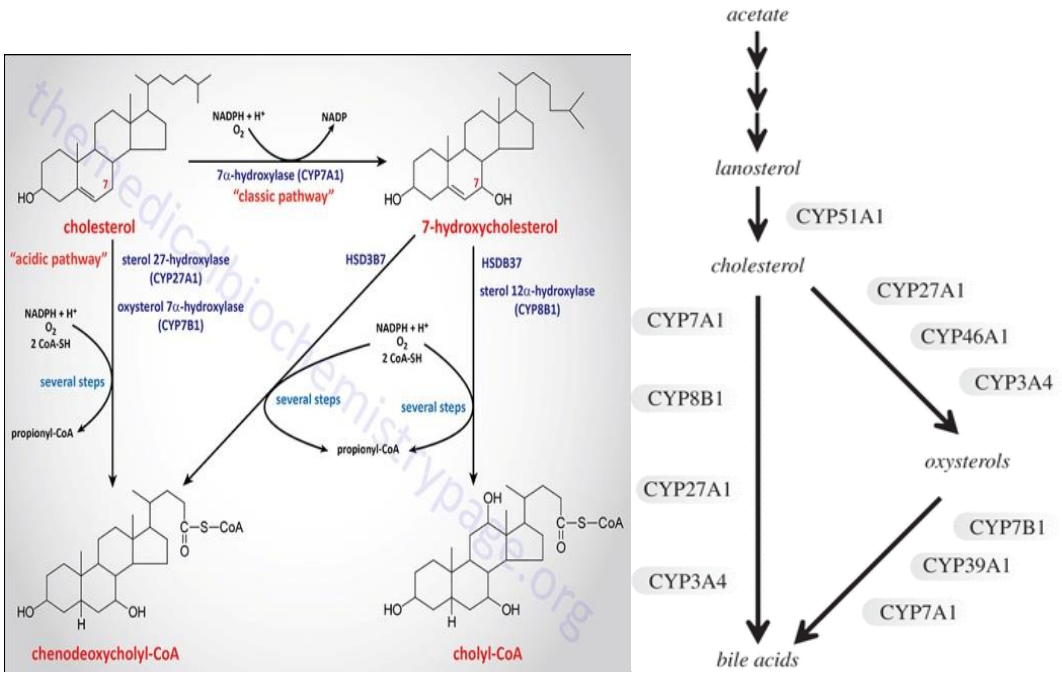


Figure 1.13: Enzymes involved in bile acid biosynthesis (The Medical Biochemistry Page, 2015; Nebert *et al.* 2013).

Enzymatic activity of CYP7A1 depends also on the diet, drugs, age, obesity and hormones (Pikuleva, 2006). Gene transcription is thought to be the main mechanism of CYP7A1 activity regulation because the changes in CYP7A1 enzymatic activity are parallel to the changes in its mRNA levels (Chiang, 1998).

1.2.4 CYP7B1

CYP7B1 being present in different tissues such as lung, liver, prostate, and kidney but also the brain (Stapleton *et al.* 1995), catalyzes hydroxylation reactions that occurs in 7-alpha-hydroxylation of 25- and 27-hydroxycholesterol which is an alternative pathway of bile acid synthesis. It has been reported that CYP7B1 gene mutation may lead to severe hypercholesterolemia and neonatal liver disease (Nebert and Russell, 2002). Apart from its role in cholesterol metabolism and homeostasis, CYP7B1 is also postulated to function as neurosteroid hydroxylase (Lathe, 2002) and be implemented in some brain and immune functions (Nebert *et al.* 2013).

1.2.5 CYP2B1/2

There are three different isoforms CYP2B1, CYP2B2 and CYP2B3 expressed in rats. CYP2B1 and CYP2B2 are structurally 97% identical (Waxman, 1988). It is an inducible CYP (by i.e. phenobarbital) and compared to its human orthologue (CYP2B6) it has higher expression in liver and extrahepatic tissues (Gonzalez and Gelboin, 1994). Rat CYP2B isoforms are involved in the activation of arenes, arylamines and nitrosamines (Mori *et al.* 1995). The metabolism of the tobacco-specific nitrosoamine 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) results in DNA addition and methylation of guanine at the 6O position therefore leading to transition of GC to AT (Miller *et al.* 1992). Hormones such as glucocorticoids, sex and thyroid hormones affect expression of phenobarbital-inducible CYPs (Honkakoski and Negishi, 1997).

There is a sexual dimorphism in CYP2B expression and this may be explained by a sex-dependent secretion of pituitary growth hormone which suppresses CYP2B expression more in female compared to the male rats (Yamazoe *et al.* 1987).

1.3 AIM OF THE STUDY

Today among all organs of the body, brain is the biggest enigma that a scientist can deal with. Apart from the brain diseases with many question marks waiting to be answered, the basic brain processes still are not completely elucidated. One of the intriguing and important issues is the molecular basis of learning and memory. The purpose of this study was to correlate brain expression of selected cytochrome P450 isoforms (CYP2E1, CYP2D1, CYP7A1, CYP7B1 and CYP2B1/2) with the individual variation in the short-term and long-term spatial memory within a random population of young-adult Sprague-Dawley rats. The levels of different CYP isoforms were estimated at different stages of place learning in the rat hippocampus, the structure crucial for spatial memory. Until now, the role of CYP proteins in the liver and many other peripheral tissues was extensively investigated, however, it is still little known about their functions in the brain. This is the reason, why in the present study the main focus was put on the activity-dependent hippocampal expression of different CYP isoforms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Subjects

In this study, ninety 2.5 month old Sprague Dawley male rats were used. Rats were obtained from Kobay D.H.L A.Ş. At the beginning of experiments, the animals' body weight was varying between 200-250 grams (Figure 2.1). Rats were semi-randomly divided into three groups, each containing 30 animals. All animals were subjected to place learning task in partially baited 12-arm radial maze (Gökçek-Saraç *et al.,* 2012, 2013). Rats from each group were sacrificed at different stage of spatial training: first group after the first 4 days of training (4DG), second group after the 8 days of training (8DG), and third group after reaching a demanding performance criterion of 83% correct choices in the three consecutive training sessions (ACG).

Figure 2.1: Sprague Dawley rat (Janvier Labs, 2015)

2.1.2 Chemicals

Acrylamide (A-8887/Sigma Chemical Company, Saint Louis, Missouri, USA)
2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378/Sigma Chemical Company, Saint Louis, Missouri, USA)
Ammonium per sulfate (APS; A-3678/Sigma Chemical Company, Saint Louis, Missouri, USA)
5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821/MBI Fermentas, USA)
Bromophenol blue (B5525/Sigma Chemical Company, Saint Louis, Missouri, USA)
CYP7A1 (sc25536/Santa Cruz Biotechnology, Dallas, Texas, USA)
CYP7B1 (sc26087/Santa Cruz Biotechnology, Dallas, Texas, USA)
CYP2B1/2 (sc73546/Santa Cruz Biotechnology, Dallas, Texas, USA)
CYP2E1 (AB1252/E. Merck Millipore, Darmstadt, Germany)
CYP2D1 (AB1271/E. Merck Millipore, Darmstadt, Germany)
Diethanolamine (D-2286/Sigma Chemical Company, Saint Louis, Missouri, USA)
Ethylene diamine tetra acetic acid (EDTA; A5097/Applichem GmbH, Germany)
Ethylene glycol tetraacetic acid (EGTA; A0878/Applichem GmbH, Germany)
Glycerol (G5516/Sigma Chemical Company, Saint Louis, Missouri, USA)
Glycine (G-7126/Sigma Chemical Company, Saint Louis, Missouri, USA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; H3375/ Sigma Chemical Company, Saint Louis, Missouri, USA)
Magnesium chloride ($MgCl_2$; 05833/ E. Merck Millipore, Darmstadt, Germany)
ß-actin (sc47778/Santa Cruz Biotechnology, Dallas, Texas, USA)
ß-mercaptoethanol (M6250/Sigma Chemical Company, Saint Louis, Missouri, USA)
Methanol (34885/Sigma Chemical Company, Saint Louis, Missouri, USA)
Nitrotetrazolium blue chloride (NBT; A1243/Applichem GmbH, Germany)
N'-N'-bis-methylene-acrylamide (M7256/Sigma Chemical Company, Saint Louis, Missouri, USA)

N-N-dimethylformamide (D-8654/Sigma Chemical Company, Saint Louis, Missouri, USA)

Non-fat dry milk (170-6404/Bio-Rad Laboratories, Richmond, California, USA)

Phenazine methosulfate (P9625/Sigma Chemical Company, Saint Louis, Missouri, USA)

Polyvinyl difluoride membrane (PVDF; 88520/Thermo scientific, Waltham, USA)

Pre-stained protein ladder (26616/Thermo scientific, Waltham, USA)

Protease Inhibitor Cocktail Tablets (11836153001/Roche, Basel, Switzerland)

Secondary antibody AP anti-rabbit (sc2007/Santa Cruz Biotechnology, Dallas, Texas, USA)

Secondary antibody AP anti-mouse (sc2008/Santa Cruz Biotechnology, Dallas, Texas, USA)

Sodium dodecyl sulfate (SDS; L4390/Sigma Chemical Company, Saint Louis, Missouri, USA)

Sodium chloride (NaCl; 1.06400/ E. Merck Millipore, Darmstadt, Germany)

Tetra methyl ethylene diamine (TEMED; 161-0801/Bio-Rad Laboratories, Richmond, California, USA)

Tris-HCl (T3253/Sigma Chemical Company, Saint Louis, Missouri, USA)

Triton X (11869.1/E. Merck Millipore, Darmstadt, Germany)

Tween 20 (P1379/Sigma Chemical Company, Saint Louis, Missouri, USA)

Whatman paper (88600/Thermo scientific, Waltham, USA)

Zinc chloride ($ZnCl_2$; 108815/E. Merck Millipore, Darmstadt, Germany)

2.1.3 Twelve-arm Radial Maze Apparatus

Radial arm maze (RAM), an apparatus used for testing spatial learning in small rodents (Olton and Samuelson, 1976) was made of plywood and painted flat grey. It was elevated 80 cm above the floor level and surrounded by distal visual-spatial cues. The central platform of the maze was 40 cm in diameter with twelve, 60 cm long and 9 cm wide arms framed by 15 cm high walls made of clear Plexiglas which enabled rats to see visual cues in the room but prevented direct crossing from one arm to another. At the entrance to the arms, there were guillotine doors which could be raised/shut individually or collectively. Food wells (2 cm wide and 2 cm deep) placed at the end of each arm had at the middle a metal, perforated partition beneath which food pellets were put. Animals had no access to these pellets but they helped to unify food odor traces along the maze. The same chocolate-flavored food pellets were placed also on the metal floor in the six, semi-randomly chosen food wells to be used as a bait during the place learning. Dim light was used to illuminate the maze.

2.2 Methods

2.2.1 Behavioral procedure

Firstly, animals had an adaptation period to the animal facilities in the Dept. of Biological Sciences at METU. During this period, animals were daily handled by the experimenter. Since the food reinforcement was a primary incentive for animals trained in the place learning task, before the experiments, animals were subjected to a food-restricted diet with three standard food pellets per rat on a single day. When 15% of weight reduction was obtained, the experiments started (Jakubowska-Dogru *et al.*, 2003). Animals were taken into experiments always in the same order and at

the same time of the day. The daily food portion was given 20 min after completing daily training session (one trial per session). The animals' body weight was recorded on the daily basis and was kept at a stable level throughout the experiments. The animal care procedures and all experimental manipulations were conducted accordingly to the regulations of the METU Ethic Committee of the Use of Experimental Animals (No: 2014/04).

At the outset of the experiments, rats were subjected to habituation to the experimental apparatus and then to the shaping training. During shaping training which lasted 5 days, food pellets were initially scattered throughout the maze and rats were allowed to explore maze for 10 minutes. On each day, the number of food pellets was reduced and successively, they were placed closer and closer to the ends of the maze arms. On the last day of the shaping training (the fifth day), food pellets were placed in the food wells of the six semi-randomly selected arms. The baited arms remained the same throughout the whole training (Figure 2.2).

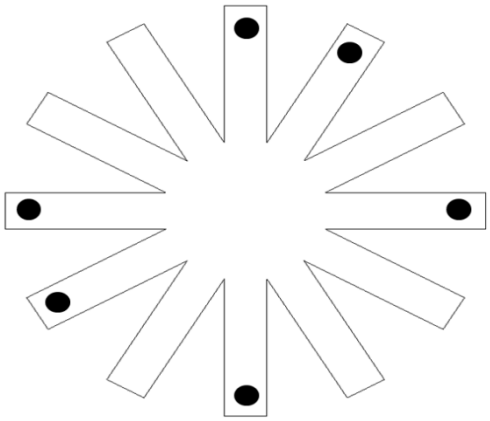


Figure 2.2. The diagram of 12-arm radial maze with black dots indicating baited arms (Gökçek-Saraç *et al*., 2012).

The partially baited RAM allows simultaneous estimation of reference memory errors (RMEs) and working memory errors (WMEs). Entry to unbaited arm was considered a RME, while re-entry to either baited or unbaited arm during a single training session was considered a WME. Making the choice was accepted only if the rat passed the midpoint of the arm with its four paws.

At the beginning of the daily sessions, rats were placed in the center of maze each time facing different direction. After 5 seconds, guillotine doors were raised and rats made their first choice. After each entry to the arms, rats returned to the center platform, guillotine doors were closed for 5 seconds, and only then rats were allowed to make the next choice. The daily session (trial) ended up with eating all food pellets, making 12 choices or when 10 minutes passed, whichever came first.

In these experiments, following measures were recorded:

1. Total number of unbaited arm entries (RMEs)
2. Total number of re-entries to both, the baited and unbaited arms (WMEs)
3. Number of choices to the acquisition criterion

According to their performance, rats were classified as "good" learners (total number of errors ≤ group mean – 3SEM) and "poor" learners (total number of errors ≥ group mean + 3SEM). The remaining rats constituted an "intermediate" group of learners.

2.2.2 Tissue Sample Preparation

Rats were sacrificed by decapitation 15 minutes after their last trial. The brains were quickly removed and placed on a dry ice for further dissection of hippocampi (left and right separately). After a quick immerse to the liquid nitrogen, tissues had been stored at -80^{o}C until the further procedures took place.

