ANALYSIS OF IMMUNOREACTIVITY OF NOS ISOFORMS (nNOS, eNOS, iNOS) IN HIPPOCAMPUS OF YOUNG RATS CLASSIFIED AS GOOD AND POOR LEARNERS

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ABSTRACT

ANALYSIS OF IMMUNOREACTIVITY OF NOS ISOFORMS (nNOS, eNOS, iNOS) IN HIPPOCAMPUS OF YOUNG RATS CLASSIFIED AS GOOD AND POOR LEARNERS

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Despite very extensive studies on molecular mechanisms of learning and memory formation it is little known about individual variation in the learning skills within a random animal population and about the differences in the brain biochemistry behind this variation. In the present study, we have focused on the expression and distribution of nitric oxide synthase (NOS), one of the molecules implemented in activity-dependent neuroplasticity, in the rat hippocampus, the structure critical for episodic memory in humans and animals. The aim of the present study was to investigate the differences in expression of three different NOS isoforms: neural (n), epithelial (e), and inducible (i), in four hippocampal subregions (CA1, CA3, DG, and hilus) between Wistar rats classified on the basis of their performance in partially baited 12-arm radial maze as "good" and "poor" learners. The NOS isoforms were visualized on coronal hippocampal sections using fluorescent

immunohistochemistry technique and n- and eNOS images were processed using ImageJ software, while iNOS immunoreactivity (IR) was assessed by counting immunoreactive cells. In this study, overall hippocampal levels of nNOS were significantly higher than those of eNOS and iNOS. The level of n and eNOS was higher in CA1 compared to DG/hilus areas, but lower than that in CA3 region. The expression of iNOS was the highest in CA1 and the lowest in hilus region. nNOS IR was significantly higher in "poor" than in "good" learners but only in CA1 region. No significant between-group differences were found in eNOS expression. iNOS expression was higher in "poor" learners but it did not reach the required significance level.

Keywords: spatial learning, nitric oxide, immunohistochemistry, nitric oxide synthase, nNOS, eNOS, iNOS

ÖZ

İYİ VE KÖTÜ ÖĞRENEN OLARAK SINIFLANDIRILAN GENÇ SIÇANLARIN HİPOKAMPUS BEYİN BÖLGESİNDE NİTRİK OKSİT SENTAZ (NOS) ENZİMİ İZOFORMLARININ İMMUNOREAKTİVİTESİNİN TAYİNİ

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Öğrenme ve bellek oluşumunun moleküler mekanizmaları hakkında pekçok çalışma bulunmasına karşın rastgele seçilmiş hayvan topluluklarında öğrenme becerileri bakımından bireyler arası varyasyon ve bu varyasyonun temelinde yatan beyin biyokimyasına ilişkin farklılıklar hakkında pek az bilgi bulunmaktadır. Sözkonusu çalışmada, aktiviteye bağlı olarak görülen nöroplastisitede rol oynadığı belirtilen nitrik oksit sentaz (NOS) enziminin sıçan hippokampüsünde ekspresyonu ve dağılımına odaklanılmıştır. Bu çalışmanın amacı, Nitrik Oksit Sentaz enziminin nöronal (nNOS), endotelyal (eNOS) ve indüklenebilir NOS (iNOS) olmak üzere üç izoformunun ekspresyonundaki farklılıkların kısmi pekiştirilmiş 12 kollu ışınsal labirentteki

performanslarına bağlı olarak “iyi” ve “kötü” öğrenen olarak sınıflandırılan Wistar sıçanların dört farklı hippokampal alt bölgesinde (CA1, CA3, dentat girus ve hilus) incelenmesidir. NOS izoformları koronal beyin kesitleri üzerinde floresan immünohistokimya tekniği ile görüntülenmiştir. nNOS ve eNOS görüntüleri Image J programı ile incelenirken iNOS immünoreaktivitesinin tayini iNOS pozitif hücrelerin sayımı yoluyla gerçekleştirilmiştir. Bu çalışmada elde edilen bulguların ışığında, nNOS ve eNOS seviyelerinin CA1 bölgesinde dentat girus ve hilus bölgelerine kıyasla yüksek olmakla birlikte CA3 bölgesine göre düşük olduğu ortaya çıkarılmıştır. iNOS ekspresyonunun en yüksek CA1 bölgesinde, en düşük ise hilus bölgesinde gözlenmiştir. nNOS immünoreaktivitesi, kötü öğrenenlerin CA1 bölgesinde iyi öğrenenlere nazaran istatistiksel açıdan anlamlı derecede fazla bulunmuştur. eNOS ekspresyonunda gruplar arasında anlamlı fark bulunamamıştır. iNOS ekspresyonu ise kötü öğrenenlerde iyi öğrenenlere kıyasla fazla olmakla birlikte yeterli anlamlılık düzeyine erişmemiştir. Başka bir deyişle, iNOS ekspresyonu bakımından gruplar arasında anlamlı fark gözlenmemiştir.

Anahtar kelimeler: mekansal bellek, nitric oksit, immünohistokimya, nitric oksit sentaz, nNOS, eNOS, iNOS

To My Family,

For your endless support and love

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LIST OF ABBREVIATIONS

#	Number
°C	Degree Celsius
µm	Micrometer
mm	Millimeter
cm	Centimeter
µg	Microgram
w/v	Weight per volume
M	Molarity
a.m.	*ante meridiem*
p.m.	*post meridiem*
WME	Working Memory Error
RME	Reference Memory Error
RAM	Radial Arm Maze
MWM	Morris Water Maze
NO	Nitric Oxide
cNOS	Constitutive Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
eNOS	Endothelial Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
macNOS	Macrophage Nitric Oxide Synthase
CA1	Cornu ammonis-1
CA2	Cornu ammonis-2
CA3	Cornu ammonis-3
CA4	Cornu ammonis-4
DG	Dentate gyrus
i.p.	Intraperitoneal
PB	Phosphate buffer

PBS	Phosphate Buffered Saline
NGS	Normal goat serum
BSA	Bovine serum albumin
IgG	Immunoglobulin G
Ag	Antigen
Ab	Antibody
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
IHC	Immunohistochemistry
ICC	Immunocytochemistry
PFA	Paraformaldehyde
SEM	Standard error of mean
O.D.	Optical density
MANOVA	Multivariate analysis of variance
Ca^{2+}	Calcium ion
K^{+}	Potassium ion
Mg^{2+}	Magnesium ion
a.a.	Aminoacid
Glu	Glutamate
LTP	Long-term potentiation
LTD	Long-term depression
NMDA	*N*-Methyl-D-aspartic acid
NMDAR	NMDA receptor
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A (cAMP-dependent protein kinase)
cGMP	Cyclic guanosine monophosphate
PKC	Protein kinase C
sGC	Soluble guanylyl cyclase

mGluR	Metabotropic glutamate receptor
IEG	Immediate early gene
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
PI3K	Phosphatidylinositol 3-kinase
TyrK	Tyrosine kinase
MAPK	Mitogen-activated protein kinase
ERK	Extracellular-signal-regulated kinase
PP-1	Protein phosphatase-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NADPH-d	Nicotinamide adenine dinucleotide phosphate diaphorase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
BH-4	Tetrahydrobiopterin
PSD95	Post synaptic density protein
GTP	Guanosine-5'-triphosphate
L-NAME	N-nitro-L-arginine methyl ester
L-NA	L- nitro arginine
Arg $(NO)_2$	Nitro arginine
7-NI	7-nitroindazole
GFAP	Glial fibrillary acidic protein

CHAPTER 1

INTRODUCTION

1.1. Learning and Memory

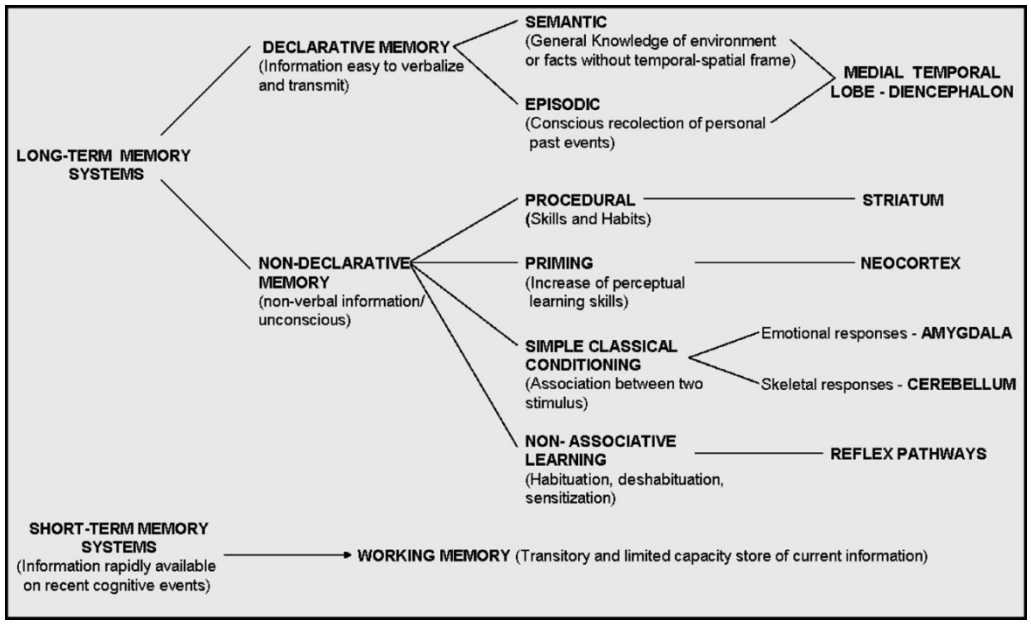
1.1.1. Definition and classification of learning and memory

From an evolutionary perspective, learning and memory are lifelong mental processes that enable organisms to adapt themselves to continuously changing environmental conditions, reaching resources with less effort and avoiding various threats that they are facing in the natural environment.

Neuroscientists describe learning as the process of acquiring knowledge about the world often resulting in more or less permanent change in behavior (Kimble *et al*., 1961). Memory is defined as the retention or storage of acquired knowledge in the central nervous system. Learning and memory are the most important means by which environment alters behavior in animals and humans.

Main categories of memories classified according to these criteria and anatomical structures thought to be responsible from these memory types are summarized in the Table 1.

Table 1. Classification of memory (after Paul *et al.*, 2009).



Memory of things that can be conventionally transmitted and expressed ("declared") are called declarative. Declarative memory is also a category that includes memory for general facts (semantic e.g. Paris is the capital of France) and memory for autobiographic, personally experienced events (episodic, e.g., I have born in August). Non-declarative memory represents the information about perceptual and motor abilities that are not necessarily orally transmitted (Paul *et al*., 2009). Due to this expression or oral transmission criteria, in some resources, declarative memory is called as "explicit memory" whereas non-declarative memory as "implicit memory".

1.1.2. Episodic memory

As a part of declarative memory, episodic memory basically deals with coding of personally experienced events and answers the basic questions such as "what happened", "where happened", and "when happened". However, the

content of episodic memory cannot be restricted to past events (retrospective encoding), processing of information encoded via past episodes and relations among them can result with planning for near future, striving for a goal (Prospective coding) (Tulving, 1972, 2001, 2002; Tulving and Markowitsch, 1998). The ability to code for both past and future events arises the question whether episodic memory is "one to one" reconstruction of the original experience or some changes should occur while an episode is encoded and then recalled. On the other hand, there is also a view that episodic memory is a reconstruction of events and these events are defined according to the relationships among different items present at the same time as a combination of multiple elements of information (Eichenbaum *et al*., 1999).

Episodic memory may be encoded by a highly distributed neuronal network including medial temporal lobe (MTL) and prefrontal cortex. On this network, studies suggest that MTL is required for the acquisition and information storage (Nyberg et al., 2000; Burgess et al., 2002) while prefrontal cortex activation is observed during searching a particular information, monitoring, outcomes of an event or choice. Furthermore, selection of a goal and pursuit of multiple subgoals, formulating plans to reach goal and behavioral monitoring and control also co-occur with activity of prefrontal cortex (Wheeler *et al*., 1995, 1997; Nyberg *et al*., 2000; Buckner and Wheeler, 2001; Burgess *et al*., 2001a, b; Burgess *et al*., 2002; Buckner, 2003; Wheeler and Stuss, 2003; Hayes *et al*., 2004). Prefrontal cortex is best known for its role in working memory and decision making at the same time as being a part of the limbic system adapting our motivated behaviors (motivated by i.e. hunger or fear) to the current stimulus conditions. For example, if we know that there is no food around, we save our energy and do not search for food.

Up to now, episodic memory is explained from the perspective of human cognition and behavior. If the relevant cognitive and behavioral properties can

be expressed as functional definitions and they are integrated in suitable behavioral tasks, animal models are also applicable for investigating the neural substrate underlying of episodic memory.

Earlier examples of animal studies of episodic memories adapted to rats involved what-when and where aspects of episodic memory (Eacott and Norman, 2004; Ergorul and Eichenbaum, 2004; Eacott *et al.*, 2005). Accordingly, tasks that use only a subgroup of features of episodic memory — what and when, what and where, or when and where— in an integrated, flexible manner and use this information prospectively or retrospectively to guide goal-oriented responses and recordings done during these tasks can still provide information about mechanisms of it. The subsets emphasized above are described in the literature as "episodic-like aspects" and memory studied in model organisms and encoding any of these pairs alone or, "what-where and when" together is named as "episodic-like memory". One of the successful examples of this approach is investigated by Clayton and colleagues with scrub jays (*Aphelocoma coerulescens*). Scrub jays are one of food-storing bird species. It is reported that food-storing birds can remember which kind of food they cached and the location of it (Clayton *et al*., 1996). Food material cached can also expire before animals consume them and therefore, these animals should adapt several strategies their caching and when recover them according to decay time of food material (Gendron *et al*., 1995). In this study, it is showed that scrub jays remembered 'when' food items were stored by recollecting perishable 'wax worms' (wax- moth larvae) and non-perishable peanuts accordingly which they had previously cached in visuospatially distinct sites. They also first searched for fresh wax worms, their favored food, when allowed to get back them shortly after caching. However, they rapidly learned to avoid searching for worms after a longer interval during which the worms had decayed. The recovery preference of jays demonstrates that they were able to recall memory of where and when particular food items were

cached, thereby satisfying the behavioral criteria for episodic-like memory in non-human animals.

What-when aspects of episodic-like memory in animals are also applicable to encode spatial memory. Especially, rodents perform these spatial memory tasks with success since their natural habitat including underground burrows reminding complex labyrinths or open meadows with landmarks such as trees and rocks requires orienting themselves in the space. In addition to this, maintenance of animal models and setup are easy and cheap. Therefore, investigation of spatial memory in rodents can provide adequate information about mechanisms of episodic memory in humans.

1.1.3. Spatial memory

All animal species have tendency to navigate especially when they are in a new environment, to explore it in order to find food and shelter or safe place to survive. This is an instinctive and highly conserved behavior across species including human. Thus, spatial memory can be delineated as that brain function responsible for recollecting, coding, storing and recalling spatial information about the arrangement of objects or specific routes (Kessels *et al.*, 2001).