Left and right hippocampi were transferred to a centrifuge tube and 800µl of Triton lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCL pH 7.4, 10 mM EDTA, 5 mM EGTA, 20 mM HEPES, 10% glycerol and protease inhibitor cocktail (Roche) was added. Samples were subjected to sonication by using a sonic dismembrator in an ice-bath with a microtip probe set to a power output of 40W by 5 seconds for three cycles. There were 15 seconds pauses between each cycle. As a last step of homogenization, samples were centrifuged at 13500 rpm for 30 minutes at 4^{o}C. Removed supernatant was stored at -80^{o}C up to further use.

2.2.3 Determination of protein concentration

Protein concentration of tissue homogenates was determined by Qubit® 2.0 Fluorometer (Life Technologies) and Qubit® Protein Assay kit (Life Technologies). Qubit Assay Kits contains concentrated assay reagent, dilution buffer, and pre-diluted standards. The reagent is diluted by provided buffer and sample is added. The concentration is read using Qubit® 2.0 Fluorometer. The assay is conducted in room temperature. Summary in Figure 2.3.

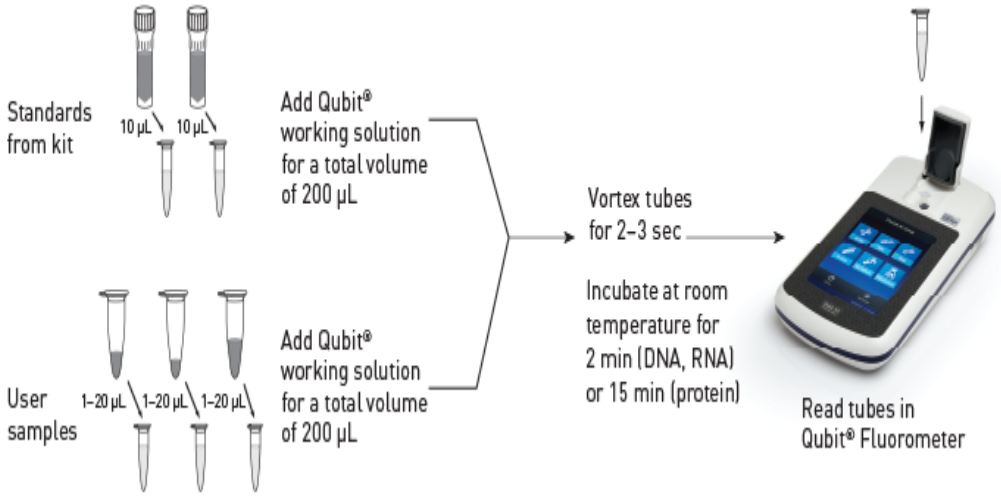


Figure 2.3: Protein concentration determination protocol summary by using Qubit® 2.0 Fluorometer (Thermofisher Scientific, 2015)

2.2.4 Protein Expression Determination

2.2.4.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Western blot technique, as described by Towbin *et al*. (1979), was used in detecting quantitative expression of enzymes of interest; CYP7A1, CYP2E1 , CYP2D1, CYP7B1 and CYP2B1/2, In the first step, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 4% stacking gel and 7.5% separating gel in a discontinuous buffer system (Laemmli, 1970). Separating and stacking gel solutions were prepared freshly according to the Table 2.1.

Table 2.1: Preparation of four gels

Content	Separating Gel	Stacking Gel
Monomer Concentration	7.5 %	4%
Gel Solution	7500 µL	1300 µL
dH_2O	14530 µL	6100 µL
Separating Buffer	7500 µL	---
Stacking Buffer	---	2500 µL
10% SDS	300 µL	100 µL
10%APS	150 µL	50 µL
TEMED	30 µL	20 µL
Total Volume	*30 mL*	*10 mL*

Reagents:

1. **Gel Solution:**

14.6 g Acrylamide and 0.4 g N'-N'-Bis-Methylene-Acrylamide were dissolved separately and then mixed to a final volume of 50 ml. Obtained mix should be filtered through filter paper.

2. **Separating Buffer:** 1.5 M Tris-HCl, pH 8.8 (Stock)

18.15 g Tris-base was dissolved with 50 ml water and titrated to pH 8.8 with 10 M HCl. After completing the volume of 100 ml with distilled water the pH was checked again.

3. **Stacking Buffer:** 0.5 M Tris-HCl, pH 6.8 (Stock)

6 g Tris-base was dissolved with 60 ml water and titrated to pH 6.8 with 10 M HCl. After completing the volume of 100 ml with distilled water the pH was checked again.

4. **SDS:** 10% (Stock)

1 g SDS was dissolved in water, at the end completing the volume to 10 ml.

5. **APS:** 10% (Fresh)

45 mg (for 4 gels) was weighed and dissolved in 450 µl distilled water.

6. **TEMED –** commercially available

7. **Sample Dilution Buffer (SDB):** 4x (stock)

2.5 ml of 1 M Tris-HCl buffer (pH 6.8), 4 ml Glycerol, 0.8 g SDS, 2 ml ß-Mercaptoethanol, 0.001 g Bromophenol Blue were mixed with a final volume of 10 ml.

8. **Electrophoretic Running Buffer (ERB):**

0.25 M Tris, 1.92 M Glycine (10x Stock should be diluted to 1x before use by adding 0.1% SDS)

15 g Tris-Base and 72 g Glycine were mixed with a final volume of 500 ml (1g SDS should be added to 1x buffer before use).

The gel sandwich was made according to the Table 2.1, between two glass plates containing 1 cm space. First, separating gel solution was prepared and poured immediately to the sandwich unit. For the fast polymerization, poured separating gel was covered by isopropanol. After the polymerization, alcohol was removed, stacking gel poured, and the comb placed instantly. When the polymerization occurred, gels were placed to the apparatus, filled with 1x ERB, and after a careful comb removal, wells were cleaned with syringe in order to get rid of the air bubbles or leftover particles of gel.

As a next step, protein samples were prepared. The necessary calculations were done and loading amount decided (Table 2.2).

Table 2.2: The amount of sample loaded into wells

	Loaded amount
CYP7A1	10 µl (2 µg/µl)
CYP2E1	10 µl (5 µg/µl)
CYP2D1	10 µl (5 µg/µl)

In order to obtain a concentration of 1mg/ml, the proteins should be diluted with distilled water calculated from the formula given below:

$$\boldsymbol{Volume}\ \mathrm{dH_2O} = \frac{[\boldsymbol{Conc.of\ Protein}]}{\mathbf{1.3333}} \times \mathbf{20} - \mathbf{20}$$

20 µl of sample was added to the obtained distilled water amount.

75 µl of sample was mixed with 25 µl of 4x SDB, and prepared sample before loading were incubated for 1.5 minutes at 100ºC heat block. Then, 10 µl of each sample and as a marker 5 µl of protein ladder (Figure 2.4) was loaded on wells. After loading the samples, tank is fully loaded with ERB. Electrophoresis was run at 15 mA–150 V in stacking gel and at 30 mA–300 V in separating gel.

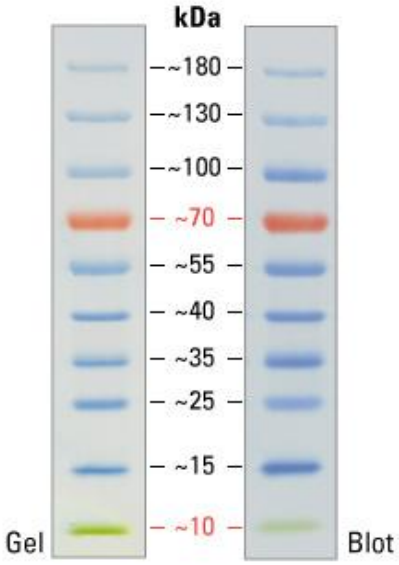


Figure 2.4: Pre-stained protein ladder image (Thermofisher Scientific, 2015)

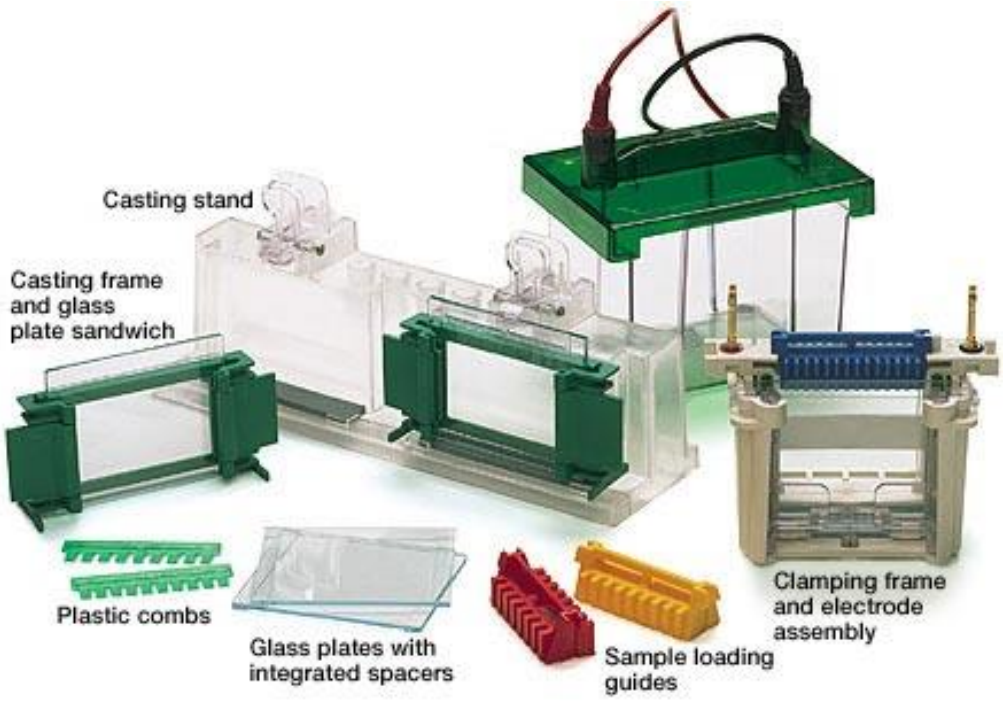


Figure 2.5: Bio-rad setup for SDS-PAGE

2.2.4.2 Western Blotting

Reagents:

1. **Transfer Buffer:** 25 mM Tris, 192 mM Glycine (Stock)

3.03 g Trisma-base, 14.4 g Glycine and 200 ml Methanol were mixed and final volume was completed to 1 L with distilled water.

2. **TBST:** 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.05% Tween 20 (Fresh)

9.5 g of NaCl and 6.5 ml of 1 M Tris-HCl Buffer pH 7.4 were mixed in some water. After 165 µl Tween 20 addition the final volume was completed to 350 ml with distilled water.

3. **Blocking Solution:** 5% Non-Fat Dry Milk (Stock)

5 g Non-Fat Dry Milk was dissolved in 100 mL TBS (TBST without Tween 20)

4. **Primary Antibody:** 1/100 to 1/1000 dilution

5. **Secondary Antibody:** 1/500 to 1/5000 dilution

6. Alkaline Phosphatase Substrate Solution:

<table>
<tr><th>SOLUTION A</th><th>SOLUTION B</th></tr>
<tr><td>2.67 ml of 1.5 M Tris-HCl, pH 8.8</td><td>2 mg of Phenazine Methosulfate in 1 mL of distilled water</td></tr>
<tr><td>4 ml of 1M NaCl</td><td>SOLUTION C</td></tr>
<tr><td>820 µl of 100 mM $MgCl_2$</td><td>5.44 mg of BCIP was dissolved in 136 µl of N-N-dimethylformamide</td></tr>
<tr><td>40 µl of 100 mM $ZnCl_2$</td><td rowspan="4">Finally,

40 ml of Solution A, 136 µl of Solution C and 268 µl of Solution B were mixed. This was prepared for 2 gels.</td></tr>
<tr><td>96 µl diethanolamine</td></tr>
<tr><td>12.2 mg NBT</td></tr>
<tr><td>pH of solution was adjusted to 9.55 and then final volume was completed to 40 ml</td></tr>
</table>

7. ECL Substrate Solution: (Pierce ECL Western Blotting Substrate)

1 ml of Peroxide solution and 1 ml of luminol enhancer solution were mixed and this was used for each membrane.

After the electrophoresis was conducted, gels were placed in transfer buffer for 10 minutes in constant shaking so that buffer salts and SDS would be cleaned up. PVDF membrane was cut according to the gel size and was immersed to methanol for a few seconds for prewetting membrane. After that, the PVDF membrane was adapted

to transfer buffer and gel, PVDF membrane, Whatman papers and two fiber pads were placed in transfer sandwich as shown in Figure 2.6. Prepared transfer sandwich was placed into Mini Trans-

Blot module (Bio-Rad Laboratories, Richmond, CA, USA). The tank was filled with transfer buffer and the transfer was carried out at 90 volt - 400 mA for 90 minutes.

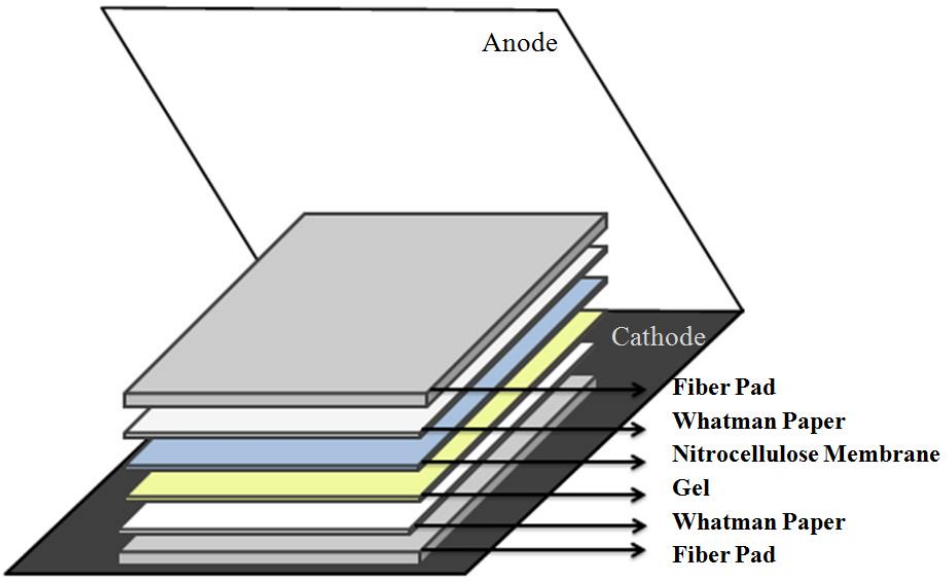


Figure 2.6: Western blot sandwich setup

Figure 2.7: Western blot transfer setup

Membrane was washed with TBST for 10 minutes after the transfer had been completed. Then membrane was incubated with blocking solution for an hour in room temperature in order to block empty space between transferred proteins so that non-specific bindings were prevented. Furthermore, the membrane was incubated with primary antibodies for 2 hours at room temperature by shaking with the dilutions given in Table 2.3. The membrane was washed with TBST for three times for 10 minutes. After removal of unbound primary antibody by washing, the membrane was incubated with alkaline phosphatase and horse reddish peroxidase secondary antibodies for an hour as given in Table 2.3. As a final step, the membrane was incubated with suitable substrate solution depending on the conjugated enzyme on the secondary antibody. In order to visualize of horseradish peroxidase conjugated secondary antibody, X-Ray Roentgen Method was used visualizing bands on the roentgen film. Image J visualization software developed by NIH was used to analyzed band intensities.

Table 2.3: Primary and secondary antibody dilutions

	PRIMARY ANTIBODY	**SECONDARY ANTIBODY**
CYP7A1	1/500	1/4000
CYP2E1	1/1000	1/3000
CYP2D1	1/350	1/3000
β-ACTIN	1/500	1/2000

2.2.5 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 6) statistical software package for Windows. All results were expressed as mean ± standard error of mean (SEM). Unpaired, two-tailed student's *t*-test, Sidak's multiple comparison test, Dunn's multiple comparison test and Mann Whitney tests were used for data evaluation.

CHAPTER 3

RESULTS

3.1 Results obtained from behavioral tests

Rats were categorized as "good" learners (total error number ≤ group mean – 3SEM) and "poor" learners (total error number ≤ group mean + 3SEM) according to their performances in 12 arm radial maze. From the three groups tested, there were 5 "good" learners and 7 "poor" learners out of total 30 animals after 4 days of training (4DG), 8 "good" learners and 11 "poor" learners out of total 30 rats after 8 days of training, (8DG), and 9 "good" learners and 11 "poor" learners out of 30 rats after reaching the arbitrary acquisition criterion of 83% of correct responses in 18 first choices (6 per session) during 3 consecutive training sessions (ACG). Remaining animals in all groups were categorized as "intermediate" learners. Total numbers of errors for individual subjects are presented in Tables 3.1, .3.2 and 3.3.

Table 3.1: Total number of errors and performance classification for individual rats from 4 Days Group

	SRAGUE DAWLEY RATS-4 DAYS GROUP	
RAT - ID	**TOTAL ERROR NUMBER**	**CATEGORY**
S4_1	2	GOOD
S4_11	22	GOOD
S4_16	22	GOOD
S4_18	7	GOOD
S4_21	22	GOOD
S4_2	26	INTERMEDIATE
S4_3	23	INTERMEDIATE
S4_4	30	INTERMEDIATE
S4_6	30	INTERMEDIATE
S4_7	24	INTERMEDIATE
S4_8	29	INTERMEDIATE
S4_12	26	INTERMEDIATE
S4_14	28	INTERMEDIATE
S4_17	26	INTERMEDIATE
S4_19	30	INTERMEDIATE
S4_20	30	INTERMEDIATE
S4_22	25	INTERMEDIATE
S4_23	29	INTERMEDIATE
S4_25	25	INTERMEDIATE
S4_26	28	INTERMEDIATE
S4_28	30	INTERMEDIATE
S4_29	30	INTERMEDIATE
S4_30	29	INTERMEDIATE
S4_5	31	POOR
S4_9	31	POOR
S4_10	31	POOR
S4_13	36	POOR
S4_15	33	POOR
S4_24	31	POOR
S4_27	33	POOR
	AVERAGE: 26.63333	
	SEM: 1.276204	

Table 3.2: Total number of errors and performance classification for individual rats from 8 Days Group

	SRAGUE DAWLEY RATS-8 DAYS GROUP	
RAT - ID	**TOTAL ERROR NUMBER**	**CATEGORY**
S8_1	50	GOOD
S8_5	51	GOOD
S8_8	51	GOOD
S8_10	34	GOOD
S8_19	50	GOOD
S8_21	51	GOOD
S8_27	52	GOOD
S8_28	36	GOOD
S8_2	60	INTERMEDIATE
S8_4	55	INTERMEDIATE
S8_7	59	INTERMEDIATE
S8_16	55	INTERMEDIATE
S8_17	48	INTERMEDIATE
S8_22	60	INTERMEDIATE
S8_23	58	INTERMEDIATE
S8_24	54	INTERMEDIATE
S8_25	60	INTERMEDIATE
S8_26	58	INTERMEDIATE
S8_30	60	INTERMEDIATE
S8_3	63	POOR
S8_6	63	POOR
S8_9	66	POOR
S8_11	61	POOR
S8_12	68	POOR
S8_13	64	POOR
S8_14	61	POOR
S8_15	62	POOR
S8_18	61	POOR
S8_20	61	POOR
S8_29	61	POOR
	AVERAGE: 56.43333	
	SEM: 1.422062072	

Table 3.3: Total number of errors and performance classification for individual rats from Acquired Criterion Group

	SRAGUE DAWLEY RATS-ACQUISITION CRITERION GROUP	
RAT - ID	**TOTAL ERROR NUMBER**	**CATEGORY**
S30_6	69	GOOD
S30_10	100	GOOD
S30_14	107	GOOD
S30_16	86	GOOD
S30_18	37	GOOD
S30_19	88	GOOD
S30_20	28	GOOD
S30_22	123	GOOD
S30_26	126	GOOD
S30_2	201	INTERMEDIATE
S30_5	184	INTERMEDIATE
S30_7	196	INTERMEDIATE
S30_9	216	INTERMEDIATE
S30_12	153	INTERMEDIATE
S30_17	170	INTERMEDIATE
S30_27	209	INTERMEDIATE
S30_28	156	INTERMEDIATE
S30_30	152	INTERMEDIATE
S30_1	229	POOR
S30_3	234	POOR
S30_8	270	POOR
S30_11	274	POOR
S30_13	235	POOR
S30_15	232	POOR
S30_21	228	POOR
S30_23	229	POOR
S30_24	305	POOR
S30_25	287	POOR
S30_29	235	POOR
	AVERAGE: 177.8965517	
	SEM: 13.98	

A percentage frequency distribution of “good”, “poor”, and “intermediate” learners in each of three training groups is presented in Figures 3.1, 3.2 and 3.3. As seen from these figures, in 4DG 16.6%, in 8DG 26.6% and in ACG 31% of subjects were classified as “good” learners and respectively, 23.3%, 36.6% and 38% of rats were classified as “poor” learners.

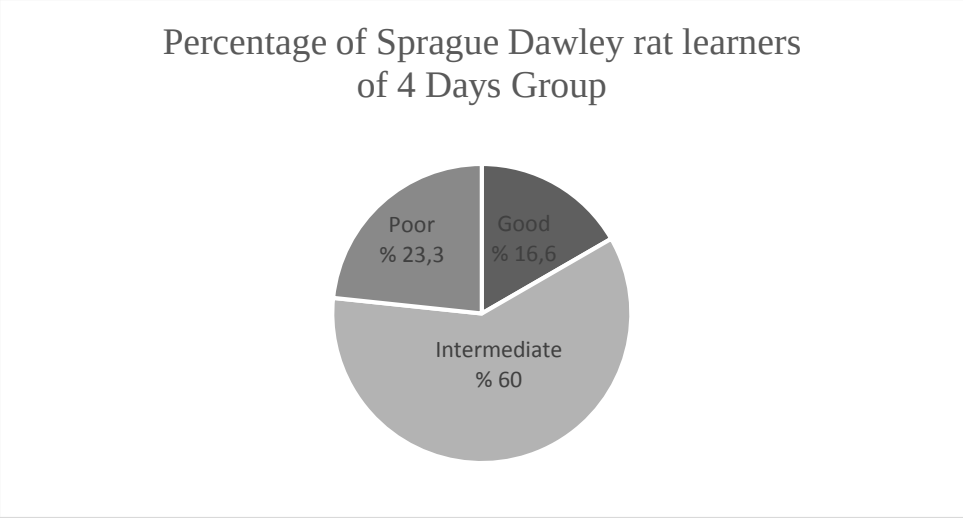


Figure 3.1: A percentage frequency distribution of “good”, “poor” and “intermediate” learners of 4 Days Group

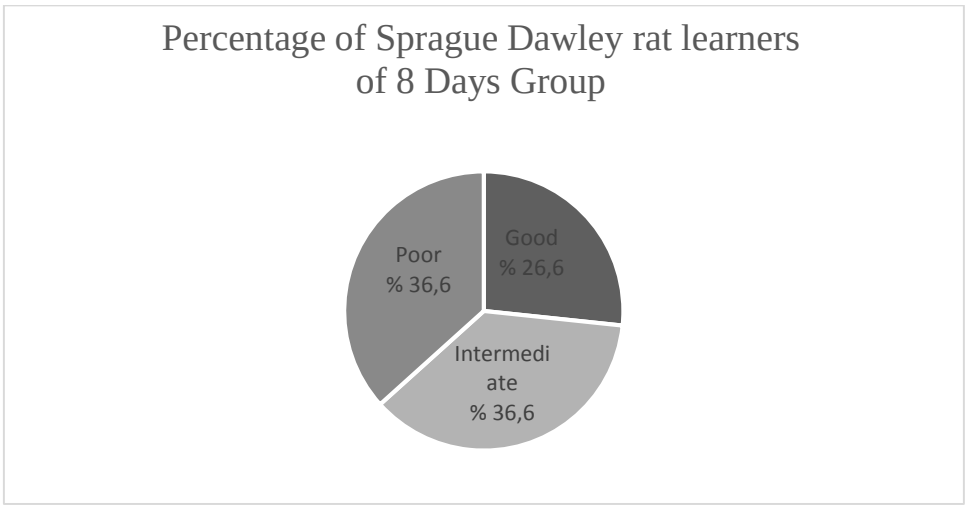


Figure 3.2: A percentage frequency distribution of "good", "poor" and "intermediate" learners of 8 Days Group

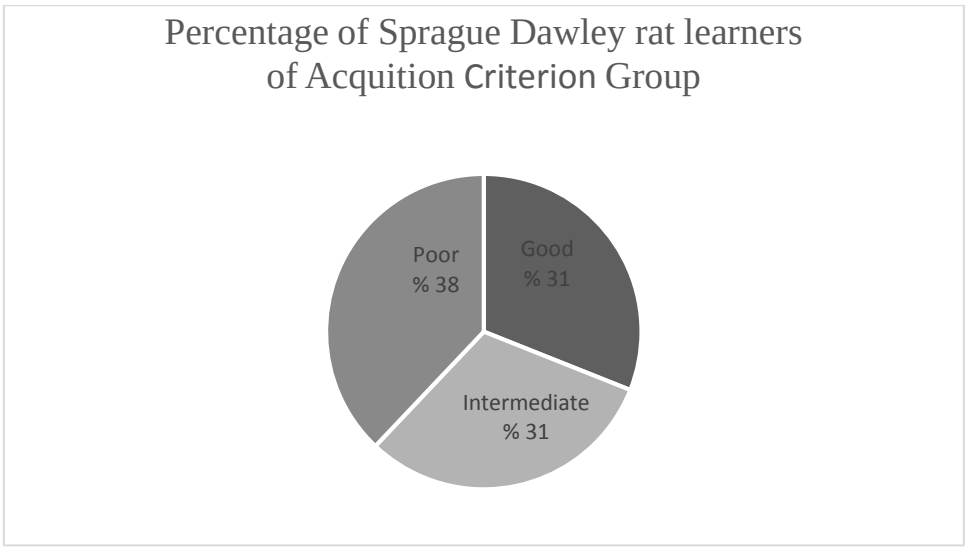


Figure 3.3: A percentage frequency distribution of "good", "poor" and "intermediate" learners of Acquisition Criterion Group

The histogram in the Figure 3.4 shows distribution of rats in ACG group according to the total number of training sessions until reaching the task acquisition criterion.