The concept of space involves at least two dimensions, the personal-corporal space—which includes the information about location of the body itself, the knowledge of the position of limbs, etc., and the external space. The information obtained from these sources is organized and used by two ways: a) *Egocentric strategy:* It is based on the information provided by bodily cues (idiothetic cues), and therefore it is independent of spatial cues. Navigation depending on this strategy is called as "path integration". b) *Allocentric*

strategy: It depends on spatial (allothetic) cues. When using this strategy, the subject memorizes the location that it wants to reach (target) in relation to the spatial position of the environmental reference landmarks that are independent of the observer (Benhamou *et al.* 1996), meaning that it is based on a spatial representation. The location of a particular target is established through a system of coordinates; this system often uses distant or closer points of reference; i.e., latitude and distance used by humans to locate places in a map (Klatzky, 1998; O'Keefe *et al*., 1978; Burgess, 2006 and Rains, 2004).

Experimental evidence revealed that processing of active memory items for short-term (spatial working memory) and a long-term storage of spatial locations (spatial reference memory) are two related but still dissociated systems (Moscovitch *et al*. 2005). The spatial working memory is a system that allows temporal storage of a limited amount of spatial information, and keeps it available for immediate access. On the other hand, the spatial reference memory system was first proposed by Olton to designate the type of memory involved in obtaining spatial information over various trials. In contrast to spatial working memory, spatial reference memory exhibits more capacity, duration and resistance to interference (Olton *et al*. 1979).

1.1.3.1. Evaluating spatial memory in rodents experimentally

Because small rodents generally have good spatial memory, mazes are the experimental devices commonly used in mnemonic studies on rats and mice. Since they may be used indoors, they allow to control experimental conditions.

For experimental paradigms designed with mazes, it is generally assumed that in their natural habitat, animals have tendency to learn locations that provide them security, food, water or other kinds of benefits or just opposite the places

that are dangerous and they remember these locations and easily revisit or avoid them. Based on this assumption, some positive (food, water, sweetened water, shelter or the opportunity to explore new objects) or negative reinforcers (exposure to water immersion, intense light, wind, a loud noise or electric shock etc.) are used in maze experiments.

Choice of maze type and reinforcers directly affect animals' performance during the task and they should be selected carefully. Other factors influencing performance of subjects can be listed as strain and species of animals, gender and age of subjects, nutritional status of the animals (directly affects motivation toward food reinforcement), stress exposure, time and schedule of the study, environmental cues, nature of the stimuli driving behaviour (whether it is appetitive or aversive) and drug administered (if any) (Jakubowska-Dogru *et al.*, 2003; Jakubowska-Dogru *et al*., 2005, Paul *et al.* 2009).

Although there are various types of mazes introduced for evaluating spatial memory, those most commonly used are the Morris water maze (Morris, 1981), the radial maze designed by Olton and Samuelson (Olton *et al.* 1979), and the Barnes' circular maze (D'Hooge *et al.* 2001; Hodges *et al.* 1996) (see Figure 1).

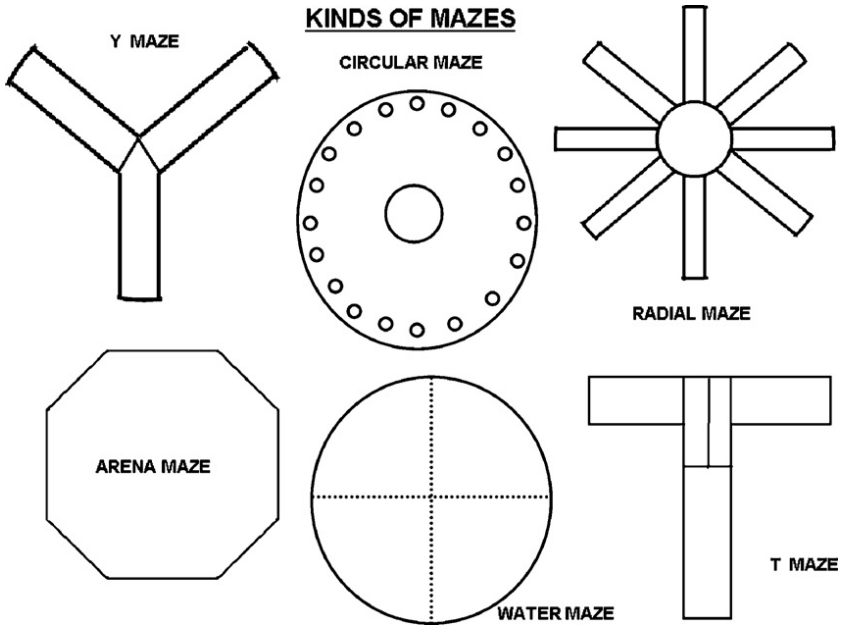


Figure 1. Types of mazes (adapted from Paul *et al.* 2009).

1.1.3.2. Radial Arm Maze

Radial arm maze is an experimental setup which consists of arm-like extensions projecting from the ontogonal central platform. Its earlier form (with 8 arms) was developed by Olton and Samuelson (Olton *et al.*, 1976), and was originally designed to evaluate spatial working memory in rats. According to needs of experimental design, 12-arm and 17-arm versions were also developed later.

There are various views about how the animals might solve the task. According to Olton and Samuelson, for solving the maze, the animals prepare a mental "list" of places already visited in order to choose only the places that have not been visited yet. Susuki and coworkers (Susuki *et al.* 1980) revealed that the

performance of the animals was significantly affected when the position of external visual cues that providing the information about the environment to recognize previously visited arms was modified during the tests. Two types of memory are thought to contribute performance in a radial maze: a memory concerning about past that informs the animal about the arms that have already been visited (retrospective component), and a memory that predicts the action for the election of new options (prospective component) (Thinus-Blanc, 1996). For example, rats trained to obtain food in each arm of a 12-arm radial maze minimize the number of items in memory by switching from a retrospective (arms already visited) to a prospective (arms yet to be visited) memory strategy (Cook *et al*., 1985).

During training, a food pellet is placed at the end of each arm; then, the animal previously subjected to food-deprivation is placed on the central platform of the maze and allowed to choose freely those arms containing food until the eight pellets have been collected. The optimum strategy implies a minimum number of visits to empty arms, or visiting a given arm only once. This is known as "win-shift" strategy (Paul *et al*. 2009).

In order to dissociate working and reference memory components of spatial learning task, radial arm maze was further modified and arm numbers increased to 12 with only 6 randomly selected arms baited. In this paradigm, the optimal performance on a single trial is to visit only once the baited arms and not to visit at all the unbaited arms. Such performance is based on win-stay strategy in the long-term and win-shift strategy in the short-term and requires both good both reference and working memory (Jakubowska-Dogru *et al*., 2003).

This dual approach for encoding arms visited fits into prospective and retrospective coding aspects of episodic memory mentioned earlier. When

considered together with the fact that episodic memory in humans can be modeled with animals studies concerning episodic-like aspects of memory, radial arm maze can be an appropriate setup to be used in studies on learning and memory requiring prospective and retrospective encoding and usage of allothetic and idiothetic cues together.

1.2. Hippocampus

The cerebral cortex (Latin for bark, rind, shell) is a multilayered, convoluted sheet of tissue overlaying the cerebral hemispheres. It is divided according the development level, structure and function into neocortex and allocortex. The periallocortex or the mesocortex are the terms used to mark the transitional zone between 6-layer neocortex and 3-layer allocortex. (El Falougy *et al*., 2006). Hippocampus is phylogenetically old structure representing the three-layered allocortex.

In amphibians, reptiles and birds, a structure homologous to the hippocampus, was characterized but its cytological composition is different from that of mammals. During the phylogenetic development of mammals, topographic changes in position of the hippocampus occurred due to the intensive development of the neocortex and corpus callosum. As a result, the hippocampal formation displaced rostrally and toward the depth of temporal lobe (West, 1990).

According to Bayer, the term hippocampal formation consists of six structures: gyrus dentatus, hippocampus proper, subiculum, presubiculum, parasubiculum and entorhinal cortex (Bayer, 1985). However, the hippocampal region is taken to include two sets of cortical structures, gyrus dentatus and hippocampus

proper on the one hand and the parahippocampal region (subiculum and area entorhinalis) on the other hand. The major defining differences between the two are the number of cortical layers present (three layers for hippocampal region whereas six layers for parahippocampal region) and the overall principles of connectivity (reciprocal or not). The subiculum is identified as a transitional area between the hippocampus proper and the entorhinal cortex (Cajal, 1911). It was also divided into the parasubiculum, presubiculum and subiculum starting from the entorhinal cortex toward the hippocampus proper (Lorente de No, 1934). Hippocampus was separated to several regions according to how thick cortical layers are and size and density of cells present.

In the literature, there are different nomenclatures for the hippocampus subregions. Lorente de NO (1934) used the term "cornu ammonis" (Ammon's horn) (CA) for the hippocampus proper. He identified four regions in cornu ammonis:

a) CA1 is the region nearest to the entorhinal cortex;
b) CA2 is a narrow region between CA1 and CA3;
c) CA3 continues until the hilus dentate gyrus (DG) and
d) CA4 is the region between the arms of DG.

All these regions of hippocampus have characteristic three-layer appearance and their connectivity is largely unidirectional (Figure 2). The dentate gyrus cells project via their distinctive mossy fibers (shown as mf in Fig.2) to the CA3 field of hippocampus. While some CA3 cells send axon collaterals to the deep or polymorphic layer of the dentate gyrus, these axons do not innervate the granule cells. A similar preferential unidirectional pattern holds for the other major intrinsic connections of the hippocampal formation: CA3-to-CA1; CA1-to-subiculum. In most cortical regions including parahippocampal regions (particularly in entorhinal cortex), reciprocal

connections are observed, but these unidirectional connections among subfields make hippocampal formation unique and help to identify its borders.

1.2.1. Hippocampus proper

The hippocampus proper can be clearly divided into two major regions, a large-celled proximal region and a smaller-celled distal region. Cajal called these two regions as regio inferior and superior, respectively. According to Lorento de No's nomenclature, CA3 and CA2 fields correspond to regio inferior of Cajal whereas CA1 is equivalent to regio superior. The cell bodies of CA3 (region inferior) are large, elongated and tightly packed. Toward the hilus (CA4), cells become more dispersed than those of CA3. The end toward CA1 (regio superior) is defined by a narrow transition zone with loosely packed pyramidal cells similar in size to those of CA1 and with distinctly smaller nuclei than those of CA3.

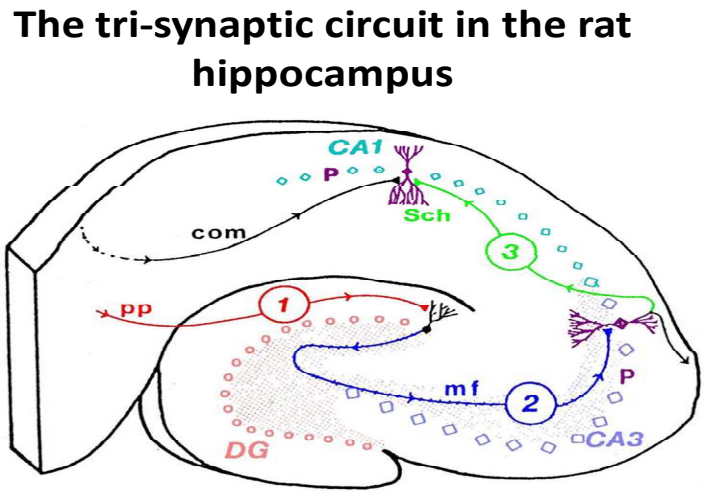


Figure 2. Schematic diagram showing intrahippocampal connections. (modified after Witter and Amaral, 2004).

The cell bodies and nuclei of the pyramidal cells of CA1 are smaller than those of CA3. At the area near the transition zone between CA1 and CA3, the cells are also tightly packed. Toward the subiculum (S), the layer becomes progressively loosely packed and the border with the subiculum is defined as the point at which cells of CA1 cease to be contiguous. The CA2 field is originally defined by Lorente de No as a narrow zone of cells interposed between CA3 and CA1 that had large cell bodies like CA3 but did not receive mossy fiber innervation like CA1 cells. It is suggested that CA2 has both connectional and perhaps even functional differences with the other hippocampal fields but its identification is much difficult than other fields. In addition to differences in the size of pyramidal cells in CA3 and CA1, there are also differences in neural connections. The major input to the CA3 pyramidal cells are mossy fibers from the dentate gyrus (indicated with 2 and shown with blue line in Fig.2) while the major input to CA1 pyramidal cells are Schaffer

collaterals from CA3 region (indicated with 3 and shown with green line in Fig.2).

For all fields of hippocampus, there is a common laminar organization. The principal cellular layer is called the pyramidal cell layer. The narrow, relatively cell-free layer located deep to the pyramidal cell layer is called ***stratum oriens*** and deep to this is the fiber-containing alveus. In the CA3 field, but not in CA1 and CA2, a narrow zone without any cell located just above the pyramidal layer is occupied by mossy fiber axons originating from the DG. This narrow layer is called ***stratum lucidum***. At the distal end of stratum lucidum, there is a slight thickening of the layer where the mossy fibers bend temporally. This "end bulb" marks the CA3/CA2 border. Superficial to the stratum lucidum in CA3, and immediately above the pyramidal cell layer in CA2 and CA1, is the ***stratum radiatum***. The stratum radiatum can be defined as the suprapyramidal region in which CA3-to-CA3 associational connections. CA3-to-CA1 Schaffer collaterals, septal and commissural connections and some interneurons are located. The most superficial portion of the hippocampus is called the ***stratum lacunosum moleculare***. It is the thin layer that perforant pathway fibers from the entorhinal cortex travel and terminate although afferents from other regions, such as those from some nuclei of thalamus, also terminate in this layer.

The principal neuronal cell type of the hippocampus is the pyramidal cell, which constitute the vast majority of neurons in the pyramidal cell layer. Pyramidal cells have a basal dendritic tree that extends into stratum oriens and an apical dendritic tree that extends to the hippocampal fissure. Dendritic organization of pyramidal cells in different parts of CA3 and CA1 differs slightly (see Fig.3). The length and organization of CA3 pyramidal cells' dendritic arbor vary consistently depending on the position along the transverse axis of CA3. Cells in the proximal part, close to DG, have the smallest overall

dendritic tree (about 10 mm in total), whereas cells in the distal part of CA3 (near CA2) have the longest dendritic tree (about 16 mm in total). CA2 contains a mixed population of both large cells and similar to those in of distal CA3 as well as cells having smaller dendritic trees resembling to CA1 pyramidal cells.

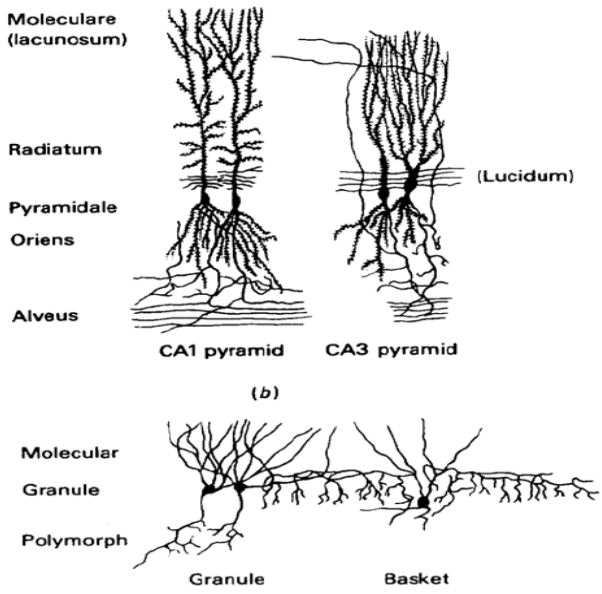


Figure 3. Examples of CA1 and CA3 pyramidal cells (upper), dentate granule cells (bottom left) and a basket cell of Cajal (bottom right). (After Cajal, 1911).

There is also a variety of non-pyramidal cell types in the stratum oriens, stratum radiatum and stratum lacunosum-moleculare of hippocampus. The vast majority of these neurons are immunoreactive for markers of GABA (Ribak et al., 1978) and most are considered to be local circuit neurons (interneurons). They provide not only inhibitory input but also regulate the precise timing of pyramidal cell activity. Overall diversity of interneurons in hippocampus is very large so that more than 20 types interneurons are described.

1.2.2. Dentate gyrus

The dentate gyrus is made up of three layers. The molecular layer which is the closest to the hippocampal fissure is a relatively cell-free. The principal cell layer or granular cell layer is found deep to the molecular layer and primarily includes granule cells. These cells are formed densely packed columnar stacks. The inner region of "U" shaped dentate gyrus constitutes the third, polymorphic cell layer known as hilus.

1.2.3. Anatomical substrate of spatial memory

First implications about role of hippocampus in learning and memory derived from observations on humans having hippocampal damage and they displayed some deficits in some of learning and memory types including explicit memories (Milner and Scoville, 1950's). Later, both in animal studies on rats and clinical studies on humans, it was shown that hippocampal damage specifically brings about impairment in the acquisition and remembering of episodic memories including spatial information. Olton and colleagues using RAM task investigated effects of the lesions of hippocampal area and associated structures in brain (Olton *et al*., 1977). In spatial learning task involving processing of external landmarks, lesioned animals failed with respect to normal animals while in cued learning task requiring formation of simple associations than spatial information processing, both groups performed similarly. Later, Morris and colleagues designed a study investigating the spatial learning in rats and mice in a circular water pool known as Morris Water Maze (MWM) (Morris *et al*., 1982). They had three experimental groups

consisting of hippocampal lesioned, neocortex lesioned and sham operated animals respectively.

According to their observations hippocampus-lesioned animals spent longer time to find platform and they were using more complicated routes meaning lack of optimization. Interestingly, when the same animals were introduced into a cued (non-spatial) learning task in the water maze, hippocampus lesioned rats had performance as good as others indicating that between-group differences in task performance cannot be attributed to motor impairments. In addition to types of tasks investigated, the timing of the lesion is another important parameter determining lesion effects. It has been revealed that when hippocampal lesion was performed before the training, acquisition of both reference (long-term) and working (short-term) memories was impaired. On the other hand, when the lesion was performed after the training the impairment was less pronounced. The time gap between training and lesion production was also shown to be effective and it was reported that when time between training and lesion is long enough, effects of lesion are much smaller (Carew, 2000). All this indicates that hippocampus plays a more important role in the acquisition then in the retention of spatial memories. It seems that over time, spatial memories become less dependent on hippocampus being consolidated via various mechanisms and stored permanently in brain areas other than hippocampus.

How spatial information is encoded inside the hippocampal networks is another important issue to be clarified. Pioneering study by O'Keefe and Dostrovsky in 1971 provided important cues to these questions. They recorded activities of hippocampal cells while animals were performing spatial learning task and observed that activities of specific cells were increased whenever rats moved into a specific location inside the experimental area. They called these specific cells as "place cells" which can be defined as class of neurons within

hippocampus that show firing patterns related to the animals' position in space. Spatial area in which the place cell is active is the cell's firing field. Since different neurons have different spatial preferences, they together construct a cognitive map of the animal's local environment. Several properties of place cells were identified to understand their preferences while working. First of all, different place cells encode different places in overall spatial field. Secondly, shape and size of firing field may differ from one cell to another. Finally, place cells can also be considered as a large subset of "complex spike cells" that were defined by Ranck and colleagues as hippocampal cells that fire in a unique and characteristic pattern (Muller, Kubre and Ranck, 1987). However, not all place cells behave like complex spike cells in all contexts. Preferences of place cells are not predetermined but flexible such that they are able to establish a new spatial firing pattern in a novel environment. However, once established, it is stabile as long as environment doesn't change. Interestingly, place cells were shown to be controlled by external (salient) cues available in the environment. When a place field is established by presentation of a specific cue to the rat and then animal is removed from the experimental area and the cue rotated by 90° counterclockwise, on return to the maze a new place field is established in accordance with cue rotation (O'Keefe and Nadel, 1978).

1.3. Cellular and Molecular Basis of Learning and Memory

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

In all organisms, brain has noteworthy capacity to apply functional and morphological remodelling process in an activity-dependent manner via plasticity mechanisms to provide memory encoding. In other words, brain is not a simple data recording unit having activity only at the mental level.

Instead, it is a dynamic system that couples mental activity with changes in its anatomical susbtrate which are neural circuits. Memories are encoded as patterns of synchronized cellular activity within widespread neural networks. This dynamic spatio-temporal activity progressively results with alterations in patterns of connectivity among the neurons that are activated together (Bruel-Jungerman *et al*., 2007). The process of encoding newly acquired information for the long-term usage is called "memory consolidation".

Main plasticity mechanisms that serve to store and update long-term memories are synaptic strengthening, synaptic elimination/weakening, synaptogenesis and neurogenesis. These mechanisms can act either independently or together for long-term memory formation.

As Hebb first suggested in 1949, during learning, memories are stabilized and stored as modifications of synaptic strength within the existing neuronal circuits. This process named as synaptic strenghtening is demonstrated by Bliss and Lomo in 1973 and reported as "long-term potentiation" (LTP). They showed that short high-frequency stimulation to perforant path which carries input to hippocampus from major cortical areas, leads to a long-lasting increase in synaptic strength at dentate gyrus granule cell synapses. Later, it was demonstrated that LTP is not specific to hippocampal area but it is observed in various cortical and subcortical regions of brain across different species.

Some physiological and pharmacological characteristics of LTP make it an important mechanism for learning and memory. First of all, LTP is long-lasting from weeks to several months, this gives enough time for formation of memory traces and consolidation to occur. Further, it is input-specific and associative, this specificity provide processing of input in a convergent manner and couple with other events as in the associative learning.

Evidences about the idea that synaptic changes brought by LTP play a crucial role in memory function are assembled by two main experimental approaches. One is to show synaptic co-activation and/or activation of specific usually diverging molecular pathways as a result of LTP induction, and the second is to manifest the blocking or enhancing effects of different pharmacological agents or genetic manipulations on LTP induction.

In hippocampus, two types of LTP are observed and they are differentiated according to their mechanisms. NMDA-independent type of LTP occurs at mossy fibers' synapses in CA3 region. It was shown to be dependent on pre-synaptic mechanism and cAMP used as a second messenger (Kessey *et al*., 1997). In contrast, LTP dependent on NMDA receptors occurs at perforant pathway synapses in DG and at Schaffer collaterals in CA1 region, and is characterized by rapid induction, input specificity and variable persistence (early-LTP and late-LTP), and has both pre- and postsynaptic mechanisms (Morris, 2006). While NMDA-independent LTP is nonassociative like i.e. sensitization, NMDA-dependent LTP is known to be associative. Associative nature of NMDA-dependent LTP is highly related with properties of NMDA receptors (NMDAR) which will be described below in detail and represents a cellular model of behavioral associative learning.

Induction of synaptic potentiation in various brain pathways begins with NMDA receptor activation. The NMDA receptor (NMDAR), is a slow-acting, ionotropic glutamatergic receptor known as the most potent Ca^{2+} ionophore selectively involved in LTP induction. Since activation of NMDAR requires simultaneous occurrence of two events, it is considered as a "coincidence detector." NMDA receptor is activated when both, glutamate binds to the receptor and the postsynaptic membrane is partially depolarized by the same or another synaptic input.

When these two conditions are met together, Mg ion (Mg^{2+}) blocking the channel is removed and Ca^{2+} enters the postsynaptic neuron. The importance of the NMDAR-dependent mechanisms in LTP induction and behavioral learning was proven by studies involving pharmacological blocking or genetic inactivation of NMDAR resulting in in learning impairment and preventing induction of LTP (Davis *et al*., 1992; Tsien *et al*., 1993).

Rise in Ca^{2+} leads to the activation of various synaptic proteins via posttranslational modification and protein-protein interaction. These two processes establish the biochemical machinery providing enduring modifications of synapse. Several signal transduction cascades involving a great diversity of molecules such as kinases and phosphatases are implicated in LTP.

Modification of synaptic receptors can occur by

- Phosphorylation/dephosphorylation of subunits
- Change in the composition of heteromeric receptors (differing types and stochiometry of subunits)
- Recruitment of extra-synaptic AMPA receptors (ionotropic glutamatergic receptors known to prime and potentiate NMDARs effects) to synaptic sites
- Mobilization of the trafficking/recycling machinery to increase the number of AMPA receptors by insertion in the synaptic membrane.

Transduction and transmission of synaptic signals may extent to the nucleus and result in *de novo* protein synthesis which is a critical mechanism for long- term stabilization of synaptic changes. Evidence suggests that antibiotics (i.e. anisomycin) that block protein synthesis or other substances that can

inhibit transcription may also disrupt LTP formation (Frey and Morris, 1997).

To modulate circuit functions activity-dependent potentiation of some synapses may be accompanied by activity-dependent depression of some other synapses. The "long-term depression" (LTD) phenomena is considered as a suitable mechanism to reduce synaptic strength in this situation. LTD is observed in two forms:

a) *de novo* LTD: It is an activity-dependent depression observed in newly formed synapses. Since this form of LTD deals with naive synapses, it is thought that de novo LTD is usually seen during development. However, there is accumulating evidence indicating de novo LTD in adults specifically in cerebellum (Ito *et al*., 1982) but also in CA1 and dentate gyrus regions of hippocampus (Thiels *et al*., 1994; Doyere *et al*. 1996; Kemp and Manahan-Vaughan, 2007).

b) Heterosynaptic LTD: The reduction of synaptic strength at nonactive synapses in association with potentiation of other neighbouring synapses is considered as "heterosynaptic LTD" (Bruel-Jungerman *et al.,* 2007).

Similar to LTP, LTD may depend on different types of receptors including NMDA receptors (with different NR2 subunits than receptors involved in NMDA-dependent LTP) or metabotropic Glutamate receptors (group I mGluRs). Key mechanisms involved in LTD formation can be summarized as dephosphorylation of specific residues on GluR1 containing AMPA receptors, lateral dispersal of receptors to extrasynaptic sites, internalization of AMPA receptors (Bruel-Jungerman *et al*., 2007).

In the literature, there is a controversy about whether LTD works for learning and memory as a cellular storage mechanism or it mainly serves for

adjustment of synaptic strength together with LTP. Most experimental data favoring first proposal came from studies examining cerebellum and hippocampus. In the cerebellum, both LTP and LTD are observed but LTD seems to be the dominant form of plasticity. Furthermore, in cerebellum-dependent forms of motor and associative learning, LTD mechanisms are implicated (De Zeeuw and Yeo, 2005). In hippocampus, LTD may be induced by low-frequency stimulation only when rats explore new environments indicating the role of LTD in complex spatial mapping (Kemp and Manahan-Vaughan, 2007). In addition, in mutant mice with a deletion of the forebrain-specific serum response factor (an enhancer site for expression of many immediate early genes, IEGs) showing deficits in memory for novel context, LTD is impaired whereas there is no alteration in LTP (Etkin *et al*., 2006). This data suggest that LTD-like mechanisms during novel context memory acquisition prepare synapses to LTP-like mechanisms expected to occur in their subsequent recruitments. It is important to note that mechanisms of hippocampal LTD differ from LTD observed in other brain areas such that both LTP and LTD require a rise in intracellular calcium concentration with other factors including amplitude, kinetics and localization of calcium influx determining whether LTP or LTD will occur. Usually, for hippocampal areas, rapid increase of Ca^{2+} due to kinase activation indicates LTP. On the contrary, a modest and more lasting increase in Ca^{2+} coupled with phosphatase activity favors LTD (Bruel-Jungerman *et al*., 2007).

1.3.2. Molecular basis of learning and memory

Cellular mechanisms that incline learning and memory are highly dependent on elaborate signal transduction pathways. A very diverse family of neuromodulatory proteins, neurotransmitters and their receptors, second

messengers and ion channels work together to establish stable memory traces in anatomical structures. Phosphorylation is a very important modification in these systems and phosphorylation/dephosphorylation states of target molecules determine their activities. Proteins responsible from phosphorylation and dephosphorylation reactions are kinases and phosphatases, respectively. The balance among activities of these two classes of proteins enables transmission of input signal from one molecule to another. Even these proteins themselves are phosphorylated or dephosphorylated to become activated. Well-characterized examples of these proteins include PKC (Protein kinase C), PKA (Protein Kinase A), CaMKII (Ca^{2+}-calmodulin dependent protein kinase), PI3K (Phosphatidylinositol-3 kinase), TyrK (Tyrosine kinase). These kinases are phosphorylated during or just after learning and pharmacological or genetic distruption of the normal activity of CaMKII, PKC, PKA, PI3K or MAPK/ERK generally end up with learning and memory impairments in various tasks.

1.4. Protein of Interest: Nitric Oxide Synthases

NO is a diffusible, free-radical containing molecule that acts on various cell and tissue types including neurons, endothelial cells, smooth muscles with a regulatory function in signal transduction mechanisms. Due to its low molecular weight and hydrophobic properties, it easily permeates across cell membranes and efficiently diffuses (Shaw and Vosper, 1977) reaching far from its site of synthesis within a very short time. Measurement of NO diffusion distance in physiological conditions with electrochemical microsensors reported 100-200 μm and reaching to steady-state concentration in 10-15 s. (Malinski *et al*., 1993). Early experimental evidence that NO acts as a intercellular messenger comes from work of Schuman and Madison (Schuman

et al., 1994). They were able to show NO-induced synaptic potentiation between paired neurons and synapses that were approximately 100 μm distant from each other in hippocampal slices.

Nitric oxide is produced by the enzyme Nitric Oxide Synthase (NOS) from conversion of L-arginine to L-citrulline in a NADPH-dependent manner (Figure 4). The synthesis reaction for NO involves a 5-electron oxidation of one of the chemically equivalent guanidino-nitrogens of L-arginine, leading to the concomitant production of L-citrulline and NO. It is accompanied by an NADPH-dependent reduction of molecular oxygen (Mayer *et al*., 1991) which is incorporated into both reaction products (Kwon *et al*., 1990; Leone *et al*., 1991). This reaction is also summarized in Figure 4 that L-arginine is N-hydroxylated to give L-NHA, which is further oxidised, yielding L-citrulline and NO. In mammalian cells, NO synthesis carried out mainly in neurons, endothelial cells and macrophages and different isoforms of NOS enyzme are identified in those cells. Biochemical characterization of these enzymes showed that they are complex enzymes and require various redox- active cofactors for proper functioning.