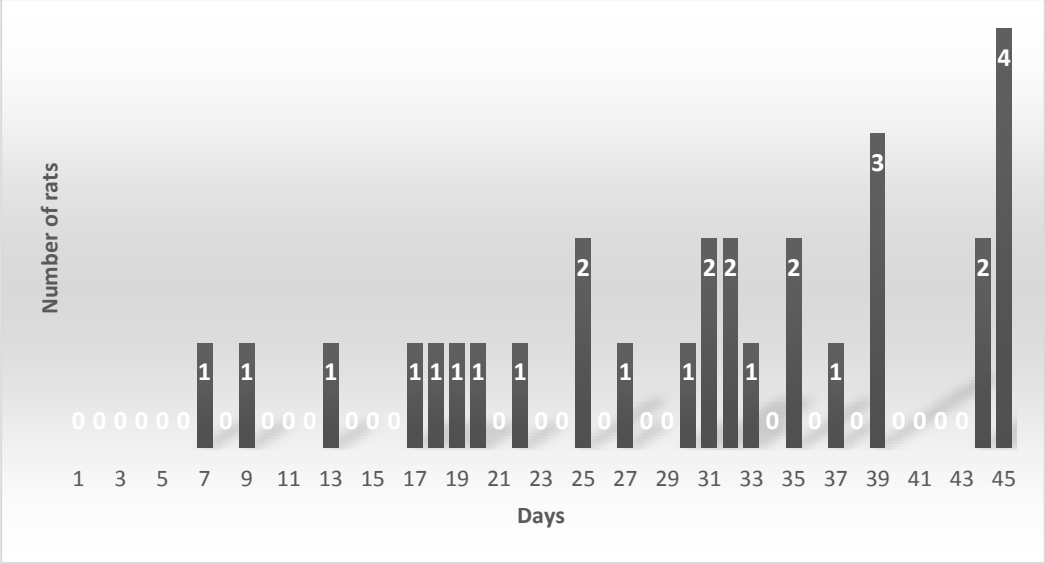
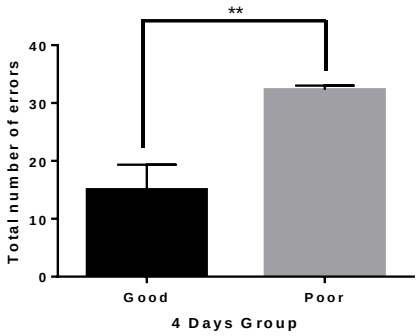


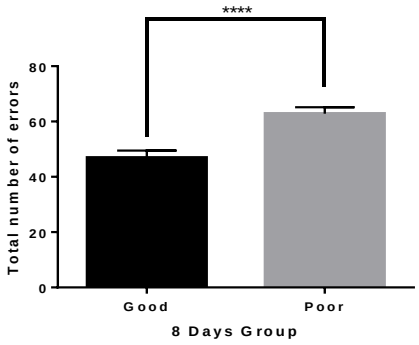
Figure 3.4: Distribution of rats in ACG group according to the total number of training sessions until reaching the task acquisition criterion

Figure 3.5 presents the mean total error numbers in “good” and “poor” learners at three different stages of place learning. Statistical evaluation of these data performed by Mann - Whitney U Test confirmed highly significant difference in task performance between .rat groups classified as “good” and “poor” learners in all three training groups. Figure 3.6 presents the mean numbers of, respectively, working and reference memory errors in “good” and “poor” learners at three different stages of place learning.

A.



B.



C.

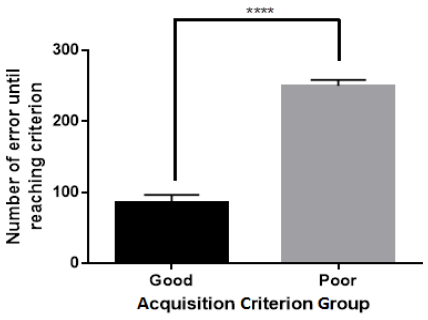
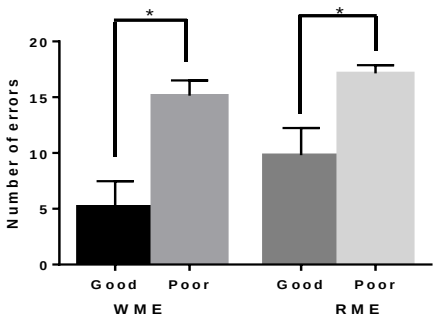
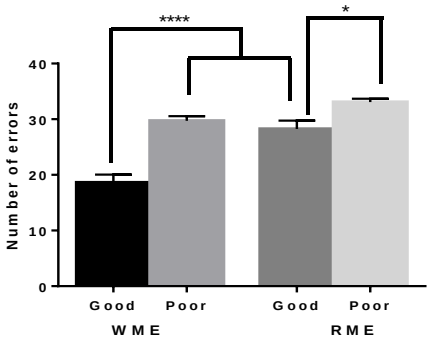


Figure 3.5: Comparison of mean numbers of total errors between "good" and "poor" learners from 4 Days Group (A), 8 Days Group (B), and Acquisition Criterion Group (C). Error bars denote ± SEM. Asterisks denote the level of significance: **p≤ 0.01, **** p≤ 0.0001.

A.



B.



C.

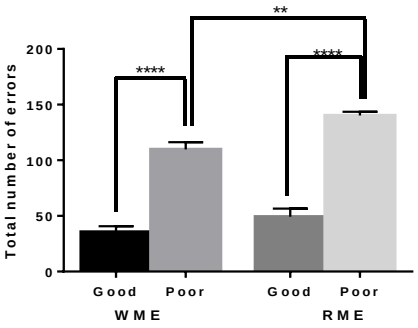


Figure 3.6. Comparison of the mean numbers of working and reference memory errors between "good" and "poor" learners at each of three stages of training (A: 4DG; B: 8DG; C: ACG), Error bars denote ± SEM. Asterisks denote the level of significance: * $p \leq 0.05$. **$p \leq 0.01$, **** $p \leq 0.0001$.

Statistical evaluation of these data performed by Sidak's and Dunn's multiple comparison tests confirmed highly significant difference in the task performance between rat groups classified as "good" and "poor" learners in all three training groups. Results of statistical analysis confirmed statistically significant difference in the number of both WMEs and RMEs between rats classified as "poor " and "good" learners in all three training groups with generally lower counts of WMEs than RMEs. The latter difference gains statistical significance when training extends. This is observed also when comparing percent WME and percent RME during the last three consecutive sessions at each learning stage, separately, for the whole groups: 4DG, 8DG, and ACG (Figure 3.7.)

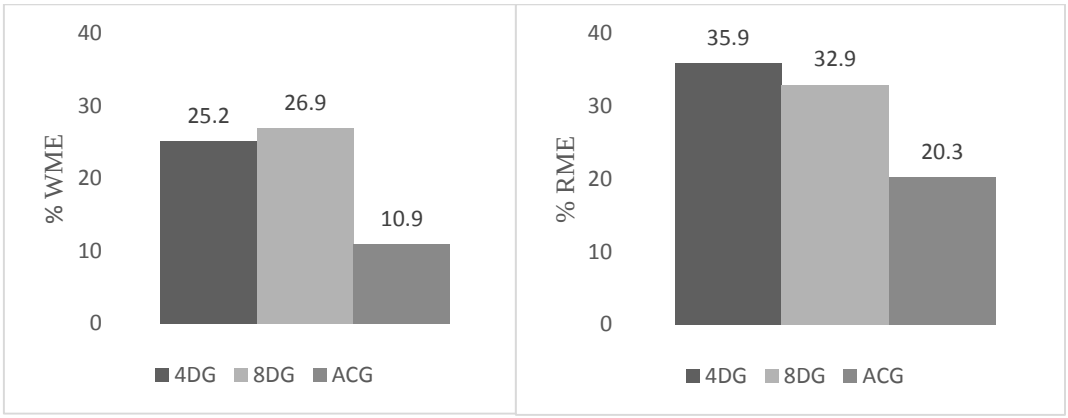


Figure 3.7. Percent WME and percent RME calculated for the last three sessions at each learning stage, separately, for the whole groups: 4DG, 8DG, and ACG.

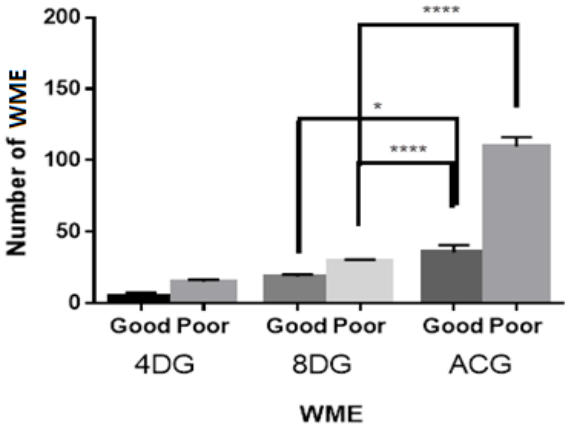


Figure 3.8: Between-group comparison of working memory errors in "good" and "poor" learners. Error bars denote ± SEM. Asterisks denote the level of significance: ****p≤0.0001 ve *p≤ 0.05.

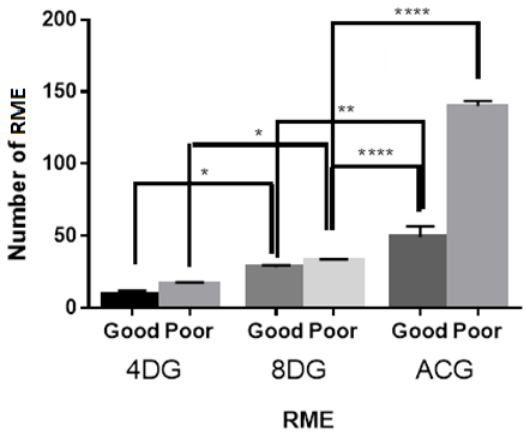


Figure 3.9: Between-group comparison of reference memory errors in "good" and "poor" learners. Error bars denote ± SEM. Asterisks denote the level of significance: * p<0.05, ** p<0.01, **** p< 0.0001.

3.2 Results obtained from biochemical assays

3.2.1 Protein concentration determination

Below given tables show protein concentrations measured in left and right hippocampi independently, for "good" and "poor" learners of all three groups.

Table 3.4: Protein concentrations (mg/ml) of average left and right hippocampi of "good" and "poor" learners of 4 days group

SRAGUE DAWLEY-4 DAYS GROUP			
"GOOD"		"POOR"	
RAT-ID	PROTEIN CONCENTRATION (mg/ml)	RAT-ID	PROTEIN CONCENTRATION (mg/ml)
S4_1	5.90	S4_5	12.3
S4_11	9.85	S4_9	13.4
S4_16L	11.5	S4_10	12.3
S4_18L	15.4	S4_13	9.50
S4_21L	16.8	S4_15	10.2
		S4_24	16.1
		S4_27	12.3

Table 3.5: Protein concentrations (mg/ml) of average left and right hippocampi of "good" and "poor" learners of 8 days group

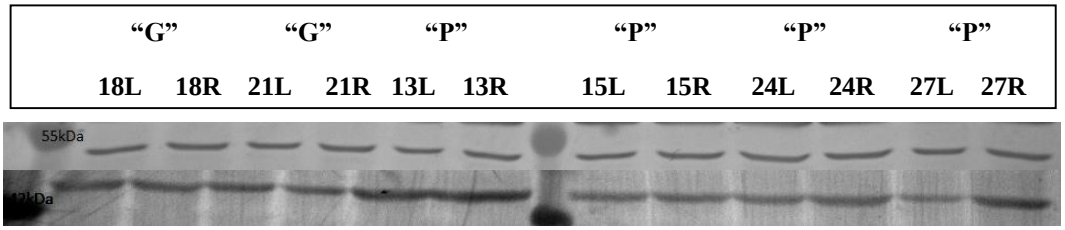
SRAGUE DAWLEY-8 DAYS GROUP			
"GOOD"		"POOR"	
RAT-ID	PROTEIN CONCENTRATION (mg/ml)	RAT-ID	PROTEIN CONCENTRATION (mg/ml)
S8_1	5.00	S8_3	12.5
S8_5	19.5	S8_6	14.0
S8_8	16.0	S8_9	6.90
S8_10	4.00	S8_11	7.00
S8_19	13.5	S8_12	6.75
S8_21	12.0	S8_13	13.5
S8_27	15.3	S8_14	12.6
S8_28	12.6	S8_15	12.2
		S8_18	13.6
		S8_20	13.4
		S8_29	13.5

Table 3.6: Protein concentrations (mg/ml) of average left and right hippocampi of "good" and "poor" learners of acquisition criterion group

SRAGUE DAWLEY-ACQUISITION CRITERION GROUP			
"GOOD"		"POOR"	
RAT-ID	PROTEIN CONCENTRATION (mg/ml)	RAT-ID	PROTEIN CONCENTRATION (mg/ml)
S30_6	14.1	S30_1	12.6
S30_10	13.7	S30_3	14.2
S30_14	4.80	S30_8	13.9
S30_16	8.20	S30_11	13.1
S30_18	21.4	S30_13	8.00
S30_19	14.2	S30_15	8.40
S30_20	16.9	S30_21	16.3
S30_22	12.5	S30_23	13.8
S30_26	10.1	S30_24	10.8
		S30_25	13.6
		S30_29	11.4

No statistical difference was observed between left and right hippocampi protein levels. Therefore, the average protein concentrations from both hippocampi were used in the following analyses.

In the western blot analysis, immunoreactive bands for CYP2E1 were observed at 52 kDa. Relative CYP2E1 level was determined by comparing intensity of this band with β-actin band (42 kDa) intensity used as internal control. Mann Whitney test revealed a significant difference in expression of CYP2E1 protein between rats classified as "good" and "poor" learners in all three training groups, with higher protein levels in "good" than "poor" learners (Figure 3.10).



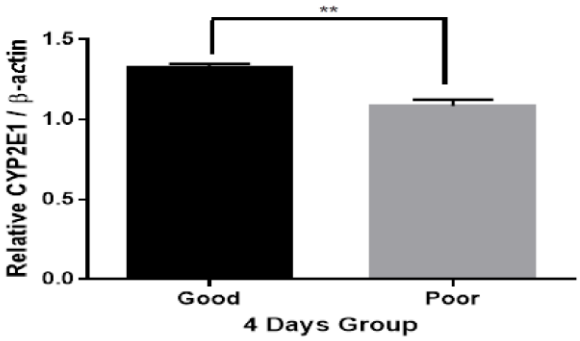


Figure 3.10: Mean relative intensity of CYP2E1 band (52 kDa) with respect to that of β-actin (42kDa), used as an internal control, for rats classified as "good" and "poor" learners in 4 Days Group. Error bars denote ± SEM. Asterisk denotes the level of significance ** $p<0.01$. Representative immunoreactive bands for both CYP2E1 and β-actin are presented above the graphic.