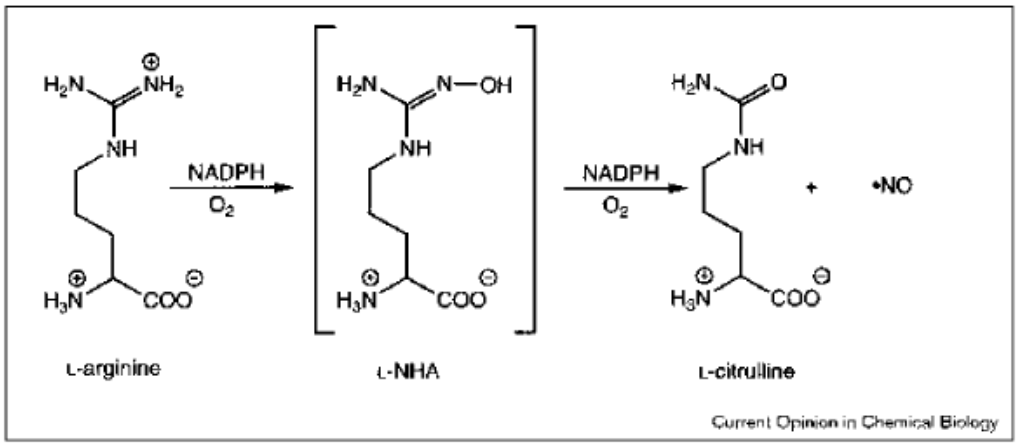


Figure 4. The reaction catalyzed by NOS (from Marletta *et al*., 1998).

The source of the diversity of NOS isoforms is alternative splicing. Different NOS isoforms are named according to cell types they are expressed in: nNOS for neuronal isoform, eNOS for endothelial isoform and iNOS (inducible NOS) mainly expressed by macrophages. While nNOS and eNOS are activated in a Ca^{2+}-dependent manner and constitutively expressed, iNOS is induced via inflammation or other kind of stimuli and do not require Ca^{2+} for activation.

All isoforms of NOS identified so far contain a C-terminal region homologous to cytochrome P450 reductase and a N-terminal region containing heme and BH4-binding sites separated by a calmodulin-binding site. Consensus-binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), calmodulin (CAL), and heme (H) are also conserved for all cloned NOSs. A phosphorylation site (P) for cAMP-dependent kinase is conserved between the neuronal and endothelial forms (shown in Figure 5).

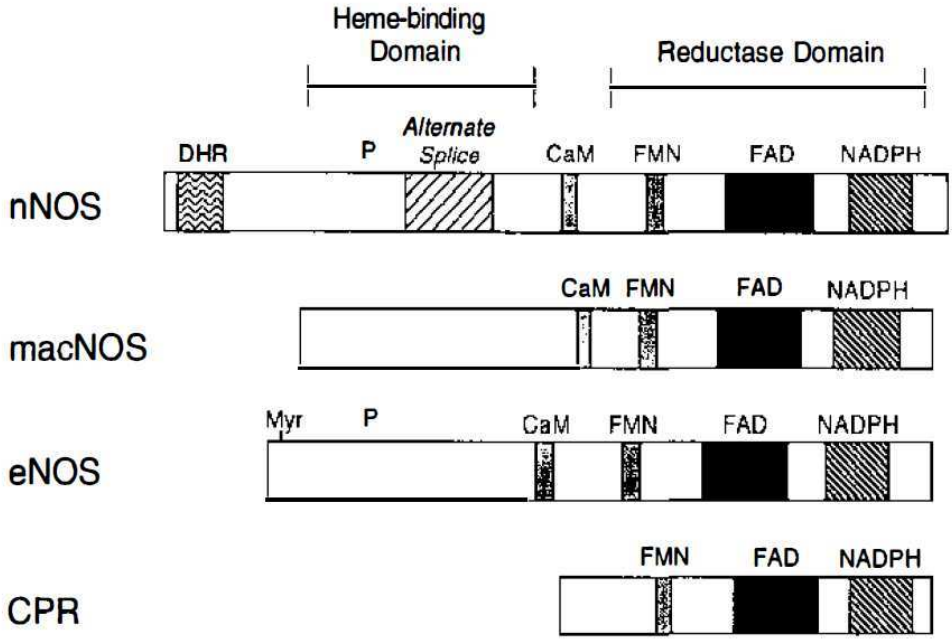


Figure 5. Sequence homologies of NOS isoforms (from Jaffrey *et al.*, 1995).

The nNOS isoform is unique in that it contains a discs-large homology region at the N-terminal (DHR in Figure 5). This approximately 220 a.a.-long leader sequence contains a PDZ binding motif that interacts with several proteins that may target the enzyme to signal transduction hot spots within a cell. Proteins having PDZ domains in their sequences were conducted to specialized cell compartments and are also believed to be important in keeping components of signal transduction pathways in contact as complexes (Chanrion *et al.*, 2007; Riefler *et al.*, 2001). Similarly, it is shown that NO signaling may be altered by anchoring nNOS to membrane or cytosolic protein via direct PDZ–PDZ domain or C-terminal-PDZ interactions. PSD95 (post-synaptic density protein-95), which is a multivalent synaptic scaffolding protein and core component of the post-synaptic density, is responsible for the efficient activation of nNOS by NMDAR stimulation and it can link nNOS to NMDAR (Sattler *et al.*, 1999). Binding of nNOS to PSD95 is a determinant of

postsynaptic targeting of nNOS. nNOS and iNOS become activated when they are in dimeric form. Dimerization is thought to be mediated by a region N-terminal to the calmodulin-binding site. The endothelial NOS has a myristoylation site (M) at its amino-terminus (Bredt & Snyder 1994b., Jaffrey 1995). This enables post-translational modification of eNOS and its translocation to plasma membrane caveolae which is necessary for its activation.

1.4.1. Role of NOS Isoforms in Synaptic Plasticity, Learning and Memory

All three isoforms of NOS enzyme are produced in different locations of both central and peripheral nervous system. In order to understand contributions of NOS isoforms to mnemonic processes, several experimental approaches are established. Among them there are pharmacological manipulation of NOS enzymes and assessment of their effects on synaptic and cognitive functions, examination of genetically modified animals with reduced or amplified NOS expression in terms of cognitive and other functions, and finally, examination of the effects of i.e. learning, training on NOS activity and expression. However, first of all, it is important to summarize mechanisms about molecular biology of NO and NOS enzymes in synaptic mechanisms.

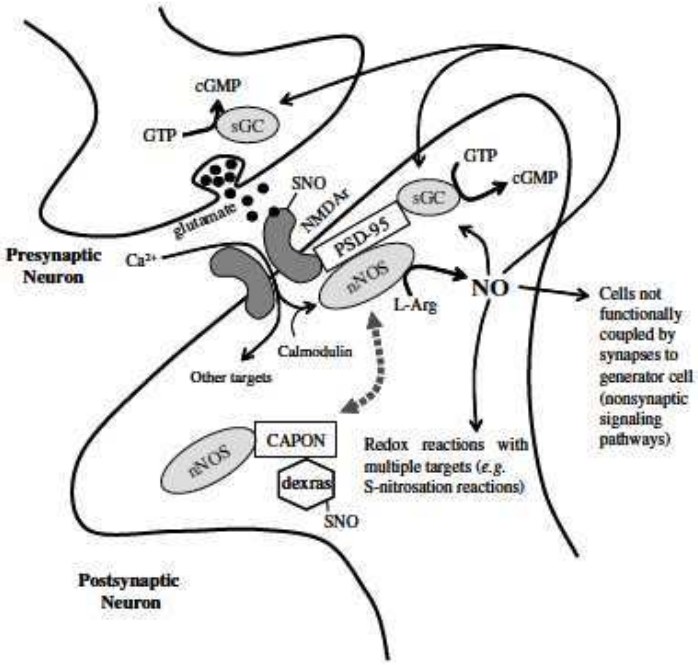


Figure 6. Diagram showing NMDAR coupled nNOS activity and NO signaling at postsynaptic density (from Ledo *et al*., 2004).

Activity of nNOS in post synaptic neuron is mostly coupled with dynamics of NMDA receptors (NMDAR). As described in Section 1.3.1, NMDAR's are voltage-sensitive glutamate receptors that enable Ca^{2+} entrance to the postsynaptic cell. Ca^{2+} in conjugation with calmodulin act as second messenger and causes activation (via interaction with PSD-95, described in Section 1.4) and translocation of nNOS from the cytoplasm to the postsynaptic membrane where NO production and release take place. NO diffuses to the nearby cells including presynatic neuron (therefore it is called a retrograde messender) and activates cytoplasmic soluble Guanylyl Cyclase (sGC) enzyme which catalyzes conversion of GTP to cGMP, an important second messenger. cGMP activates other kinases such as cGMP-dependent protein kinase (Protein kinase C-PKC) or phosphatases (PP-1) and indirectly by decreasing K^+ conductance of the presynaptic membrane and reducing re-uptake of glutamate to presynaptic

terminal increases release of Glu from the presynaptic neuron. Since NO do not require any vesicular transport mechanism, these processes happen in a very short time compared to other conventional transmitters and NO can have wider effect on more than one cell. All of these phenomena are summarized in Figure 6.

NMDAR activation actually brings an important form of synaptic plasticity which is described before, LTP. Therefore, coupling of NMDAR activation with nNOS activity can explain previous observations about elimination of hippocampal LTP with NOS inhibitors (Böhme *et al*., 1991; O'Dell *et al*., 1991; Bon *et al*., 1992) and potentiation or facilitation of LTP with NO donors (Arancio *et al*., 1995; Bon *et al*., 1992).

Effects of NO on synaptic plasticity are specified according to anatomical structures worked on. While NO helps LTP formation in hippocampus, in other brain areas it can initiate other forms of plasticity. For example, in cerebellum, learning and memory in eye-blink conditioning task is related with a long-lasting decrease in synaptic transmission (LTD) on synapses between parallel fibers and Purkinje cells. Since originally Purkinje cells have inhibitory activity, decreasing their activity promote activation of deep cerebellar nuclei. Parallel fiber activity causes the release of NO, and NO alone can substitute for parallel fiber activation (Lev-Ram *et al*., 1997). NO initiate cGMP production from sGC and eventually activation of PKG. Parallel fiber activity is responded by phosphorylation of AMPA receptors (Nakazawa *et al*., 1995) and an increase in their binding affinity (Dev and Morris 1994).

These two examples implying use of same transmitter for opposite forms of plasticity with different cellular mechanisms (hippocampal LTP and cerebellar LTD) represent unconventional property of NO signaling arising from highly reactive nature of NO which can react with different molecules.

For pharmacological manipulations, agents that are known to enhance or inhibit NO synthesis and NOS activity are administered to animals and then, possible effects of these drugs are investigated in different behavioral tasks. The biological effects of NO can be controlled via drugs in different ways. These drugs may either inhibit the uptake of L-arginine into the cell, reduce the cellular availability of necessary cofactors i.e. (tetrabiopterin) BH4 by preventing their formation or promoting their breakdown, scavenging NO once formed or inhibiting the cellular mechanisms leading to induction of the particular NOS isoform (Moore *et al*., 1997). Over recent years effects of the inhibition of NOS enzymatic activity have been the most thoroughly investigated. In 1993, Böhme and colleagues administered Arg $(NO)_2$ (an arginine analog) intraperitoneally to inhibit endogenous NO production and studied its effects on hippocampal LTP formation and behaviorally, on spatial learning, social memory and shock-avoidance learning (Böhme *et al.,* 1993). They showed that NOS inhibitor Arg $(NO)_2$ given systematically is able to penetrate the brain to block hippocampal LTP, slow down spatial learning and impair olfactory recognition in social memory but not shock-avoidance learning which depends on simple associations instead of hippocampal computing. Chapman and colleagues (Chapman *et al.,* 1992) have also shown that injections of another arginine analog L-NAME (75 mg/kg) impaired acquisition of spatial reference memory in the water maze but did not affect the retention of previously acquired spatial information. However, one year later, another study by Bannerman et al. (Bannerman *et al.,* 1994) argued against these results and reported that inhibiton of NOS doesn't impair spatial learning. They delivered L-NAME intraperitoneally to naive rats during acquisition of spatial learning task in Morris Water Maze (MWM) and also to rats that previously learned the task and, after a delay, were subjected to the memory retention test and reversal training. The same groups were also tested in a visual discrimination task. Rats receiving NOS inhibitor during the acquision training showed an impaired performance in both a spatial reference memory

and a visual discrimination task, but the same dose of L-NAME (75 mg/kg) was without effect on performance in retention test, reversal learning, the learning of a new spatial environment in water maze by experienced animals. On the contrary, Blokland and coworkers (1999) delivered L-NA, another NOS inhibitor, directly to hippocampus instead of i.p. administration. They reported that L-NA administration into dorsal hippocampus affected the way in which animals were searching the hidden platform during training in MWM task but it didn't significantly affect learning the platform place. Based on these data, they concluded that hippocampal NO is not critically involved in spatial learning in rats (Blokland *et al*., 1999). On the other hand, Zou and coworkers (Zou *et al.*, 1998) is an example of this approach. They examined the effects of systemic administrations of non-selective NOS inhibitor L-NAME, selective nNOS inhibitor 7-nitroindazole (7-NI) and NO precursor L-Arg on performance of spatial learning task performed with partially baited radial arm maze. It was observed that 7-NI impairs spatial reference memory formation in the radial arm maze task while L-NAME inhibits both reference and working memories. Furthermore, it has been demonstrated that an NO precursor, L-Arg, increased the choice accuracy, by reducing reference memory errors in the late phase of training. Based on these results, it is suggested that NO has a significant role in spatial memory formation, especially in reference memory. The controversial results of experiments employing NOS inhibitors may arise from differences in drug administered, the drug dose and route of administration, the task used in behavioral tests, and even the strain of the animal.

As mentioned earlier, transgenic animals were also used in the studies on the role of NO and different NOS isoforms in learning and memory formation. These studies are advantageous because using pharmacological inhibitors, it is difficult to find an isoform-selective inhibitor. Besides with genetical manipulation, it is possible to restrict its effects to a limited brain area and even

specific time (i.e. during or after the training). Genetic manipulation may be achieved by complete knock out of the gene encoding candidate protein or reducing/ blocking its activity. NOS gene knockout animals are compared with wild-type animals to see knock-out effects (see Huang *et al*., 1999 and Mashimo *et al*., 1999 for detailed review).

Distruption of genes encoding different NOS isoforms affects behavioral parameters in rodents differently. When cognitive skills of nNOS knockout mice (nNOS-KO) was investigated, they displayed hyperactivity on locomotor activity testing. On context-dependent fear conditioning task, nNOS-KO mice failed performing the task and received much more shock than wild-type mice. To investigate whether NOS KO mice's performance are affected by their hyperactive profile, cued version of same task applied. As a result, both nNOS-KO and wild-type mice didn't differ in cued version of fear conditioning implying that hyperactivity do not affect performance of nNOS-KO in previous task and nNOS-KO mice have an impaired working memory (Zoubovsky *et al.,* 2011). In another study performed by Frisch and colleagues, eNOS-deficient and wild-type mice were compared in terms of their Morris water maze (MWM) performance. Interestingly enough, eNOS deficient-mice displayed better performance than wild-type mice in MWM task and this superiority continued in long-term retention and reversal trainings. In a cued version of MWM task, wild-type and eNOS deficient-mice do not differ in terms of task performance. This latter information also excluded the effect of sensorymotor capacities on MWM performance (Frisch *et al*., 2000).

In addition to genetic and pharmacological manipulations, learning and memory themselves may affect molecular substrate that help to produce them. Learning-induced changes were also investigated for NOS isoforms. In the study conducted by Zhang and colleagues, rats in the training group (T) and sham-training group (ST) were subjected to water-rewarded spatial alternation

task. Naïve control (N) group was not participated into task. After task, all rats were investigated for nNOS immunoreactivity via immunohistochemistry and the number of nNOS positive neurons were determined in dentate gyrus and frontal cortex. Analysis of data revealed that in training group, up-regulation of nNOS positive neurons in the brain areas investigated observed (Zhang *et al*., 1998).

Effect of exercise on cognitive performance and nitrergic system was studied by Pietrelli and coworkers, they applied aerobic training for 18 months to a group of rats (AT) and compared their performance on 8-arm radial maze with not trained, sedentary control group (SC). It is observed that AT group performed better at radial maze with less numner of working memory errors than SC group. After, NADPH-d reactivity of neurons in parieto-temporal cortex, CA1 region of hippocampus and dorso-medial striatum and co-localization of NADPH-d positive neurons with nNOS and iNOS were examined. In all areas studies, NADPH-d staining intensity was higher in trained group. As expected, no co-localization for NADPH-d/iNOS was observed whereas number of nNOS/NADPH-d double stained neurons were higher in trained group. To sum up, aerobic training improved cognitive status of animals followed by up-regulation of nitrergic neurons (Pietrelli *et al*., 2011).

To summarize, it is observed that parameters that enhance cognitive skills usually cause up-regulation of nNOS in relevant brain structures. At the same time, parameters that inhibit nNOS activity and NO synthesis reduce cognitive performance. Whereas increased eNOS activity is not positively correlated with improved learning and memory skills. Genetically manipulated animal models also provide information supporting these observations. Changes in expression and activity of NOS can be detected in various molecular methods. Expression of NOS isoforms under different circumstances can be measured

directly from tissue homogenates via immunoblotting (Nava-Ruiz *et al.*, 2010; Gokcek-Saraç *et al*., 2012) or enzymatic activity can be detected by performing a radiochemical assay that measures the conversion of L-[3H] Arg to L-[3H] Citrulline (Cunningham *et al.,* 1998). Direct detection of NO is also possible with electrochemical method (with sensors and electrodes) (Allen *et al.*, 2005) or fluorescent probes labeling NO (von Bohlen und Halbach *et al.*, 2003). However, none of these methods can give information about distribution of NOS isoforms among brain areas thought to be affected from condition or learning paradigm applied or metabolism of drug administered. Investigation of NOS isoforms via histochemistry or immunohistochemistry (IHC)/immunocytochemistry (ICC) provides information about cellular and subcellular localization of NOS isoforms in brain areas and comparing this localization info under different circumstances with the one obtained from control groups to determine whether the condition applied causes transport or translocation of NOS from one compartment to another. NADPH-diaphorase (NADPH-d) histochemistry is widely used to assess NOS activity and cellular localization (Endoh *et al*., 1994). However, it is also reported that different staining patterns were observed with NADPH-d histochemistry when fixation parameters changed (Gonzalez-Hernandez *et al*., 1996). Detection of NOS isoforms on brain sections by applying specific antisera or by NADPH-d histochemistry which relies on NADPH-dependent enzymatic activity of NOS isoforms has been widely used in the NOS research.

1.4.2. Distribution of NOS isoforms in hippocampal regions

Distribution of different isoforms of NOS protein and their mRNA's among different brain areas has been previously investigated in animal studies via IHC or in situ hybridization methods either under normal physiological conditions

or after drug administration, in different pathological states, and also when animals were subjected to a behavioral task.,. Although results of these studies are often discrepant which can be attributed to the animal strain used in the experiments, the brain area under study, or different parameters used for molecular assays i.e. PFA fixation parameters (weak (0,5-2%) vs. heavy (4-10%) and staining protocols (please see Section 2.3.3) they may still provide a general framework for NOS positive structures and cell/neuron types. Since in the present study, the region of interest was limited to the dorsal dorsal hippocampus (the part of hippocampus known to especially implemented in spatial learning and memory), the literature survey presented beneath will focus on the distribution of NOS isoforms in the hippocampus.

Rodrigo and colleagues (Rodrigo *et al*., 1994) investigated localization of constitutive isoforms of NOS (nNOS and eNOS together) in adult rat brain and reported constitutive NOS immunoreactivity (cNOS-IR) in all areas of Ammon's horn. In sections obtained from rostral part of brain, cNOS-IR neurons were found in pyramidal layer of CA1, CA2 and CA3 with extensions toward stratum oriens and dendritic tufts into stratum lacunosum moleculare. The number of cNOS-IR cells were found to be higher in CA1 than CA2 and CA3. In caudal sections, in CA1 and subiculum, the number of cNOS-IR cells in pyramidal layer were higher with respect to rostral part. Small, scattered cells in multipolar form were found out in stratum oriens, radiatum and lacunocum moleculare. In dentate gyrus also, cNOS positive cells were more numerous in caudal than in rostral part. In the study by Wendland et al. (1994), the presence of nNOS immunoreactivity was shown in CA1 pyramidal cells under weak (0.5%) PFA fixation. In another study done by Dun et al. (1994), small nNOS immunoreactive neurons were identified as scattered in stratum pyramidale, stratum oriens and stratum radiatum layers of CA1 and CA3 areas. In dentate gyrus, small and large polymorphic nNOS-IR cells were found in hilus and as a single plate at hilar border, in the region known as subgranular

zone (SGZ). Also in the study by Dinerman and coworkers (1994), nNOS IR in CA1 region was shown as limited to interneurons with cell bodies faintly stained whereas intense eNOS IR was in CA1 pyramidal layer and DG granular layer. In addition to above studies describing hippocampal profiling of nNOS distribution, quantitative analysis of nNOS positive cells were performed in mouse brain by Jinno and coworkers (1999) using stereological optical dissector method. They revealed that both principal and non-principal neurons in hippocampus showed nNOS-IR. The nNOS-IR nonprincipal neurons (interneurons) were scattered throughout all layers in all hippocampal areas and were especially numerous in the subgranular zone of the DG and stratum pyramidale, stratum radiatum, and at the border between the stratum stratum radiatum and striatum lacunosum-moleculare of the CA regions. In contrast to this, principal neurons in dorsal CA1 region (pyramidal neurons) lacked nNOS-IR, whereas CA3 pyramidal neurons adjacent to the hilus showed nNOS IR of moderate intensity. In the ventral region, CA1 pyramidal neurons adjacent to the subiculum showed nNOS IR of moderate intensity, and CA3 pyramidal neurons adjacent to the hilus showed weak nNOS IR. Granule cells in the DG were weakly stained for nNOS throughout dorsoventral axis.

Table 2. Table showing numbers and numerical densities of nNOS positive neurons in mouse hippocampal subregions (from Jinno *et al*., 1999).

TABLE 7. Numerical Densities ($\times 10^3/mm^3$) of nNOS-IR Neurons in Each Subdivision[1]

Sub-division	Dorsal	Middle	Ventral	Statistical analysis: Dorsal vs. middle	Middle vs. ventral	Dorsal vs. ventral
CA1	1.6 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	ns	ns	ns
CA3	1.2 ± 0.0	1.5 ± 0.1	2.3 ± 0.3	*	*	**
DG	1.9 ± 0.2	2.3 ± 0.2	3.0 ± 0.3	*	**	**
Total	1.6 ± 0.1	1.7 ± 0.1	2.2 ± 0.2	ns	*	*

[1]Values represent the means ± SD among animals (n = 4). The dorsoventral difference is determined by Welch's t-test (*P < 0.05 or **P < 0.01). nNOS-IR, neuronal nitric acidsynthase–immunoreactive; DG, dentate gyrus.

TABLE 5. Numbers of Disector-Counted nNOS-IR Neurons in Each Subdivision[1]

Subdivision	Dorsal	Middle	Ventral
CA1	649	481	491
CA3	214	370	1042
DG	536	639	970
Total	1399	1490	2503

[1]nNOS-IR, neuronal nitric oxide synthase–immunoreactive; DG, dentate gyrus.

As shown in Table 1.1, at the dorsal level, nNOS-IR neurons were more numerous in CA1 than CA3 and DG. nNOS was also localized in non-neuronal structures in the brain like endothelial and glial cells (Seidel *et al*., 1997; Gabbott *et al*., 1996; Catalan *et al*., 1996).

Lastly, it is important to emphasize the observation of Endoh and coworkers (1994) about the remarkable discrepancy between the levels of nNOS mRNA and nNOS protein in CA1 hipppocampal neurons which might indicate possible involvement of post-translational regulation of nNOS expression (Endoh *et al*., 1994). Later, it was also reported that rostro-caudal gradients in expression of nNOS mRNA in CA1 and CA3 in rat with substantially higher expression in caudal than in rostral areas. Distribution of nNOS mRNA was also different among hippocampal regions such that in rostral portion of rat brain, the highest nNOS mRNA expression was observed in CA3 whereas the lowest expression was observed in CA1. On the contrary, in caudal portion,

nNOS mRNA expression was highest in CA1 while the expression was lowest in CA3 (Blackshaw *et al*., 2003).

Distribution of endothelial isoform of eNOS was investigated by Töpel and colleagues (Töpel *et al*., 1998) both in adult and developing rat brain. This study revealed that eNOS protein was mainly localized in endothelial lining of arteries and arterioles to a lesser extent in veins, venules and capillaries. In neurons and glias, no eNOS immunoreactivity was observed in this study. Stanarius *et al*. (1997) using tyramide signal amplification (TSA) technique demonstrated eNOS IR in the endothelium of almost all arteries, but also veins and blood capillaries. Similarly, Seidel *et al*. (1997) detected eNOS mRNA via in situ hybridization only in the brain blood vessels but not in neurons or glial cells. According to comparative work by Blackshaw and colleagues (2003), it was reported that the distribution and levels of eNOS in mouse and rat brain were similar and when examined at high power, a scattered pattern representing blood vessels was observed (Blackshaw *et al*., 2003). However, in the study performed by Dinerman and colleagues (1994), extensive staining of dendritic processes and perikaryons revealed strong eNOS IR in CA1 pyramidal layer and in DG granular layer.

In opposite to previous evidence, presence of eNOS in astrocytes was also confirmed by examining co-localization of eNOS protein with glial-acidic fibrillary protein (GFAP) in coronal rat brain sections (Iwase *et al*., 2000). According to the latter data, obvious but less intense staining was found in hippocampus, compared to other areas. Immunoreactivity was mainly located in the stratum oriens and stratum radiatum layers but it was lacking at pyramidal layer.

While histological, physiological, and behavioral studies bring accumulating evidence about possible roles of neuronal and endothelial isoforms of NOS in

learning and memory, there is much less data about distribution of iNOS in hippocampus in healthy subjects. As the name implies, inducible NOS was mostly observed in the brain near the neuronal injury or inflammation sites after the induction of its expression via neuronal damage mainly in microglia (Hoehn *et al.,* 2003; Iadecola *et al*., 1995). However, recently, there is more and more evidence for iNOS expression and activation also in neurons and astrocytes. Rodrigo and coworkers (2000), reported the presence of iNOS positive neurons in the ectal (dorsal) and endal (ventral) portions of dentate gyrus, in the basal part of granular layer (Rodrigo *et al.,* 2000). There are also some examples also reporting that iNOS may affect synaptic release and short-term dynamics in mouse neocortex (Buskila *et al*., 2010). Nevertheless, a prevailing opinion is that there is no or very little NOS expression in the healthy brain.

In summary, we can say that all three NOS isoforms are expressed in the mammalian brain including hippocampus. According to the literature presented in this chapter, a constitutive expression of nNOS seems to prevail in the dorsal hippocampus, more in the interneurons and less in the principal neurons, the eNOS expression takes place predominantly in the blood vessels' endothelium but some eNOS expression has been observed also in neurons and glia, while the iNOS expression is induced upon the brain trauma in microglia but also in neural cells and in astrocytes.

1.5. Aim of the study

Considering our poor knowledge about the potential correlation between hippocampal expression of NOS isoforms and the animals' learning skills, the aim of the present study was to investigate the variation in the expression of n, e, and iNOS, in four different regions of hippocampus including CA1, CA3, DG, and hilus areas, in young adult Wistar rats classified as "good" and "poor"

learners based on their performance in the spatial learning task, the 12-arm radial maze, which allows to monitor both, reference and working memory. To evaluate not only the expression levels but also the distribution of three NOS isoforms within the hippocampus, NOS isoforms were visualized using immunohistochemistry methods.

CHAPTER 2

MATERIALS AND METHODS

2.1. Subjects

In the present study, 4 month-old inbred male albino Wistar rats (n=30) obtained from the GATA Animal Facility, Ankara, Turkey were used. During the experimental procedures, rats were kept in home cages in groups of three under a constant temperature (21°C) and a 12/12 hour light/dark cycle (lights on at 7.00 a.m, lights off at 7 p.m.), with *ad libitum* access to water in the animal house, in the Department of Biological Sciences at METU. Before rats were taken into experiments, their ad libitum body weight was reduced by 15% (Jakubowska-Dogru *et al*., 2003). Animals' body weights were determined at each day and recorded daily.

2.2. Behavioral Experiments

2.2.1. Apparatus

2.2.1.1. Activity Boxes

In order to determine animals' locomotor activity, activity test was applied using MAY ACT 508 Animal Locomotor Activity Meter, Commat Ltd, Turkey. The activity box (30x30x30 cm) had a grid floor and was surrounded

by plexiglass walls with two arrays of photocells one at the floor level and the second 8 cm above the floor which were detecting animals' horizontal (ambulation) and vertical (rearing) movements. The activity scores were recorded by a computer software.

2.2.1.2. Twelve-arm Radial Maze Apparatus

Radial arm maze (RAM) is a test used for assesment of spatial memory in small rodents, mice and rats (Olton et al., 1979). In the present study, 12-arm radial maze was used. It was made of painted plain wood and consisted of a central platform 40 cm in diameter and 60 cm long/9 cm wide 12 radial arms. All of arms were surrounded by 15 cm high transparent plexiglass walls. At the entrance to each of the arms there was a sliding door. The doors could be open by the experimenter one by one or all at the same time. At the end of the arms, there were food cups (2 cm in diameter and placed in 2 cm deep) where chocholate flavored rice puffs were put as a reinforcement. In the present study, the RAM was partially baited: only 6 semi-randomly chosen arms (always the same) were baited while the remaining 6 arms were empty. This allowed to assess both long-term (reference) ans short-term (working) memory. To ensure equal distribution of food odour traces inside the maze and prevent formation of intramaze cues by this way, two chocholate flavoured rice puffs (same pellets that were used as a bait during training) were placed inside of all food cups (in both baited and unbaited ones) and a perforated partition was inserted 1 cm from the bottom of all food cups to make the puffs other than bait inaccessible for rats.