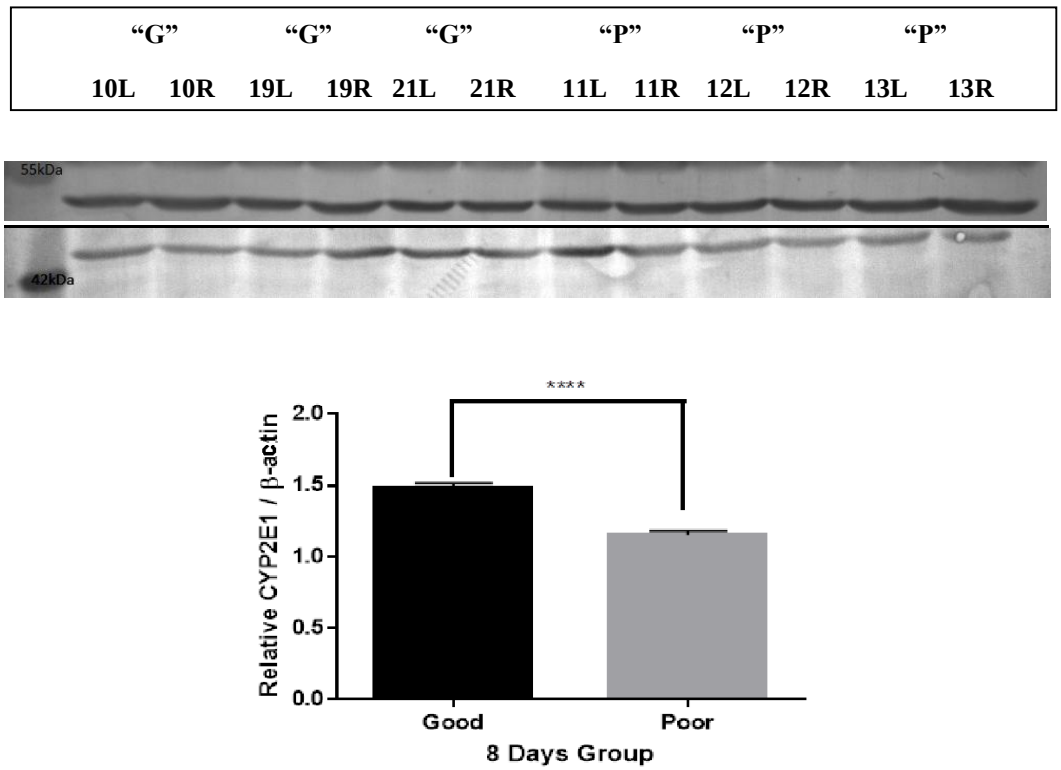


Figure 3.11: Mean relative intensity of CYP2E1 band (52 kDa) with respect to that of β-actin (42kDa), used as an internal control, for rats classified as "good" and "poor" learners in 8 Days Group. Error bars denote ± SEM. Asterisk denotes the level of significance **** $p<0.0001$. Representative immunoreactive bands for both CYP2E1 and β-actin are presented above the graphic.

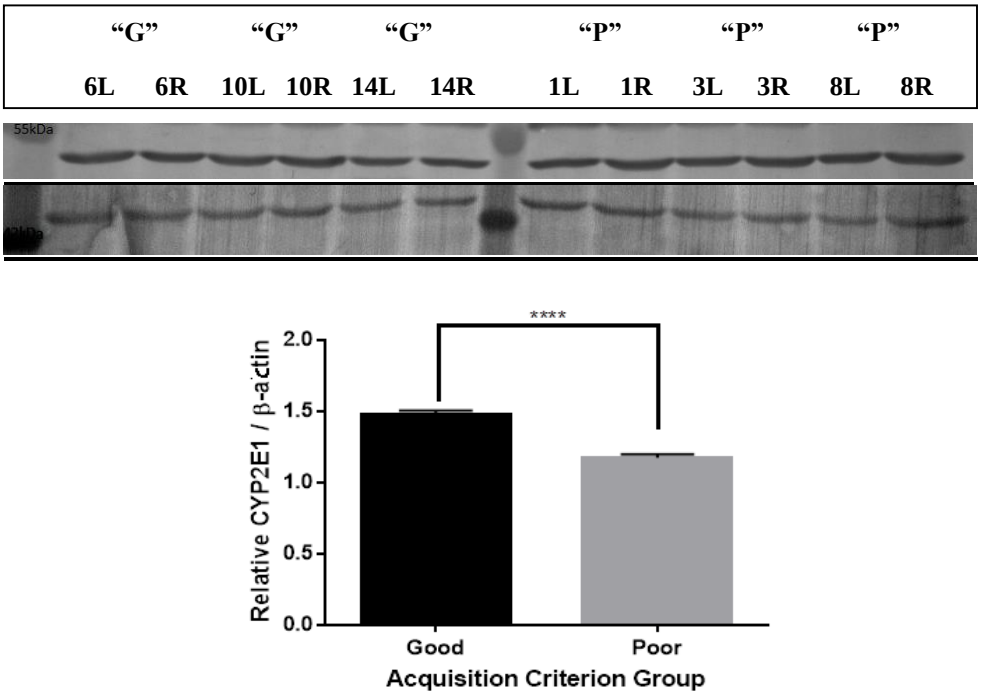


Figure 3.12: Mean relative intensity of CYP2E1 band (52 kDa) with respect to that of β-actin (42kDa), used as an internal control, for rats classified as "good" and "poor" learners in Acquisition Criterion Group. Error bars denote ± SEM. Asterisk denotes the level of significance **** $p<0.0001$. Representative immunoreactive bands for both CYP2E1 and β-actin are presented above the graphic.

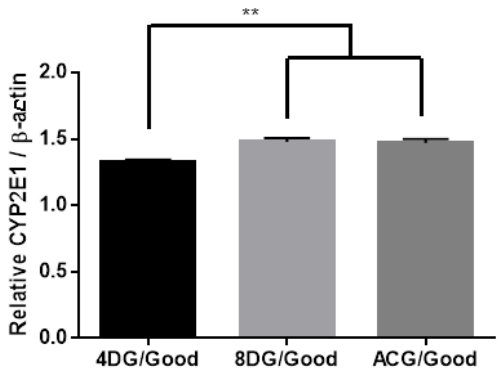


Figure 3.13: Between-group comparison of the levels of CYP2E1 in rats classified as "good" learners. Error bars denote ± SEM. Asterisk denotes the level of significance ** $p<0.01$.

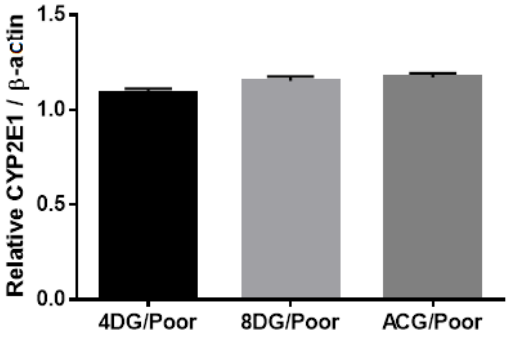
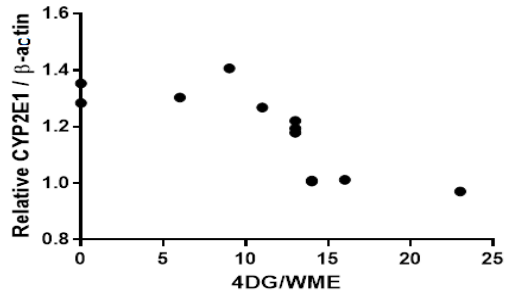


Figure 3.14: Between-group comparison of the levels of CYP2E1 in rats classified as "poor" learners. Error bars denote ± SEM. No significant differences were observed.

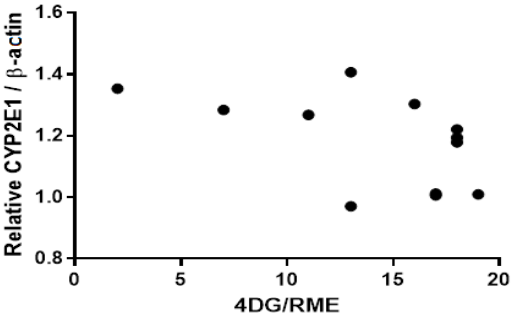
To evaluate the correlation between hippocampal CYP2E1 levels and animals' learning skills, Pearson's correlation analyses with number of different types of errors (WME, RME, Total) as the covariate were performed. These analyses were

conducted for each stage of spatial training and each group independently. The regression plots are presented in Figure 3.15.

A.



B.



C.

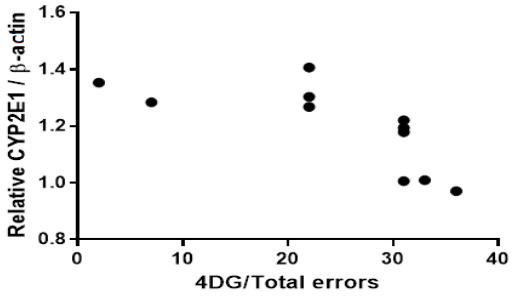
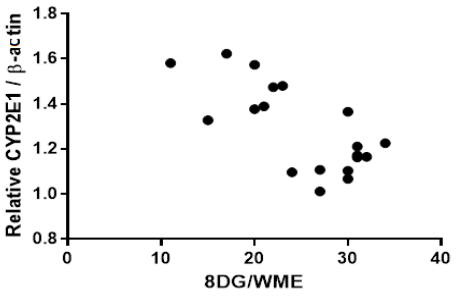
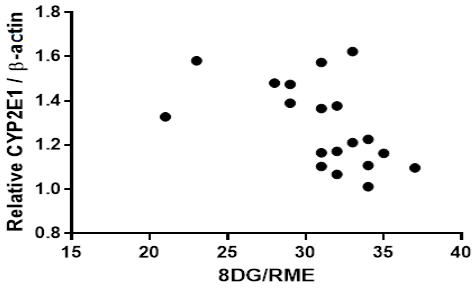


Figure 3.15: Regression plots based on individual data showing the correlation between the hippocampal expression of CYP2E1 protein (52 kDa) and the number of working memory errors (A), reference memory errors (B), and total number of errors (C) in 4 days group. R^2=0.6074**, p=0.0015, R^2=0.2744*, p=0.0402, and R^2=0.5276**, p=0.0037, respectively.

A.



B.



C.

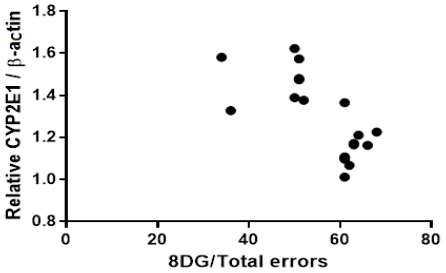
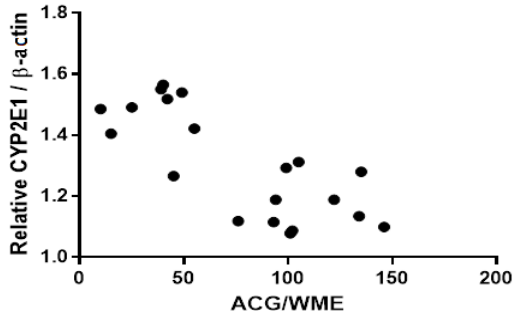
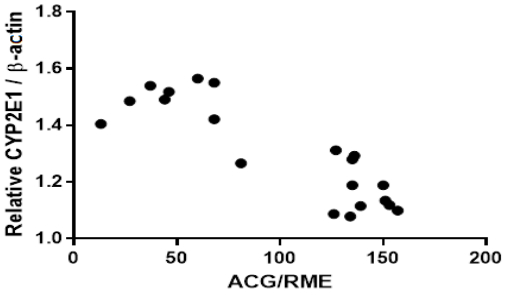


Figure 3.16: Regression plots based on individual data showing the correlation between the hippocampal expression of CYP2E1 protein (52 kDa) and the number of working memory errors (A), reference memory errors (B), and total number of errors (C) in 8-day group. R^2=0.5168***, p=0.0003. R^2=0.2613*, p=0.0127, and R^2=0.4943***, p=0.0004, respectively).

A.



B.



C.

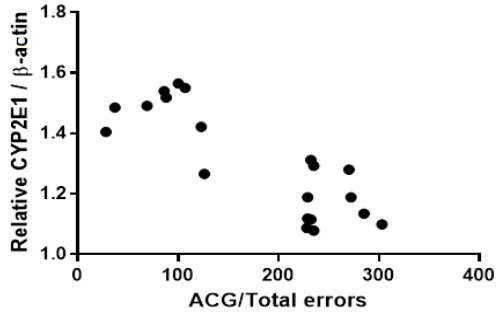


Figure 3.17: Regression plots based on individual data showing the correlation between the hippocampal expression of CYP2E1 protein (52 kDa) and the number of working memory errors (A), reference memory errors (B), and total number of errors (C) in Acquisition Criterion Group., R^2=0.5928****, p<0.0001, R^2=0.7443****, p<0.0001, R^2=0.7007****, p<0.0001,respectively).

As seen from the Figures 3.15-17, a significant negative correlation between the frequency of both working and reference memory errors and the hippocampal expression of CYP2E1was confirmed for all three stages of RAM learning.

The next enzyme to be estimated by the western blot analysis in the rat hippocampus was CYP7A1. CYP7A1 immunoreactive bands were observed at 70 kDa. Relative expression level of CYP7A1 was determined by comparing the band intensity with that of internal control β-actin (42 kDa) (Figures 3.18-20).

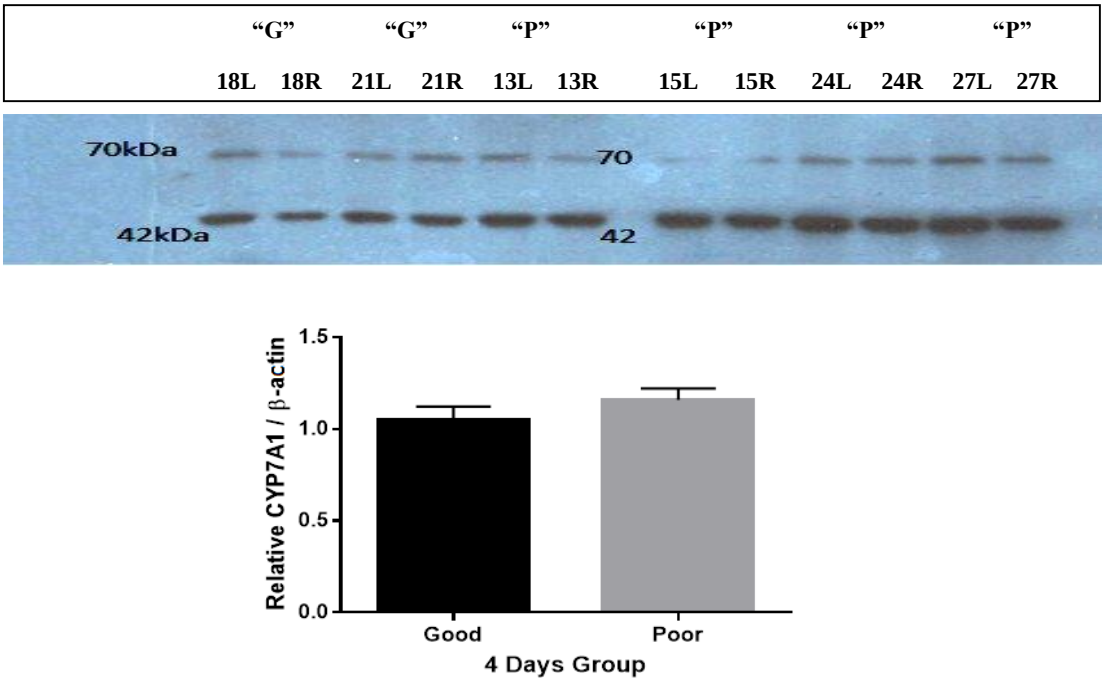


Figure 3.18: Mean relative intensity of CYP7A1 band (70 kDa) with respect to that of β-actin (42kDa), used as an internal control, for rats classified as "good" and "poor" learners in the 4-Days Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP7A1 and β-actin are presented above the graphic.

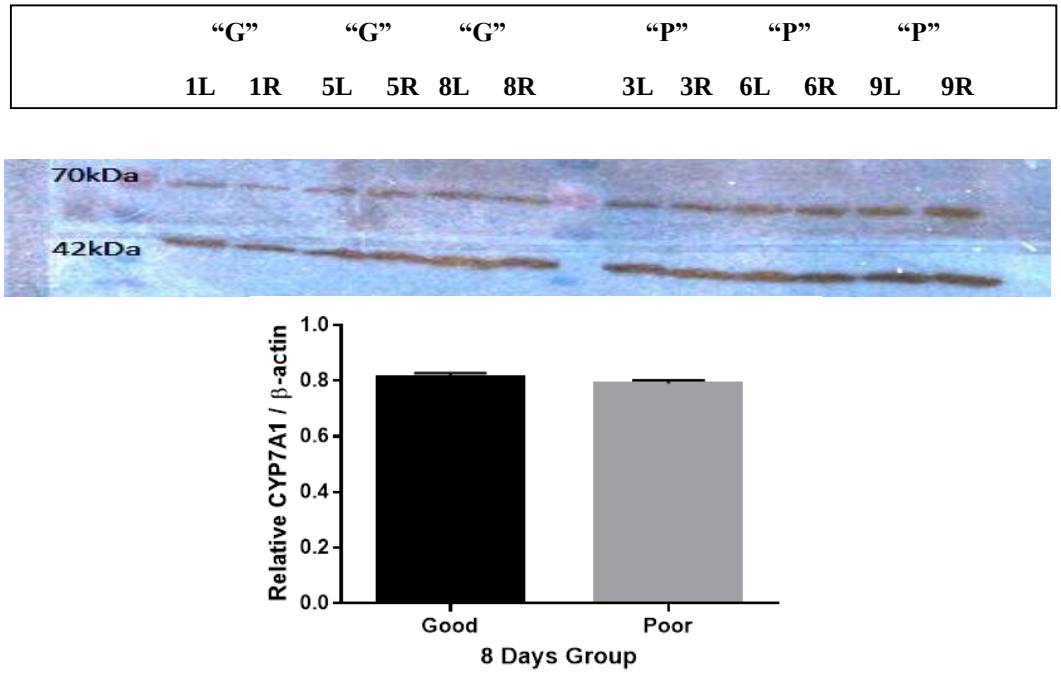


Figure 3.19: Mean relative intensity of CYP7A1 band (70 kDa) with respect to that of β-actin (42kDa), used as an internal control, for rats classified as "good" and "poor" learners in the 8 Days Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP7A1 and β-actin are presented above the graphic.

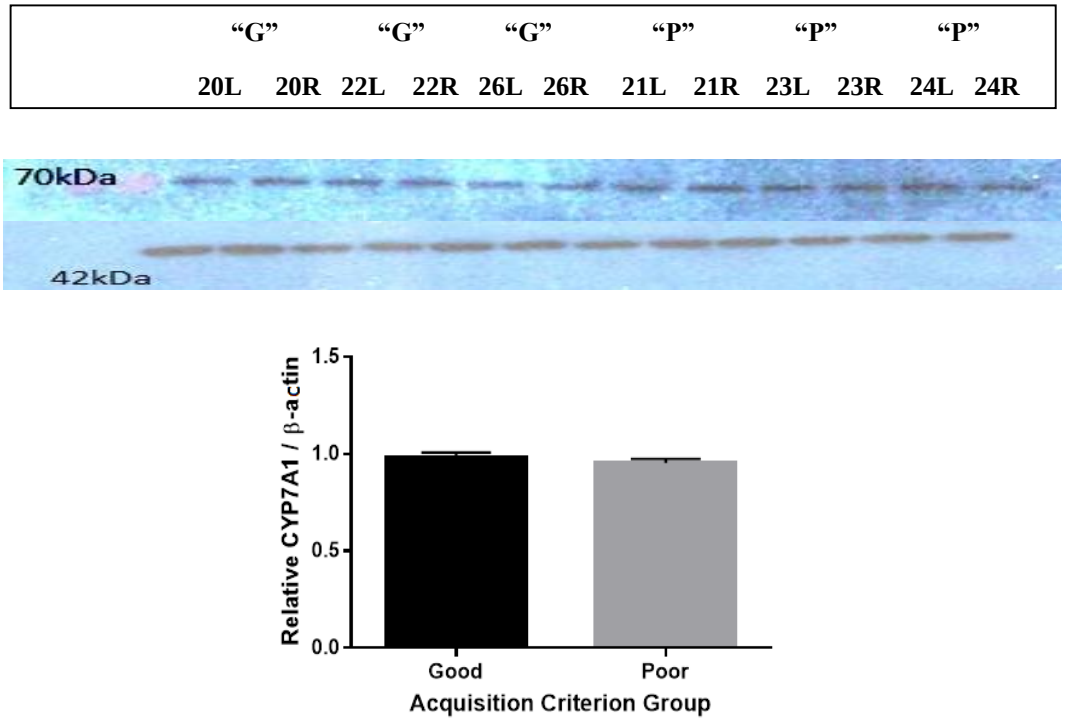


Figure 3.20: Mean relative intensity of CYP7A1 band (70 kDa) with respect to that of β-actin (42kDa) used as an internal control, for rats classified as "good" and "poor" learners in the Acquisition Criterion Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP7A1 and β-actin are presented above the graphic.

Mann Whitney test did not reveal a significant relation between animals' performance and the hippocampal CYP7A1 levels at any of the three stages of place learning in the RAM task. No difference in the CYP7A1 expression was also observed between the experimental groups and thus, between different stages of place learning.

The third enzyme considered in this study was CYP2D1. CYP2D1 immunoreactive bands were observed at 52 kDa. Relative CYP2D1 expression amount was determined by comparing the band intensity with internal control β-actin having a molecular weight of 42 kDa (Figures 3.21-23).

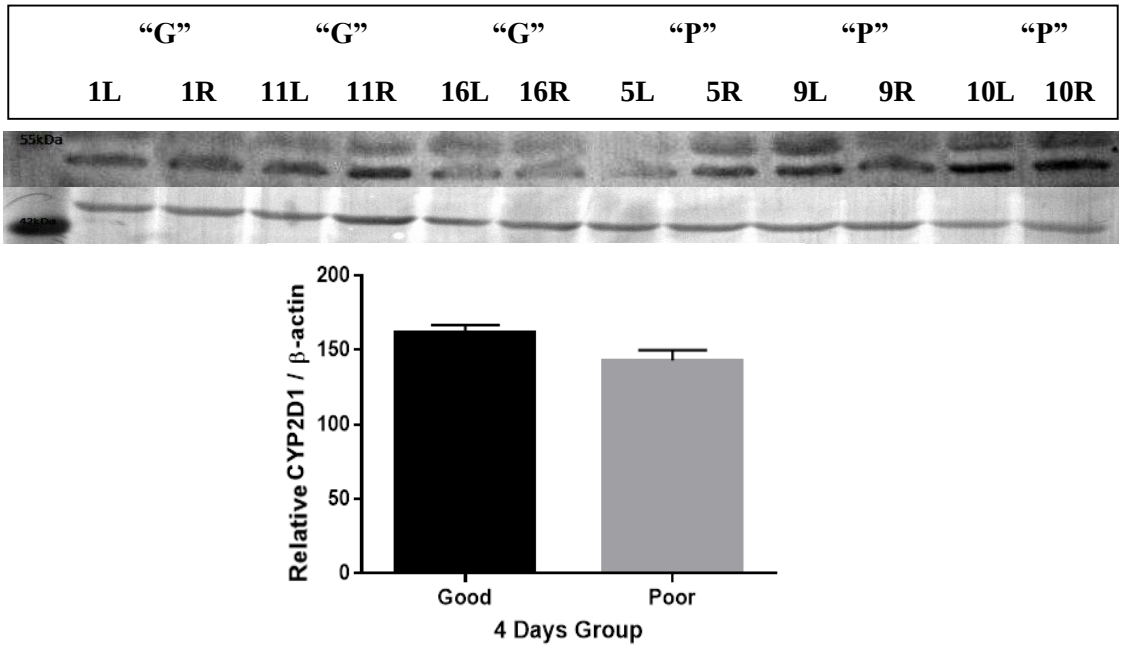


Figure 3.21: Mean relative intensity of CYP2D1 band (52 kDa) with respect to that of β-actin (42kDa) used as an internal control, for rats classified as "good" and "poor" learners in the 4 Days Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP2D1 and β-actin are presented above the graphic.

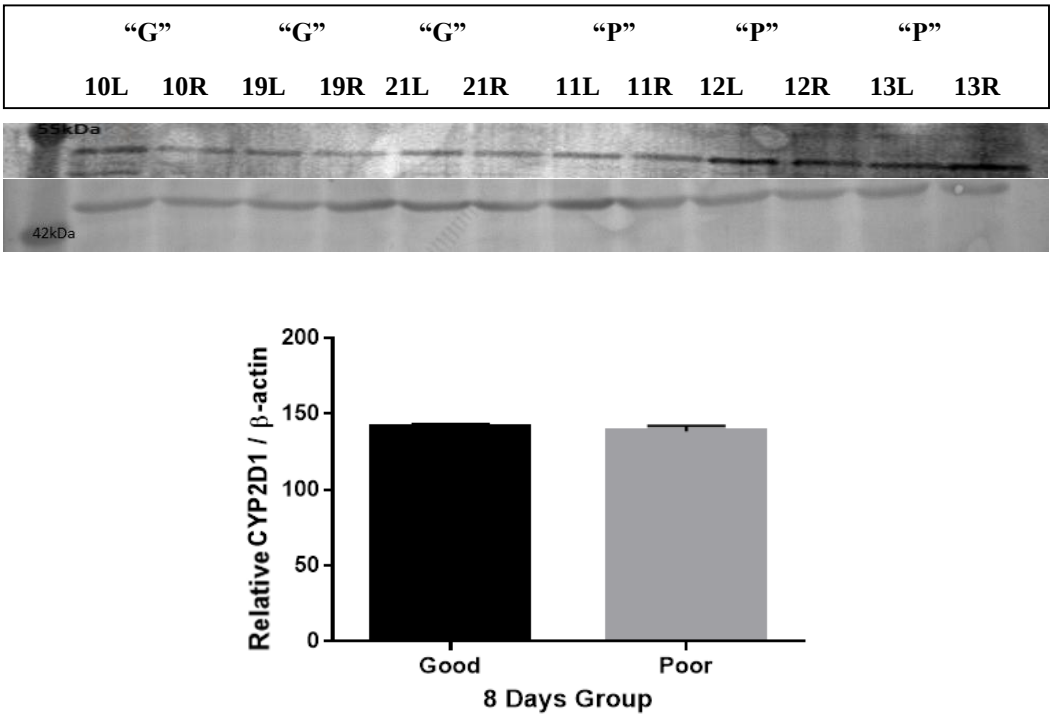


Figure 3.22: Mean relative intensity of CYP2D1 band (52 kDa) with respect to that of β-actin (42kDa) used as an internal control, for rats classified as "good" and "poor" learners in the 8 Days Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP2D1 and β-actin are presented above the graphic.

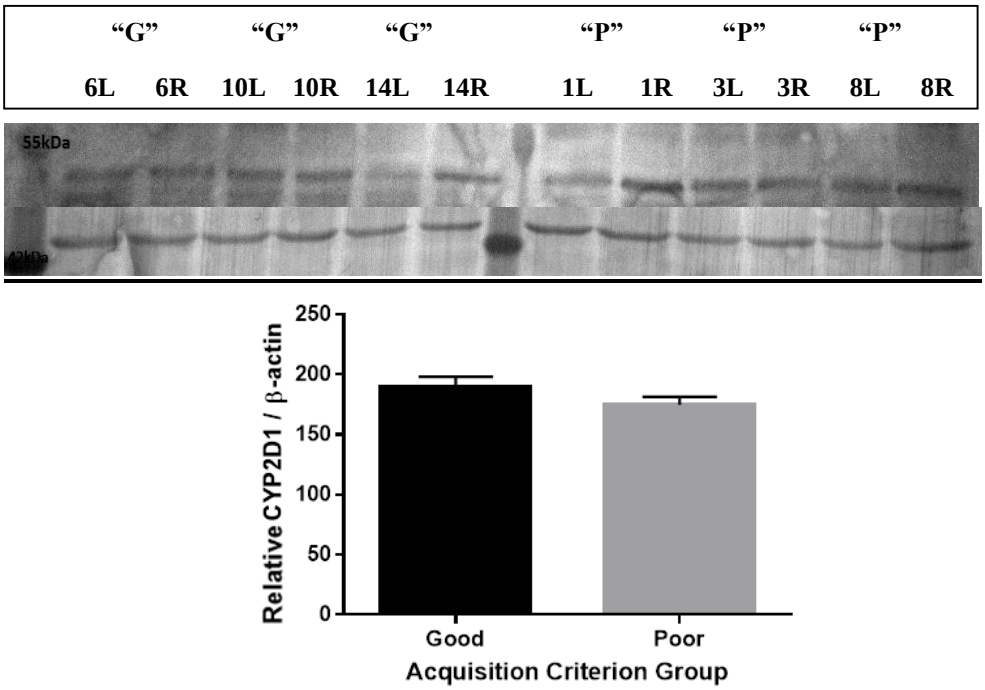


Figure 3.23: Mean relative intensity of CYP2D1 band (52 kDa) with respect to that of β-actin (42kDa) used as an internal control, for rats classified as "good" and "poor" learners in the Acquisition Criterion Group. Error bars denote ± SEM. Representative immunoreactive bands for both CYP2D1 and β-actin are presented above the graphic.