2.2.2. Behavioral Procedure

2.2.2.1. Activity Measurements

Since the RAM performance depending primarily on the animals' cognitive skills is also affected by their spontaneous locomotor activity, animals displaying very low ambulatory activity should be excluded from the experiments. Therefore, basal activity levels of animals were determined using activity boxes described above. Each animal was kept inside activity box for 15 minutes. The experimental room in which activity measurement were carried out was silent and dimly illuminated. Ambulatory (locomotor) and vertical (rearing) activities of each animal were recorded via the apparatus software.

2.2.2.2. Place Learning in the RAM Task

The behavioral procedure was adopted from Jakubowska-Dogru et al. (2003). For 6 consecutive days prior to the beginning of experiments, all rats were daily handled for 5 min each to get used animals to the experimenter. Before starting the acquisition training, animals were subjected to habituation and shaping training. During 3 shaping sessions, rats were allowed to explore the maze for a 10 min period each day, and eat all the food pellets. During the first day of shaping training, food pellets were scattered throughout the maze. During each successive day, the number of pellets was reduced, and they were placed closer to the ends of the arms. At the end of the shaping training, food was placed only in the food cups of pre-selected arms. Figure 7 shows general structure of 12-arm radial maze apparatus and indicates pre-selected arms that

were baited at the last day of shaping trial and during the whole acquisition training.

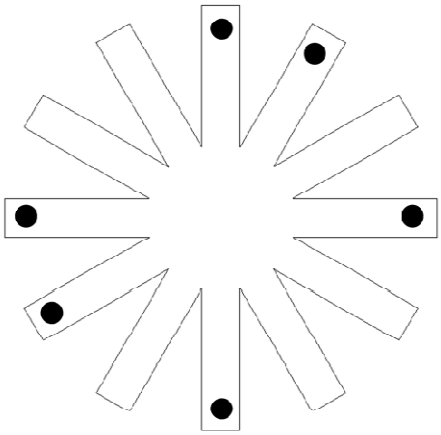


Figure 7. 12-arm radial maze apparatus. Black dots at the end of the arms indicate partially baited arms during task. (Modified after Gokcek Sarac, 2012).

Experimental room was furnished with several extra-maze cues immobile throughout the entire experimental period. Indirect illumination was provided by diffused light coming from the sides of the room. A camera was mounted to the ceiling above the RAM apparatus and was connected to a microprocessor. Computerized video tracking system (EthoVision System by Noldus Information Technology, Holland) was used to track the animal in the apparatus. On each daily trial, rats were placed on the central platform facing different directions. The guillotine doors to the arms were raised and rats were allowed to make their first choice by entering one of the arms. Each time, the rat returned to the central platform after making a choice, the guillotine doors were shut for 5 s, and only then the animal was allowed to make the next choice.

Partially baited radial arm maze apparatus enables rats to follow two different strategies between trials and within a daily trial in order to solve task effciently and reach food reward. Between trials, rats should use a win-stay strategy based on a long-term reference memory to learn and memorize constantly baited arms whereas within a daily trial, they should prefer a win-shift strategy based on a short-term or working memory to adjust its orientation based on distant cues when they were introduced to the maze from different locations in each day. Therefore, entries of rats to unbaited arms implies an impairment of long-term (reference) memory and considered as a Reference Memory Error (RME). When rats re-enter arms (either baited or unbaited) that already have been visited during a current daily trial, this is classified as Working Memory Error (WME).

During experiment the following measures were recorded:

1. Total number of choices to reach the acquisition criterion of 3 consecutive daily trials with maximum 3 out of the first 18 entries (6 on each trial) being to unbaited arms considered RMEs, This corresponds to a high performance level of 83% correct choices.
2. Total number of entries to unbaited arms (reference memory errors, RMEs);
3. Total number of re-entries to either baited or unbaited arms (working memory errors, WMEs).

Daily trial was ended if animal made 12 choices, or visited all the baited arms and ate all 6 food rewards (baits), or 10 min elapsed, which one came first. The experiment continued until the all animals reached the acquisition criterion. Animals were classified as “good” and “poor” learners based on their total number of choices to reach the criterion. Rats having scores 3 SEM below the group mean were considered as “good” learners (total number of

choices to the acquisition criterion ≤ group mean - 3SEM) whereas those having scores 3SEM above the mean (total number of choices to the acquisition criterion ≥ group mean + 3SEM) were considered as "poor" learners.

Before being sacrificed all rats were subjected to a 5 min retraining session during which all the conditions (baited arms, opening and closure of doors etc.) were kept same as before. Rats were sacrificed 15 min after the retraining session.

2.3 Histological Studies

2.3.1. Tissue Sample Preparation

For the preparation of the brain tissues, rats were deeply anesthetized with intraperitoneal (i.p.) injection of 5 mg/ml chloral hydrate per kg of animal, and the perfusion of the brain was carried out when all the reflexes to cutaneous stimulation disappeared. Brain tissue was cleaned from the blood and partially fixed in situ by intra-aortic perfusion with 0.1 M phosphate buffer (PB) pH 7.4, followed by 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4). Afterwards, brains were removed and, kept overnight at 4 ºC, in 4% PFA in 0.1 M PB for the post-fixation. Cryoprotection was provided by immersion of brains for one day in 10 % sucrose solution in 0.1M PB and for the another day in 30 % sucrose solution. Before being sectioned brains were frozen with liquid nitrogen and kept at -80 ºC until sectioning.

To obtain 20 µm thick coronal brain slices, frozen brains were sectioned on a cryostat microtome (SHANDON cryotome Thermo Fischer Scientific INC., USA). The sections were collected inside the 24-well plates containing a

cryoprotectant solution. Every 12th section through the hippocampus area (total 6 sections) was processed for neuronal NOS (nNOS) immunoreactivity (IR). Second set of 6 sections was processed for endothelial NOS (eNOS) IR, while the third set of 6 sections was processed for inducible NOS (iNOS) IR. Fourth set of 6 sections for each animal was Nissl (cresyl-violet)-stained to examine morphology of hippocampal area. Remaining sets were stored at +4ºC as a reserve.

2.3.2. Nissl Staining

0.1% cresyl violet acetate solution was prepared and used in this staining. The sections were mounted onto poly-lysine coated slides and allowed to dry overnight. Staining procedure basically includes dehydration of tissues by exchange of water inside with alcohol to enable penetration of xylene in later steps, defatting of tissue with xylene, rehydration step by replacement of xylene with alcohol to prepare them for staining, incubation of sections in cresyl violet solution until the dye is absorbed, differentiation step with ethanol mixed with glacial acetic acid to remove excess dye and finally dehydration by exchange of remained water with ethanol and then xylene for permanent storage. Finally, sections were covered with Permount and coverglass. The protocol applied in this study was adapted from Dr. Jurgen Wenzel's protocol. (2008).

2.3.3. A Brief Overview Of Immunohistochemistry Method

The main principle of IHC is the exposure of antigens (Ag) found within tissue sections to antibodies specific to these antigens and after maintaining antigen-

antibody interaction, development of histochemical reaction product (Ag-Ab complex) with the help of several detection systems. These detection systems are responsible of labeling Ag-Ab complex and formation of colored reaction product to make the complex visible by light microscopy. Some detection systems involve fluorochrome labeled Ab's and they directly give fluorescent signal when excited under epifluorescent or confocal laser microscopy instead of color development due to chemical reaction. This method is also named as "immunofluorescence" in some research papers and publications. Binding of Ab's to their target Ag's require some conditions to be met together and these conditions contribute specificity of assay in different ratios in different tissues. Major factors affecting formation of Ag-Ab complex can be listed as follows: method of tissue/cell fixation (type of fixative, fixation procedure, postfixation solution and duration), tissue processing (cryoprotection, freezing, embedding, sectioning) and detection method (enzymatic reaction for instance AP or peroxidase staining or immunofluorescence) and last but not least, quality of the tissue to be processed (Lorincz and Nusser, 2008). Tissue fixation aims to preserve cellular components (both soluble and structural ones), prevent autolytic activity of enzymes and spill out cell content (including Ag's and enzymes), stabilize cellular materials against procedures during staining and facilitate staining. Fixatives are solutions that carry out fixation step, they may act on tissues in either cross-linking proteins via methylene bridges (ex:aldehyde-derivatives such as formaldehyde, glutaraldehyde) or precipitating proteins by breaking hydrogen bonds among them (ex: ethanol, acetone). Most common example of fixative is the paraformaldehyde (PFA) (usually 2% or 4% w/v solution in PB). It is the dry, highly polymerized form of formaldehyde. It forms cross-links or covalent bridges between and within proteins and nucleic acids forming hydroxymethyl groups from side groups that contain an active hydrogen and/or primarily tertiary amines. This offers a rapid, stable fixation that maintains excellent tissue morphology, however it may mask the epitope by chemically modifying the proteins (i.e.

Epitope masking). Detection of relevant Ag may not be always available due to inaccesibility by the Ab's. Epitope masking due to other molecules or methylene cross-bridges after PFA fixation is the one of the most prominent causes of this. Unmasking of epitopes may be achieved via pretreatment of tissue with some detergents (1% sodium dodecyl sulfate (SDS)) to increase penetration of Ab's or co-application of heat and highly acidic (ex: Sodium citrate buffer pH 6.0) or basic (ex: Tris-EDTA buffer pH 9.0) buffers to the tissues for a pre-optimized period of time.

It is also important how the fixative solution is delivered to the tissue sample. For small rodents, usually transcardiac perfusion under heavy anaesthesia is prefered to replace blood circulating first with a neutral buffer (0.1 M PB or serum physiologic) then with fixative by using heart that is still beating as a pump. Although it is widely used, this way of delivery can have some confounds because physiological variables can affect vascular flow and therefore the speed and strength of fixation (Fritschy, 2008). Rarely, high flow rate may also bring about mechanical shear stress on vascular tissue and reduce antigeneity of some Ag's on vascular lining (Stanarius et al. 1997). In several studies, immersion fixation of tissue just after removal of it from the organism suggested to preserve antigeneity.

Some tissue types need additional fixation to maintain their integrity before further processing, this is called as "postfixation". Postfixation is usually carried out by immersion of tissue sample in the same fixative solution that has been used in fixation period. Rather than content of postfixative, time of incubation seems to be the determining factor for this process and it should be carefully determined for Ag of interest via optimization assays. There are some studies available investigating differential effects of different postfixation times on tissue antigeneity. Prolonged postfixation time may sometimes reduce IHC

signal for some Ag's (Gonzalez-Hernandez *et al*., 1996) while in some Ag's it may enhance staining pattern (Fritschy *et al.,* 2008).

Long-term storage of tissue samples is another issue of concern. If it is not prefered to use samples just after the fixation and decided to keep them in cold, samples should be secured against the destroying effects of freezing and thawing by cryoprotection process. Proper cryoprotection maintains tissue integrity, morphologies of cellular structures and antigeneity by removing water inside the tissues which crystallize while freezing and replacing it with high-concentration sucrose solution which has a freezing temperature much lower than pure water and doesn't crystallize. This step usually involves immersion of tissue on sucrose solutions serially (by gradually increasing the concentration, ex: from 10% (w/v) sucrose solution in PB to 20% and finally to 30% for several days in each). After this step, tissues are kept in cold (preferably at -80°C) for long-term storage either in sectioned form and mounted onto slides or in the form of intact tissue block.

For freezing, liquid nitrogen or dry ice are used but the contact of tissues in any of them is problematic. This freezing step may render fixed tissues more fragile and bring about cracking of tissue after thawing.

On the other hand, usually, it is expected that when exposed to Ab, part of the tissue missing the relevant Ag is not stained. However, sometimes, immunoglobulins (IgGs) that are responsible of Ag (epitope) recognition can go and attach to numerous unspecified targets with low affinity and produce high background when color developed during detection. In this case, blocking of non-specific targets before exposing to Ab with normal serum of the species that antibody was raised in (ex: NGS-normal goat serum for Ab raised in goat), bovine serum albumin (BSA), non-fat dry milk or casein is considered. This "blocking" step (incubation time, nature of blocking agent-reactivity) is also

found to be improving effect on the quality of some of IHC staining. However, casein, BSA, and dry milk can all contain bovine IgG. Many secondary Abs, such as anti-bovine Ig Ab, anti-goat Ig Ab, and antisheep Ig Ab, will react strongly with bovine IgG. Therefore, the use of BSA, dry milk or casein as a blocking agent may actually increase background and reduce antibody titer by competing with Ab's for epitope sites available (Buchwalow *et al*. 2011).

Another important step in immunohistochemistry assays is detection of Ag-Ab complexes via detection systems. These systems include chemical reactions of several reagents with labeled Ab's that give colored products or by-products that indicate each of Ag-Ab complexes available. Identification of localization of signals coming from detected complexes and their measurements may be achieved via light microscopy and quantitative (ex: counting of separate entities donating signals (ex: cells) showing immunoreactivity in the selected tissue) and semi-quantitative methods (colorimetric methods-analysis of signal intensities via different softwares) of analyses are available. Since they can directly affect interpretation of data, selection and application of detection complexes are particularly important. Some color developing substances in detection system may cause excess background and render signal to noise ratio higher. For example, peroxidase detection system may also develop signals from endogenous peroxidases available in samples and localization of signals from Ag-Ab complexes becomes difficult. To prevent this, samples are treated with dilute solutions of hydrogen peroxide (15% v/v or 30% v/v) to block endogenous peroxidases. In each detection system, this kind of pitfalls may be possible and these should be searched and optimized before obtaining data. Detection of antibodies labeled with fluorescent probes (fluorochromes) is achieved by epifluorescent or confocal microscopy. Enzymatic detection systems are not usually needed. Besides the properties of equipment (filters and light source of microscope, efficiency of camera), those of the fluorochrome should be considered. Excitation-emission spectra of fluorochrome should

correspond the filters' detection range. Fluoro-labeled material (Ab's and Ab-treated samples) should be kept away from light to prevent bleaching of fluorochrome before detection.

2.3.4. Immunohistochemistry Method Applied In The Present Study

Immunohistochemistry procedure used in this study was modified from Kilic et al., (2005), Kilic et al. (2006) and Ramos-Vara et al. (2005). For immunohistochemical determination of three isoforms of NOS, polyclonal primary antibodies specific for each isoform were used (Chemicon Millipore rabbit polyclonal to nNOS; Abcam rabbit polyclonal to eNOS, Abcam rabbit polyclonal to iNOS).

To determine the optimum conditions for immunohistochemistry assay, a preliminary optimization procedure was designed. In the Table 3, the different conditions applied for each NOS protein during optimization are shown and the appropriate ones are indicated in bold.

Table 3. Conditions applied during optimization of immunohistochemistry assay

Name of protein	1°Ab dilution	2°Ab dilution	Rinsing after 1°Ab and 2°Ab
nNOS	1/100	1/200	3x5 min
	1/100	1/500	3x5 min
	1/100	**1/500**	**3x5+3x15 min**
eNOS	1/100	1/200	3x5 min
	1/100	1/200	3x5+2x15 min
	1/100	**1/500**	**3x5+2x15 min**
iNOS	1/100	1/200	3x5 min
	1/100	1/200	3x5+2x15 min
	1/100	1/1000	3x5+2x15 min
	1/100	**1/500**	**3x5+2x15 min**

First of all, tissue sections were mounted on poly-lysine coated glass microscope slides. Then, all slides were dried at 37 ºC inside an incubator for approximately 15 min to ensure proper attachment of sections onto slides.