As seen from Figures 3.21-23 and confirmed by Mann Whitney test, no difference in the hippocampal expression of CYP2D1 was found either between groups (training stages) or between the "good" and "poor" learners within the groups.

Despite of many trials and optimization of parameter we did not manage to visualize immunoreactive protein bands for CYP 2B1/2 and CYP 7B1 in the rat hippocampus.

CHAPTER 4

DISCUSSION

In this study, each of the experimental groups was decapitated at different stage of place learning: the 4 Days Group at the early stage of task acquisition, the 8 Days Group at the more advanced learning stage corresponding to the memory consolidation phase, and the last Acquisition Criterion Group when the animals were over trained to the performance level of 83% or more correct choices. The difference between rats classified on the basis of their performance as "good" and "poor " learners was growing as training progressed. That is because at the early stage of learning, equivalent to the learning of the task's rules, the initial rats' choices are highly random. Consistently with the previous reports, an overall frequency of RMEs was higher than that of WMEs (Gökçek-Saraç *et al.* 2012, 2013) at all three stages of training (Figure 3.6). This difference got statistical significance at the intermediate stage of training in "good" learners and at the final criterion stage in "poor" learners.

Expression of different CYP isoform was estimated in the brain region related to spatial memory hippocampus. In this study, hippocampal expression of CYP2E1, CYP2D1 and CYP7A1 has been shown by the western blot analysis using specific antibodies. The presence of these three isoforms of CYP in the rat hippocampus was

earlier reported by Miksys and Tyndale, (2012). In contrast, despite of many attempts to optimize Western blot parameters, CYP2B1/2 and CYP7B1 immunoreactivity was not found in the studied hippocampal samples.

From the obtained results it is observed that hippocampal CYP2E1 expression is positively correlated with the learning skills in the RAM task, being higher in "good" than in "poor" learners. CYP2E1 expression showed a negative correlation with both, working and reference memory errors at all three stages of place learning. In the course of training, along with memory consolidation, CYP2E1 expression increased. As shown previously, CYP2E1 has a constitutive expression but it can be also induced by different neuroactive xenobiotics like ethanol or nicotine (Upadhya *et al.* 2000). According to our results, it can be also induced in the hippocampus in the course of place learning with hippocampal levels of CYP2E1 positively correlating with animals' learning skills. However, the mechanism underlying potential effects of CYP2E1 on cognitive learning and performance are highly speculative. Barhwal and colleagues (2015) demonstrated dendritic arborization increases and spatial learning and memory improvement in salidroside-treated hypoxic rats which was linked among the others to the increased activity of the class III histone deacetylase Sirtuin 1 (SIRT1) known to affect glucose metabolism, stress reactions and aging process. SIRT1 activity in turn was reported to be dependent on the cellular redox state regulated by CYP2E1 (Barhwal *et al.* 2015).

It is also known that in the brain, CYP2E1 is involved in the metabolism of arachidonic acid, an important signaling molecule in the brain, into two major metabolites: 19-HETE (19- hydroxyeicosatetraenoic acid) and 18-HETE (ω-2 hydroxylated metabolite) (French *et al.* 1997). On the other hand, eicosanoids are postulated to play important roles in some neural functions including sleep induction, but also long term potentiation, spatial learning and synaptic plasticity

(Tassoni *et al.* 2008). Cis-epoxyeicosatrienoic acid (EET) was reported to stimulate neuropeptide release from the pituitary gland and hypothalamus (Qu *et al.* 2001). Eicosanoids are also known as potent vasodilators contributing to the regulation of the local cerebral blood flow by enhancing outward K^+ current so hyperpolarizing membrane of pericytes and/or smooth muscle cells in the blood vessels (Qu *et al.* 2001; Iliff *et al.* 2009; Hamilton *et al.* 2010). On the other hand, it is known that the neural activity is tightly coupled to the cerebral blood flow.

However, there are also contradictory data concerning the potential positive effect of CYP2E1 activity on cognitive processes. It was shown for instance, that at least in some brain regions i.e. substantia nigra there is a negative correlation between CYP2E1 activity and extracellular DA (Nissbrandt *et al.* 2001), a well-documented memory consolidation enhancer in the hippocampus (Bethus *et al.* 2010).

CYP2D1 (human homolog CYP2D6) has also been detected in the hippocampus. This result is compatible with previous studies confirming CYP2D1 expression in different brain regions including hippocampus (Norris *et al*. 1996). According to the literature, CYP2D1 has a role in the metabolism of exogenous psychotropic substances such as antidepressants, tranquilizers, and some neurotoxins but it is also involved in the biosynthesis of neurotransmitters such as dopamine, norepinephrine, epinephrine, and serotonin (Hiroi *et al.* 1998; Srivastava *et al.* 2012). Apart from its postulated role in serotonin and dopamine metabolism, in the present study, no correlation was observed between CYP2D1 expression and learning skills. The lack of such a correlation may be either due to a negligible role of this CYP isoform in monoamine metabolism in the hippocampus or relatively minor role of serotonin and dopamine neurotransmitters in the functional regulation of hippocampal circuitry compared to glutamate, GABA and acetylcholine.

A similar result was obtained for CYP7A1. The expression of CYP7A1 isoform was documented in the rat hippocampus but no correlation with the learning skills was observed. CYP7A1 has a role in converting cholesterol to the 24S-hydroxycholesterol and maintaining a cholesterol homeostasis in the brain (Russell *et al.* 2009). Cholesterol is an important constituent of cell membrane and myelin sheets in neurons, but it is also an important precursor of several neuroactive hormones such as pregnalone, testosterone and estrogen (Ramirez, 2009). Some researchers associate the disturbance of cellular cholesterol homeostasis with neurogenerative diseases such as Alzheimer's disease (Wollmer, 2010). However, some other authors postulated that CYP7A1 might not be involved in the cholesterol metabolism in the brain since cholesterol levels and synthesis in Cyp7a1 knockout mice were the same as those of wild-type animals (Xie *et al*. 2003).

CYP7B1 has a nearly 40% homology with CYP7A1 (Nebert *et al.* 2013). It was first observed by Stapleton and coworkers (1995) in rat hippocampal cDNA library. Alike CYP7A1, it plays a role in the steroid metabolism, cholesterol homeostasis, and also in neurosteroid synthesis (Rose *et al.* 1997; 2001). It is shown that CYP7B1 mutation leads to disruption of cholesterol metabolism leading to neuron degeneration (Tsaousidou *et al.* 2008). In the present study, the failure in demonstrating the CYP7B1 expression in the rat hippocampus could be due to insufficient sensitivity of applied antibodies especially that the reported by other authors hippocampal expression of this CYP isoform was very low (Miksys and Tyndale, 2002).

CHAPTER 5

CONCLUSION

This study provided an insight into individual variation in the learning skills and memory within a random young animal population pointing a potential molecular basis of this variation. To our knowledge, this is the first study investigating the correlation between hippocampal expression of five different CYP isoforms and the individual variation in the learning skills in a random population of young rats.

In the present study, hippocampal expression of selected CYP isoforms (CYP2E1, CYP2D1, CYP7A1, CYP7B1 and CYP2B1/2) was examined in young, healthy rats classified as "good" and "poor" learners depending on their performance in the hippocampus-dependent learning task: partially baited 12-arm radial maze requiring both short- and long-term memory. Obtained results have demonstrated a correlation between CYP2E1 expression levels and the animals' spatial task performance, with CYP levels progressively increasing in the course of learning. A negative correlation between hippocampal levels of CYP2E1 and the frequency of both working and reference memory errors was noted. In contrast to this, despite of their presence in the hippocampal tissue, CYP2D1 and CYP7A1 levels did not correlate with the rats' performance in the spatial memory task. These findings manifest the differential effects of the cellular redox machinery on cognitive processes.

REFERENCES

Anzenbacher, P., Anzerbacherova E., (2003). Cytochrome P450: Review on their basic principles. *Proc. Indian natn Sci Acad.,* 6, 883-991.

Awh, E., Jonides, J., (2001). Overlapping mechanisms of attention and spatial working memory. *Trends Cognitive Science,* 5, 119–26.

Bailey, C.H., Bartsch D., Kandel, E.R., (1996). Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci*., 93, 13445-13452.

Barhwal, K., Das, S.K., Kumar, A., Hota, S.K., Srivastava, R.B. (2015). Insulin receptor A and Sirtuin 1 synergistically improve learning and spatial memory following chronic salidroside treatment during hypoxia, *J Neurochem*, 135(2), 332-46.

Bartsch, T., Butler, C., (2013). Transient amnesic syndromes. *Nat. Rev. Neurol.,* 1-12.

Bethus, I., Tse, D., Morris, R.G.M., (2010). Persistence Dopamine and Memory: Modulation of the of Memory for Novel Hippocampal NMDA Receptor-Dependent Paired Associates. *The Journal of Neuroscience*, 30(5), 1610 –1618.

Boustead, C., Taber, H., Idle, J.R., Cholerton, S., (1997). CYP2D6 genotype and smoking behavior in cigarette smokers. *Pharmacogenetics*. 7, 411-4.

Chen, X., Xia, Y., Alford, M., DeTeresa, R., Hansen, L., Klauber, M.R., Katzman, R., Thal, L., Masliah, E., Saitoh, T., (1995). The CYP2D6B allele is associated with

a milder synaptic pathology in Alzheimer's disease. *Annals of Neurology*, 38(4), 653–658.

Chiang, J.Y., (1998). Regulation of bile acid synthesis. *Front Biosci.*, 15, 3, 176-93.

Davidson, A.N., (1965). Brain Sterol Metabolism. *Adv Lipid Res*., 3: 171-196.

Ferguson, C.S., Tyndale, R.F., (2011). Cytochrome P450 enzymes in the brain: emerging evidence of biological significance. *Trends Pharmacol Sci.,* 32, 708-14.

French, S.W., Morimoto, M., Reitz, R.C., Koop, D., Klopfenstein, B., Estes, K., Clot, P., Ingelman-Sundberg, M., Albano, E., (1997). Lipid peroxidation, CYP2E1 and arachidonic acid metabolism in alcoholic liver disease in rats, *J Nutr.* 127(5 Suppl), 907-911.

Funae, Y., Kishimoto, W., Cho, T., Niwa, T., Hiroi, T., (2003). CYP2D in the brain. *Drug Met. Pharmacokin*., 18(6), 337-349.

Fyhn, M., Hafting, T., Treves, A., Moser, M.B. Moser, E.I., (2007). Hippocampal remapping and grid realignment in entorhinal cortex. *Nature* 446(7132), 190–194.

Goelet, P., Castelluci, V.F., Schacher, S., Kandel, E.R., (1986). The long and the short of long-term memory-a molecular framework. *Nature vol.,* 332, 419-422.

Gökçek-Saraç, Ç., Adalı, O. Jakubowska-Doğru, E., (2013). Hippocampal levels of chat, PKA, phospho-PKA and phosho-camkiiα but not camkiiα positively correlate with spatial learning skills in rats. *Neuroscience letters*, 545, 112-116.

Gökçek-Saraç, Ç., Karakurt, S., Adalı, O., Jakubowska-Doğru, E., (2012). Correlation between hippocampal levels of neural, epithelial and inducible NOS and spatial learning skills in rats. *Behav Brain Res.,* 235(2), 326-333.

Gonzalez, F., Gelboin, H., (1994). Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev,* 26, 165–183.

Guengerich, F.P., (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chemical Research in Toxicology*, 14(6), 611–50.

Hamilton, N.B., Attwell, D., Hall, C.N., (2010). Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. *Frontiers in Neuroenergetics,* 2(5).

Hawkins, R.D., Kandel, E.R. Bailey, C.H., (2006). Molecular mechanisms of memory storage in Aplysia. *Biol Bull*., 210, 174-191.

Hedlung, E., (2001). Cytochrome P450 in the brain: a review. *Curr. Drug metab,* 2, 245-263.

Hiroi, T., Imaoka, S., Funae, Y., (1998). Dopamine formation from tyramine by CYP2D6. *Biochem Biophys Res Commun.,* 249, 838-43.

Hiroi, T., Imaoka, S., Chow, T., Funae, Y., (1998). Tissue distributions of CYP2D1, 2D2, 2D3 and 2D4 mRNA in rats detected by RT-PCR. *Biochimica et Biophysica Acta.*, 1380, 305–312.

Hiroi, T., Chow, T., Imaoka, S., Funae, Y., (2002). Catalytic specificity of CYP2D isoforms in rat and human. *Drug Metab Dispos.,* 30(9), 970-6.

Hodges, H., (1996). Maze procedures: the radial-arm and water maze compared. *Cog Brain Res,* 3, 167‑81.

Honkakoski, P., Negishi, M., (1997). Characterization of a phenobarbital-responsive enhancer module in mouse P450 Cyp2b10 gene. *J. Biol. Chem*. 272, 14943–14949.

Iliff, J.J., Wang, R., Zeldin, D.C., Alkayed, N.J., (2009). Epoxyeicosanoids as mediators of neurogenic vasodilation in cerebral vessels. *Am J Physiol Heart Circ Physiol*. 296(5), 1352-1363.

Ingelman-Sundberg, M., (2004). Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn-Schmiedeberg's Arch Pharmacol.*, 369, 89‑104.

Izquerdo, I., Medina, J.H., Vianna, M.R.M., Izquierdo, L.A., Barros, D.M., (1999). Separate mechanisms for short- and long-term memory. *Behavioural Brain Research*, 103, 1–11.

Jakubowska, D.E., Gümüşbaş, U., Kara, F., (2003). Individual variation in the spatial reference and working memory assessed under allothetic and idiothetic orientation cues in rat. *Acta Neurobiol. Exp.*, 63, 17-23.

Kandel, E.R., (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294, 1030-1038.

Kandel, E.R., Schwartz, J.H., Jessell, T.M., Siegelbaum, S.A., Hudspeth, A.J., (1991). Principles of Neural Science. *McGraw Hill*.

Komori, M., (1993). A novel P450 expressed at the high level in rat brain. *Biochem Biophys Res Commun.*, 196, 721-8.

Kubik, S., Miyashita, T., Guzowski, J.F., (2007). Using immediate-early genes to map hippocampal subregional functions. *Learning & Memory*, 14, 758–770.

Kurth, M.C., Kurth, J.H., (1993). Variant cytochrome P450 CYP2D6 allelic frequencies in Parkinson's disease, *Am J Med Genet*, 15, 48(3), 166-8.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227, 680–685.

Lathe, R., (2002). Steroid and sterol 7-hydroxylation: ancient pathways, *Steroids*, 67(12), 967-77.

Lieber, C.S., (2004). Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol*, 34(1), 9–19.

Lømo, T., (2003). The discovery of long-term potentiation. *Philos Trans R Soc Lond B Biol Sci.* 358(1432), 617–620.

Lynch, M.A., (2004). Long-term potentiation and memory. *Physiol Rev*., 84, 87-136.

Martignoni, M., Groothuis, G.M.M., de Kanter, R., (2006). Species differences between mouse, rat, dog, monkey and human cytochrome P450-mediated drug metabolism. *Expert Opinion on Drug Metabolism et Toxicology*, 8-44.

Miksys, S., Tyndale, R.F., (2002). Drug-Metabolizing Cytochrome P450s in the brain. *J Psychiatry Neurosci.*, 27(6), 406-15.

Miller, M., Warner, S., Jorquera, R., Caston-guay, A., Schüller, H., (1992). Expression of the cytochrome P4502E and 2B gene families in the lungs and livers of nonpregnant, pregnant, and fetal hamsters. *Biochem. Pharmacol*. 44, 797–803.

Mori, Y., Iimura, K., Furukawa, F., Nishikawa, A., Takahashi, M., Konishi, Y., (1995). Effect of cigarette smoke on the mutagenic activation of various carcinogens in hamster. *Mutat. Res*. 346, 1–8.

Morris, R.G.M., (2006). Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. *European Journal of Neuroscience*, 23, 2829–2846.

Nebert, D.W., Russell, D.W., (2002). Clinical importance of the cytochromes P450, *Lancet*, 12, 360(9340), 1155-62.

Nebert, D.W., Wikwall, K., Miller, W.L., (2013). Human cytochrome 450 in health and disease. *The Royal Society*, 368, 1-22.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W., (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature, *Pharmacogenetics*, 6(1), 1-42.

Nissbrandt, H., Bergquist, F., Jonason, J., Engberg, G., (2001). Inhibition of cytochrome P450 2E1 induces an increase in extracellular dopamine in rat substantia nigra: a new metabolic pathway?, *Synapse*, 40(4), 294-301.

Niznik, H.B., Tyndale, R.F., Sallee, F.R., Gonzales, F.J., Hardwick, J.P., Inaba, T., Kalow, W., (1990). The dopamine transporter and cytochrome P450IID1 (debrisoquine 4-hydroxylase) in brain: Resolution and identification of two distinct [^{3}H]GBR-12935 binding proteins. *Archives of Biochemistry and Biophysics*, 276(2), 424–432.

Norlin, M., Wikwall, K., (2007). Enzymes in the Conversion of Cholesterol into Bile Acids. *Current Molecular Medicine*, 7(2), 199-218.

Norris, P.J., Hardwick, J.P., Emson, P.C., (1996). Regional distribution of cytochrome P450 2D1 in the rat central nervous system, *J. Comp. Neurol*., 366(2), 244-58.

Novak, R.F., Woodcroft, K.J., (2000). The alcohol-inducible form of cytochrome P450 (CYP2E1): Role in toxicology and regulation of expression. *Archives of Pharmacal Research*, 23(4), 267-282.

O'Keefe, J., Dostrovsky, J., (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Research*, 34(1), 171-5.

O'Keefe, J., Nadel, L., (1978). The hippocampus as a cognitive map. *Oxford: Clarendon Press*.

Okano, H., Hirano, T., Balaban, E., (2000). Learning and Memory. *PNAS*, 97, 12403-12404.

Olton, D.S., Samuelson, R.J., (1976). Remembrance of place passed. Spatial memory in rats. *Journal of Experimental Psychology: Animal Behavior Processes*, 2, 97–116.

Olton, D.S., Becker, J.T., Handelmann, G.E., (1979). Hippocampus, space and memory. *The Behavioural and Brain Sciences*, 2, 313-365.

Packard, M. G., Hirsh, R., White, N. M., (1989). Differential effects of fornix and caudate nucleus lesions on two radial maze tasks: evidence for multiple memory systems. *Journal of Neuroscience*, 9, 1465-1472.

Paratore, S., Alessi, E., Coffa, S., Torrisi, A., Mastrobuono, F. Cavallaro, S. (2006). Early genomics of learning and memory:a review. *Genes, brain and behavior*, 5, 209-221.

Paul, C.M., Magda, G. Abel, S., (2009). Spatial memory: Theoretical basis and comparative review on experimental methods in rodents. *Behavioral Brain Research*, 203, 151-164.

Pikuleva, I.A., (2006). Cholesterol- metabolizing cytochromes p450. *Drug metabolism and disposition*, 34, 513‑520.

Qu, W., Bradbury, J.A., Tsao, C.C., Maronpot, R., Harry, G.J., Parker, C.E., Davis, L.S., Breyer, M.D., Waalkes, M.P., Falck, J.R., Chen, J., Rosenberg, R.L., Zeldin, D.C., (2001). Cytochrome P450 CYP2J9, a new mouse arachidonic acid omega-1 hydroxylase predominantly expressed in brain, *J Biol Chem.*, 6, 276(27), 25467-79.

Quillfeldt, J.A., (2006). Behavioral Methods to Study Learning and Memory in Rats. 1-42.

Quinn, M., DeMorrow S. (2012). Bile in the brain? A role for bile acids in the central nervous system, *J Cell Sci Ther*, 3(7).

Ramirez, D.M.O., (2009). Role of cholesterol 24-hydroxylase in hippocampal long-term potentiation, PhD. thesis.

Rose, K., Allan, A., Gauldie, S., Stapleton, G., Dobbie, L., Dott, K., Martin, C., Wang, L., Hedlund, E., Seckl, J.R., Gustafsson, J.A., Lathe, R., (2001). Neurosteroid hydroxylase CYP7B: vivid reporter activity in dentate gyrus of gene-

targeted mice and abolition of a widespread pathway of steroid and oxysterol hydroxylation, *J Biol Chem*, 276, 23937–23944.

Rose, K.A., Stapleton, G., Dott, K., Kieny, M.P., Best, R., Schwarz, M., Russell, D.W., Björkhem, I., Seckl, J., Lathe, R., (1997). Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7alpha-hydroxy dehydroepiandrosterone and 7alpha-hydroxy pregnenolone, *Proc Natl Acad Sci*, 94(10), 4925-30.

Russell, D.W., Halford, R.W., Ramirez, D.M.O., Shah, R., Kotti, T., (2009). Cholesterol 24-Hydroxylase: An Enzyme of Cholesterol Turnover in the Brain, *Annu Rev Biochem*, 78, 1017–1040.

Seliskar, M., Rozman, D., (2007). Mammalian cytochromes P450--importance of tissue specificity. *Biochimica et Biophysica Acta*, 1770(3), 458–66.

Squire, L.B., Knowlton, B., Musen, G., (1993). The Structure and Organization of Memory. *Annual Review of Psychology*, 44, 453-495.

Squire, L.R., Kandel, E.R., (1999). Memory from minds to molecules. *Scientific American Library*.

Srivastava, G., Dixit, A., Yadav, S., Patel D.K., Prakash, O., Singh, M.P., (2012). Resveratrol potentiates cytochrome P450 2 d22-mediated neuroprotection in maneb- and paraquat-induced Parkinsonism in the mouse, *Free Radical Biology & Medicine*, 52, 1294-1306.

Stapleton, G., Steel, M., Richardson, M., Mason, J.O., Rose, K.A., Morris, R.G., Lathe, R., (1995). A novel cytochrome P450 expressed primarily in brain, *J. Biol. Chem*, 270(50), 29739–45.

Suzuki, W.A., Amaral, D.G., (2004). Functional neuroanatomy of the medial temporal lobe memory system. *Cortex*, 40(1), 220-2.

Tassoni, D., Kaur, G., Weisinger, R.S., Sinclair, A.J., (2008). The role of eicosanoids in the brain. *Asia Pac J Clin Nutr*, 17(S1), 220-228.

Tindberg, N., Ingelman-Sundberg, M., (1996). Expression, catalytic activity, and inducibility of cytochrome P450 2E1 (CYP2E1) in the rat central nervous system, *J Neurochem*, 67(5), 2066-73.

Towbin, H., Staehelin, T., Gordon, J., (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc Natl Acad Sci*, 76(9), 4350-4.

Tsaousidou, M.K., Ouahchi, K., Warner, T.T., Yang, Y., Simpson, M.A., Laing, N.G., Wilkinson, P.A., Madrid, R.E., Patel, H., Hentati, F., Patton, M.A., Hentati, A., Lamont, P.J., Siddique, T., Crosby, A.H., (2008). Sequence alterations within CYP7B1 implicate defective cholesterol homeostasis in motor-neuron degeneration, *Am J Hum Genet*, 82(2), 510–5.

Upadhya, S.C., Tirumalai, P.S., Boyd, M.R., Mori, T., Ravindranath. (2000). Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization, *Arch Biochem Biophys*, 373(1), 23-34.

Venhorst, J., ter Laak, A.M., Commandeur, J.N., Funae, Y., Hiroi, T., Vermeulen, N.P., (2003). Homology modeling of rat and human cytochrome P450 2D (CYP2D) isoforms and computational rationalization of experimental ligand-binding specificities, *J Med Chem.* 2, 46(1), 74-86.

Waxman, D.J., (1988). Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression, *Biochem Pharmacol*, 37, 71-84.

Web page of Laboratory tools (available online at https://www.thermofisher.com/order/catalog/product/26616, last access date 12.11.2015).

Web page of Neuroscience related articles (available online at http://www.neuroscientificallychallenged.com/blog/2014/5/23/know-your-brain-hippocampus, last access date 17.11.2015).

Web page of medical biochemistry (available online at http://themedicalbiochemistrypage.org/bileacids.php, last access date 20.11.2015).

Web page of Rodent research models (available online at http://www.janvier-labs.com/rodent-research-models-services/research-models/per-species/outbred-rats/product/sprague-dawley.html, last access date 12.11.2015).

Web page of Protein modal portal (available online at http://www.proteinmodelportal.org, last access date 19.11.2015).

Whishaw, I.Q., Brooks, B.L., (1999). Calibrating space: exploration is important for allothetic and idiothetic navigation. *Hippocampus*. 9(6), 659-67.

Wollmer, M.A., (2010). Cholesterol-related genes in Alzheimer's disease, *Biochimica et Biophysica Acta*, 1801, 762 –773.

Xie, C., Lund, E.G., Turley, S.D., Russell, D.W., Dietschy, J.M., (2003). Quantitation of two pathways for cholesterol excretion from the brain in normal mice and mice with neurodegeneration. *Journal of lipid research*, 44(9), 1780-1789.

Yamazoe, Y., Shimada, M., Murayama, N., Kato, R., (1987). Suppression of levels of phenobarbital-inducible rat liver cytochrome P-450 by pituitary hormone, *J Biol Chem*, 25, 262(15), 7423-8.

Yoshida, K., Oka, H., (1995). Topographical projections from the medial septum-diagonal band complex to the hippocampus: a retrograde tracing study with multiple florescent dyes in rats. *Neuroscience Research*, 21(3), 199-209.

Zuber, R., Modrianský, M., Dvořák, Z., Rohovský, P., Ulrichová, J., Šimánek, V., Anzenbacher, P., (2002). Effect of Silybin and its congeners on human liver microsomal cytochrome P450 activities. *Phytotherapy Research*, 16(7), 632–638.

APPENDIX

ORTA DOĞU TEKNİK ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURUL KARARLARI

KARAR SAYISI : 04	**KARAR TARİHİ: 04.03.2014**

ODTÜ Biyolojik Bilimler Bölümü Araştırma Görevlisi Doç. Dr. Ewa Doğru'nun Etik-2014/4 no'lu "Sıçan Hipokampusunda Mekansal Öğrenmenin Farklı Safhalarında Bazı Sitokrom P450 (CYP) Enzim Tiplerinin Ekspresyon Seviyelerinin İncelenmesi" başlıklı araştırma projesinin etik başvurusu değerlendirilmiştir.

Etik Kurul'un 03.03.2014 tarihinde yapılan toplantısında, yukarıda adı geçen projenin araştırmadaki deney hayvanlarının tür, sayı ve kullanım amaçlarının ve projenin deney hayvanlarına ilişkin yönlerinin O.D.T.Ü. Hayvan Deneyleri Yerel Etik Kurul Yönergesinde belirtilen "Hayvan Deneyleri ile İlgili Etik İlkeler" dikkate alınarak hazırlandığı saptanmıştır. Bu değerlendirme sonucu çalışmanın yapılmasının hayvan etiği açısından uygun olduğu oy çokluğu/oy birliği ile onaylanmıştır.

Prof. Dr. Gülay Özcengiz Başkan	Doç.Dr.Mesut Muyan Üye,Başkan Vekili
Prof. Dr. Orhan Adalı Üye	Prof. Dr. Vasıf Hasırcı Üye
Prof. Dr. İnci Togan Üye	Jale Esin Sivil Üye
Doç. Dr. Senih Gürses Üye	Doç. Dr. Barış Parkan Üye
Doç.Dr.Sreeparna Banerjee Üye	Şakir Yıldıran Vet. Hekim, Üye