After rinsing with 1X phosphate buffered saline (PBS) once, antigen retrieval procedure is applied to uncover epitope sites blocked due to paraformaldehyde fixation.

Antigen retrieval is carried out with 10 mM sodium citrate buffer (pH 6.0) solution, sections were kept inside the preheated citrate buffer for 15 minutes then sections were kept for another 15 minutes inside citrate buffer slowly cooling down. After rinsing in PBS (3 times, 5 min for each), sections for nNOS and iNOS were incubated for 1 h at room temperature with blocking solution containing 10% normal goat serum with 0.3% Triton-X-100 in PBS.

For endothelial NOS immunoreactivity (eNOS IR), an alternative blocking solution that contained 3% BSA dissolved in 0.3% Triton-X-100 in PBS was applied for 1 h at room temperature.

Next, sections that were rinsed with 1X PBS 3 times (5 min for each) were incubated in primary antibody diluted with 2% NGS and 1X PBS for 24 hrs at 4 °C. On the following day, after being rinsed in 1X PBS (3 times for 5 min then 3 times for 15 min), sections were incubated with fluorescent- conjugated secondary antibody (Invitrogen Cy3 conjugated goat anti-rabbit IgG (1:500)) diluted with 2% NGS and 0.3% Triton-X-100 in PBS solution for 2 hrs at room temperature. Since secondary antibody is conjugated with light-sensitive fluorescent chromophore, incubation period is spent in the dark. Negative control for IHC is provided for each staining via omission of primary antibody in antibody dilution buffer.

Finally, sections were rinsed with 1X PBS (3 times for 5 min then 3 times for 15 min) and counterstain was applied to precisely locate nucleus of neurons and incubated for 15 min. (Hoescht 33342 trihydrochlorate trihydrate (Invitrogen), 1µg/ml). After rinsing sections with 1X PBS once (5 min), slides were coverslipped with an anti-fading mounting media. (Fluoromount, Sigma Aldrich Cat. No: K024). To ensure long-term storage, edges of cover slip were sealed with nail polish and slides were stored at 4°C.

Negative controls were included to test specificity of staining by omitting secondary antibody and no unspecific staining was observed.

2.3.5. Visualization and Analysis of Images

Visualization and image analysis procedure was modified after Gingerich et al. (2005) and Kajitani et al. (2010). Coronal brain sections obtained from subjects were visualized using a Leica DM6000 Microscope equipped with a fluorescent attachment containing appropriate filters. For each brain section, left and right hippocampi were visualized separately. For the analysis of nNOS and eNOS immunoreactivity, four regions of hippocampus including CA1, CA3, dentate gyrus and hilus were visualized at 40X magnification with 3 representative pictures for each region from each hippocampus. After visualization of images, they recorded as JPEG format and their analysis was performed with Image J (available from NIH website) programme. The procedure of the editing and analysis of obtained images was given as follow:

The images obtained in JPEG format were converted to grayscale and 8-bit which enable to convert the image to 256 shades (8-bit) of gray. In this scale zero represents pure black while 255 represents pure white (From the program menu choose File →Open→Browse then select the file then click OK). To convert image to 8-bit choose Image →Type →8-bit. To assign lower (0) and upper (255) threshold for measurement scale, calibration of program is required. To do this, from the menu tool bar, choose Analyze→Calibrate. A new window opened, then 1) Choose Function as "Straight line" from the Function menu; 2) Fill the empty Calibration box by writing 0 to the left side and 255 to the right side; 3) Inside same box, on the bottom row and fill the box by writing 255 to the left side and 0 to the right side; 4) Check "Global calibration" option and then click OK. A Calibration Function (G) window opened. After these steps, calibration curve was obtained as shown in Figure 8.

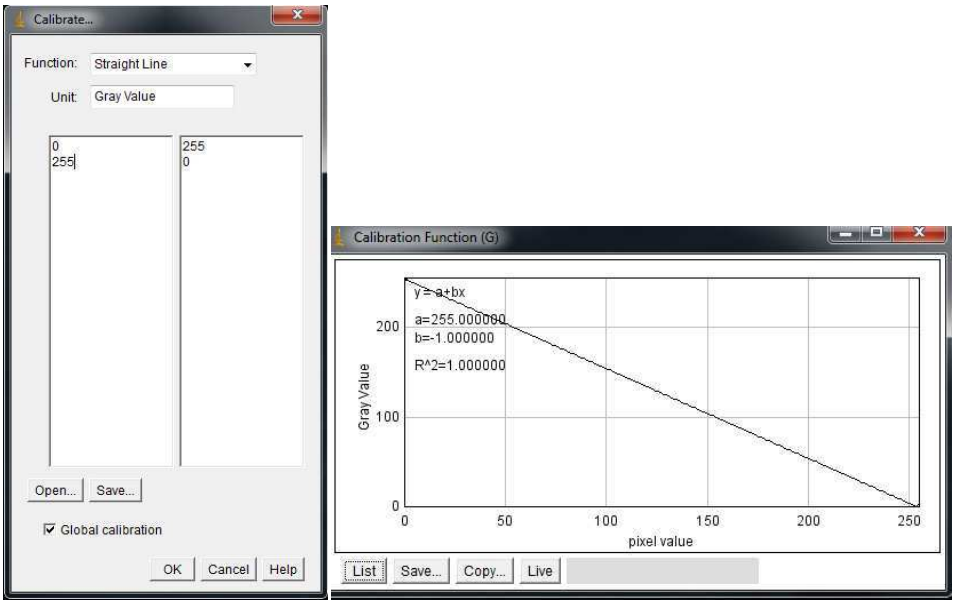


Figure 8. Calibration box (left) and Calibration Function (G) (right) of Image J window.

After calibration of program to 0-255 scale for measurements, optical densities were measured. For each image obtained, 5 representative areas (100x100 pixel of each) were chosen using "rectangular tool" from menu tool bar of programme and their optical densities (O.D.) were determined by pressing "M (measure)" on keyboard. A "Results" window showing O.D. values as the title of "Mean" opened, then these mean values were recorded for further analysis (Figure 9). The average of 5 O.D. values obtained from each picture was assigned as the region intensity value of that picture and named as "mean O.D.".

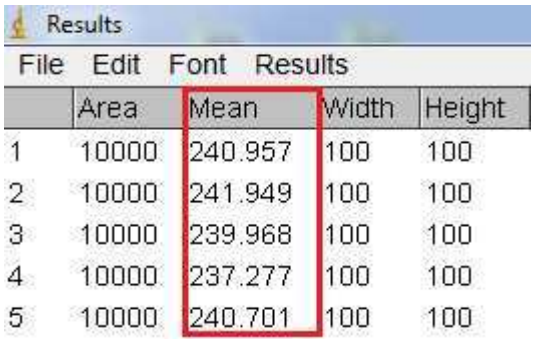


Figure 9. "Results" window showing area measured and its dimensions and mean intensity values obtained (red rectangular).

The Image J software is adapted to measure dark areas on a light background and while 0 as O.D. value represents "black" color, the value of 255 represents "white" color. In this case, this actually measure color intensity of each pixel "as how dark it is". Since the grayscale image obtained from our samples are light areas on a dark background to measure region intensity of each pixel in terms of "how white it is", the direct O.D. measurement (darkness) of each pixel was substracted from the total 255 to find out "how white it is". Then, the obtained value is called as "corrected O.D." (Corrected O.D. = 255- Mean O.D.).

To eliminate contribution of background fluorescence from obtained images, background fluorescence subtraction was applied. To do this, a representative picture was taken from "corpus callosum" region of each brain section (as shown in the Figure 10) which is known to be deficient from NOS isoforms (Kajitani *et al*., 2010). The above procedures were also applied to the background images and mean O.D. values of each background images was obtained. Background images were also corrected in the same way described above for sample images and resulting values are named as "BGR O.D". "BGR O.D" value obtained for each section was subtracted from all "corrected O.D." values obtained from the same section to get "FINAL OD" value. In other

words, every section had its own “BGR O.D.” value and evaluated according to this value. “FINAL O.D.” values for each picture were recorded and then averaged to find region intensities of CA1, CA3, DG and H regions of each experimental subjects separately. The image analyses of nNOS and eNOS immunostained sections were performed as described above.

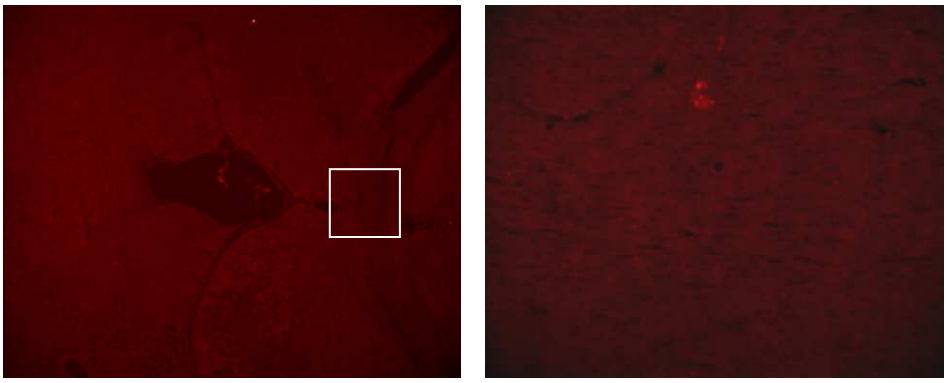


Figure 10. Photomicrographs taken from nNOS immunostained coronal section through corpus callosum region (considered as background). *Left:* *Magnification:*4X. White square (100x100 pixel dimensions in original) shows the position of the region on which representative images for background were taken. *Right:* The white square area under 40X objective. (*Total magnification*: 400X).

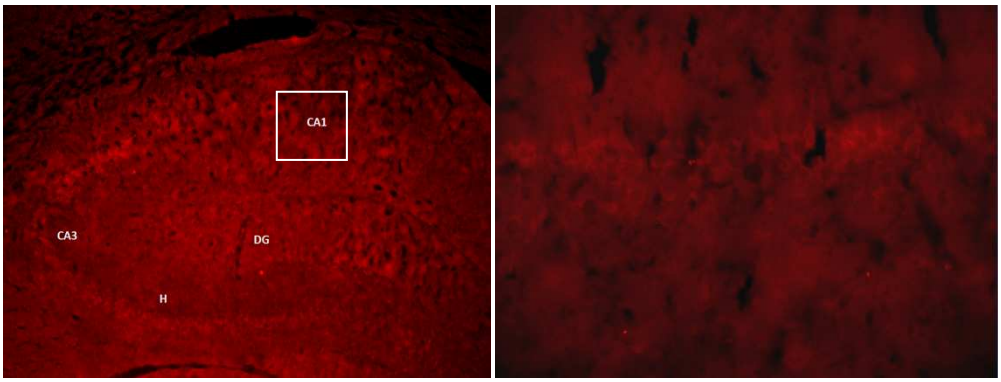


Figure 11. Photomicrographs showing nNOS immunoreactivity on a coronal section of rat hippocampus. *Left:* Hippocampal regions CA1, CA3, DG and H (*Magnification:* 4X). White square (100x100 pixel dimensions in original) shows the position of the representative region used in the image analysis. *Right:* White square area under 40X objective. (*Total magnification*: 400X).

Since the number of iNOS positive neurons was shown to be very scarce in various studies using subjects similar to our experimental subjects, the analysis of iNOS immunoreactivity was acquired via cell counting method (Zhu *et al.*, 2006). For this purpose, coronal brain regions obtained from subjects were visualized by using a Leica DM6000 at 20X magnification for four regions of hippocampus including CA1, CA3, dentate gyrus and hilus with 3 representative pictures for each region. To determine the number of iNOS immunoreactive cells, heavily stained and clearly identifiable neurons were marked by "cell counter" option available under the Plugin option of menu tool bar of Image J software (Plugins →Analyze →Cell counter). The results of three representative images were summed and named as the number of iNOS positive cells in that region of that hippocampi. Images obtained with appropriate filter of nuclear counterstain were used to determine whether a heavily stained area in immunostaining image is a real nucleus or not.

Visualization of Nissl stained sections were done under light microscope, the images obtained were used to examine the types of neurons showing NOS immunoreactivity on IHC slices.

2.4. Statistical analyses

All data were expressed as means ± SEM. Immunohistochemistry results were analyzed by Image J software (available at: http://rsb.info.nih.gov/ij/). Pixel intensity data were grouped and analyzed by SPSS software (SPSS v.18). Data from region intensities for nNOS and eNOS and the number of iNOS positive cells among hippocampal regions of experimental groups including "good" and "poor" learners (pooled data) were analyzed by using paired *t*-test. Region intensities for nNOS and eNOS IR in different hippocampal regions were compared between "good" and "poor" learners using Multivariate Analysis of Variance (MANOVA). A *p* value less than or equal to 0.05 was considered as statistically significant.

This study has been approved by the METU Ethical Comittee (*Protocol No: 2009/01*). The animal care procedures and all experimental manipulations were pursued in accordance with the METU Ethic Committee Regulations.

CHAPTER 3

RESULTS

3.1 Behavioral Results

3.1.1. Activity measurement results of Wistar rats

As described in Chapter 2, activity measurements were applied to all rats to determine their basal locomotor activity. After analysis of activity measurements data, one rat was excluded from radial arm maze task due to its low level of ambulatory score compared to the rest of the group (Ambulatory activity score for total of 15 min for W20=770). Therefore, the number of rats used in the RAM task was reduced to 29.

3.1.2. Radial arm maze task results

Table 4 presents total number of choices to the acquisition criterion, total number of WMEs (re-entries into either baited or unbaited arms), total number of RMEs (entries into unbaited arms), and total number of both errors for each rat, separately.

Table 4. Total numbers of choices to the acquisition criterion, total number of WME's, total number of RME's, and total number of both errors in RMA task for each rat, separately.

RAT ID	TOTAL number of CHOICES	TOTAL number of WME	TOTAL number of RME	TOTAL number
W1	247	50	73	123
W2	181	15	53	68
W3	288	68	96	164
W4	182	35	53	88
W5	182	45	64	109
W6	176	39	56	95
W7	334	93	99	192
W8	314	77	96	173
W9	217	37	74	118
W10	219	43	60	110
W11	192	46	53	99
W12	155	35	38	73
W13	303	64	98	158
W14	210	58	56	114
W15	192	49	60	109
W16	311	80	80	156
W17	218	32	78	110
W18	116	15	33	48
W19	152	30	39	69
W21	144	35	43	78
W22	162	36	47	83
W23	212	40	62	102
W24	137	27	43	70
W25	156	31	43	74
W26	157	46	47	93
W27	119	17	41	58
W28	156	25	43	68
W29	143	32	44	76
W30	103	25	35	60

Basing on the total number of choice to reach performance criterion of 83% of correct choice during 3 consecutive training days in RAM, rats were classified as "good" learners (G) (n=11) with total number of choices to the acquisition criterion ≤ group mean - 3SEM, "poor" learners (P) (n=6) with total number of choices to the acquisition criterion ≥ group mean + 3SEM, and the remaining "intermediate" learners (Figure 12).

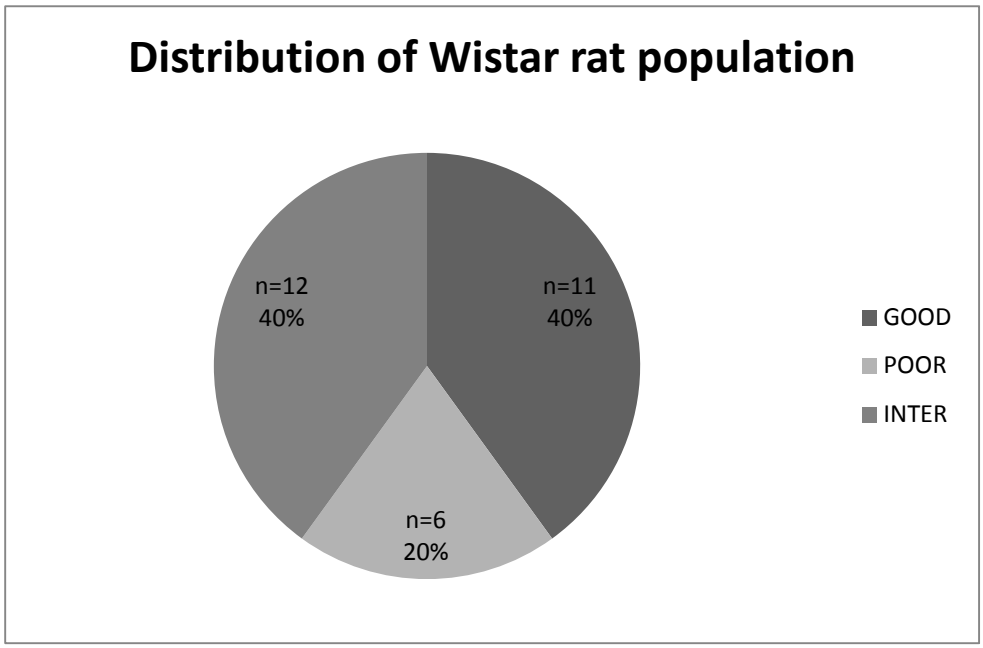


Figure 12. Distribution of "good", "poor" and "intermediate" learners among 29 Wistar rats participating in the RAM task. "n" denote number of individuals in each group.

Table 5 presents ambulatory scores of "good" and "poor" learners.

Table 5. Ambulatory activity scores of "good" and "poor" learners.

GOOD (n=11)		POOR (n=6)	
RAT ID	ACTIVITY	RAT ID	ACTIVITY
W12	1233	W1	1475
W18	1491	W3	1265
W19	2207	W7	2223
W21	1303	W8	1605
W24	972	W13	1515
W25	1542	W16	1277
W26	1545		
W27	1798		
W28	1215		
W29	1704		
W30	1023		

The mean ambulatory score for good learners was 1458 ± 99,69 whereas the mean ambulatory score for poor learners was 1560 ± 125,49. One-way ANOVA analysis applied to these data confirmed lack of a statistically significant difference between activity scores for good and poor learners ($F_{(1,16)}$= 0,317; p>0,05). In other words, individual variations of rats' performance in RAM task cannot be explained with their basal locomotor activity levels.

Figure 13 shows mean number of choices to criterion (A.) and mean number of all errors (B.) between good and poor learner groups. One-way ANOVA analysis confirmed significantly lower number of choices to the acquisition criterion and lower total number of errors in "good" learners compared to "poor" learners ($F_{(1;16)}$= 185,077; p<0.001, $F_{(1;16)}$=121,951; p<0.001, respectively).

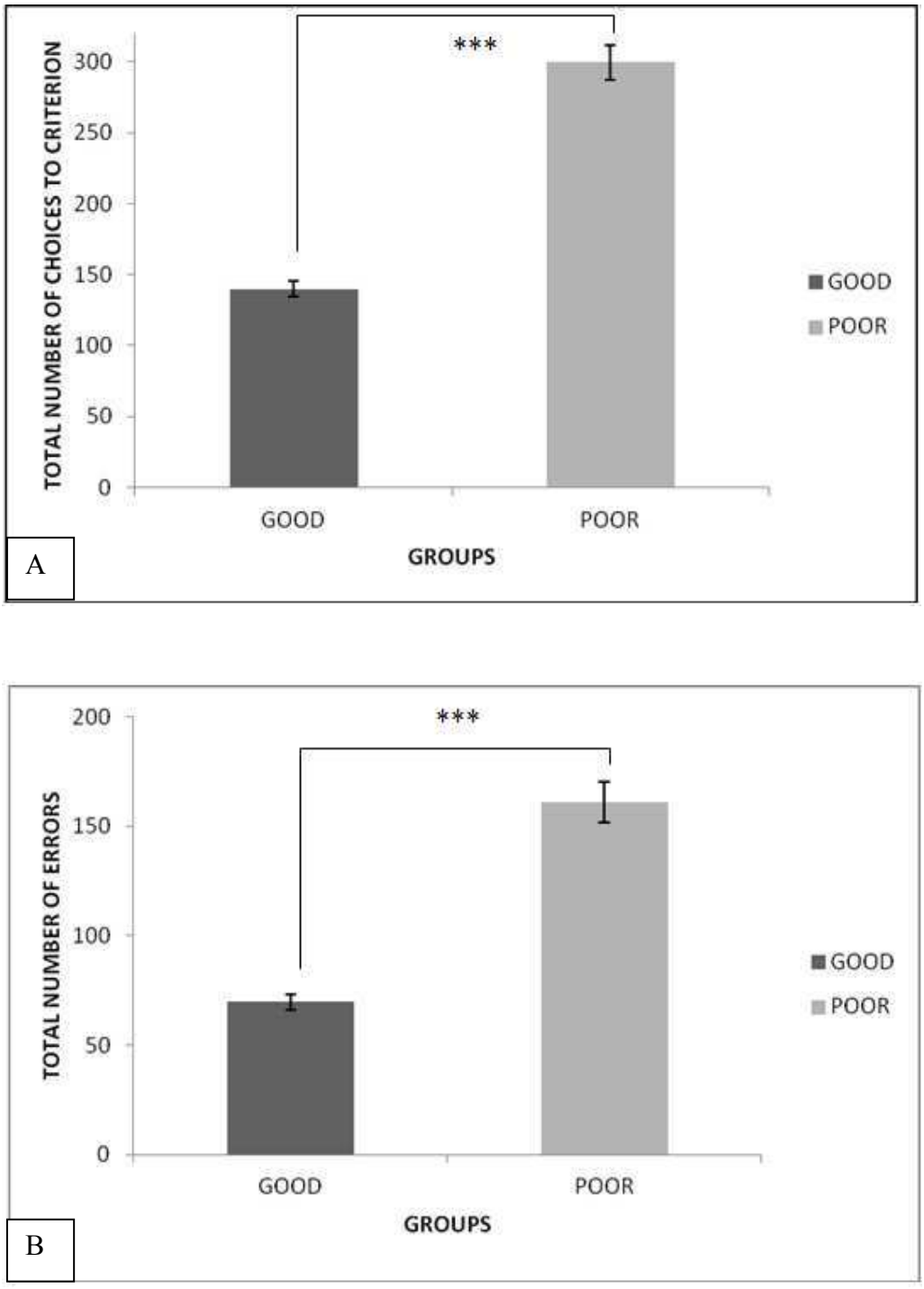


Figure 13. . Mean values of total number of choices to criterion (A.) and total number of errors (B.) between good and poor learner groups. Error bars denote ± SEM. Asteriks denote the level of significance: *** p<0,001.

Partially baited radial arm maze task enables to dissociate between working and reference memory errors and thus to examine both types of memory. Figure 14 presents total numbers of working and reference memory errors emitted by "good" and "poor" learners, separately. As seen from this figure, in both animal groups the frequency of RMEs was significantly higher than the frequency of WMEs (for good learners $F_{(1,23)}$=17,624; p<0,001 and for poor learners $F_{(1,11)}$=5,934; p<0,05) and poor learners emitted significantly more both working and reference memory errors compared to good learners.

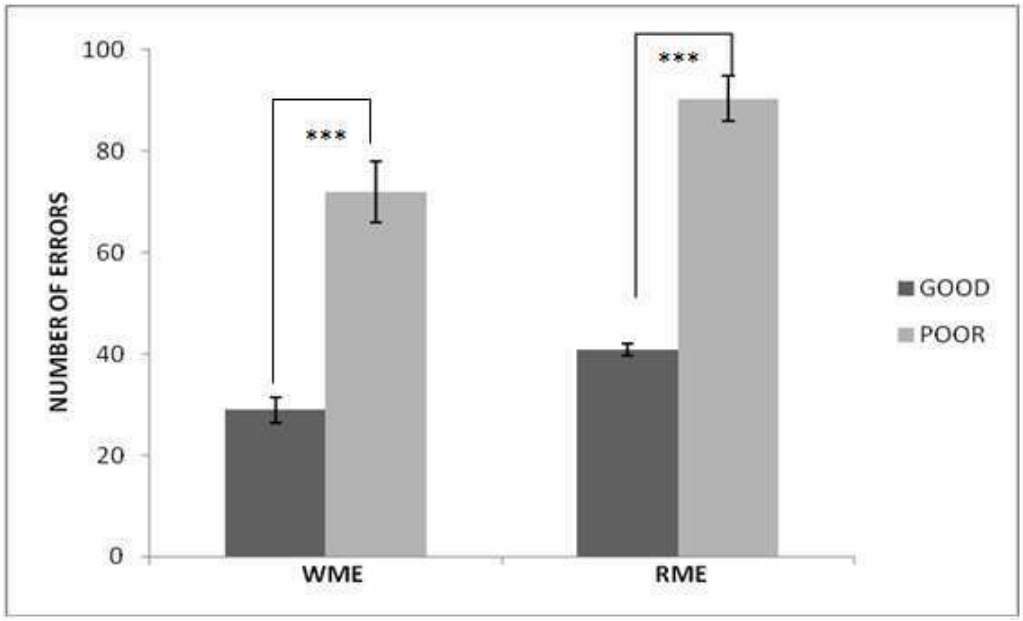


Figure 14. Mean number (± SEM) of working and reference memory errors emitted by "good" and "poor" learners. Error bars denote ± SEM. Asterisk denote the level of significance: ***p<0.001.

One-way ANOVA performed on these data yielded the between-group differences in WMEs and RMEs significant ($F_{(1;\ 16)}$= 58,611; p<0.001, and $F_{(1;16)}$=183,291 ; p<0.001 respectively).

3.2. Immunohistochemistry Results of NOS Isoforms

To examine whether there is a relationship between animals' spatial learning capacities and expression of different NOS isoforms in different hippocampal subregions, immunoreactivities for nNOS, eNOS and iNOS isoforms were assayed in cornu ammonis (CA) 1 and 3 regions, dentate gyrus (DG) and hilus (H). Since sections obtained from rat labeled as W25 and W27 lost tissue integrity after freezing with liquid nitrogen and cracked while mounting onto slides, they were excluded from following histological assays.Therefore, the number of individuals in each experimental group were rearranged as following: n=9 for "good" learners whereas n=6 for "poor" learners.

3.2.1. Distribution of nNOS and eNOS immunoreactivities in different hippocampal regions

The data obtained from "good" and "poor" learners were pooled to analyze distributions of nNOS and eNOS immunoreactivity (IR) among hippocampal subregions. The results of this analysis are presented in Figure 15.

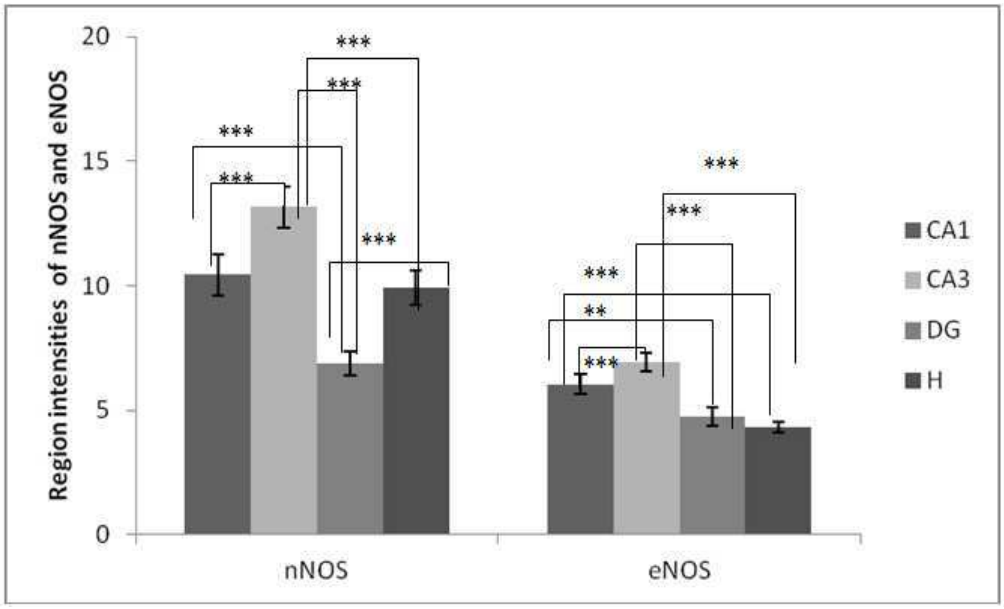


Figure 15. Distributions of nNOS and eNOS immunoreactivities expressed as mean signal intensities among hippocampal regions of cornus ammoni (CA) 1 and 3 regions, DG (dentate gyrus) and H (hilus). Error bars denote ± SEM. Asterisks denote the level of significance: ** p<0.01 and ***p<0.001.

A paired-samples t-test yielded nNOS IR in CA3 region significantly higher compared to CA1, DG and H regions ($t(14)$ =-4,801, p<0,001, ($t(14)$=11,101, p < 0,001, and $t(14)$ = 5,823, p < 0,001, respectively). IR in CA1 region was significantly higher than in DG region ($t(14)$ = 6,040, p < 0,001) and that in DG region was significantly lower than in the hilus region ($t(14)$ = -7,038, p < 0,001). In summary, the highest nNOS IR was found in CA3 region and the lowest in DG region with no significant difference between CA1 and H regions. Also eNOS IR was significantly higher in CA3 region compared to CA1, DG, and H regions (t(14) = -3,092, p < 0,01, t(14) = 3,973, p = 0,001, t(14) = 7,454, p= 0,001 respectively). eNOS-IR detected in CA1 region was significantly higher as compared to DG and H regions (t(14) = -2,734, p < 0,05; and t(14) = 5,059, p < 0,001, respectively) with no significant difference between DG and H regions. Interestingly, in all the hippocampal regions, the levels of eNOS expression were significantly lower than those of nNOS.

3.2.2. Distribution of iNOS immunoreactive cells among hippocampal regions

Since the number of iNOS positive neurons were reported to be very few, it is not suitable to analyze iNOS-IR via region intensity measurement method. Therefore, the number of iNOS positive neurons were determined by cell counting. The data obtained from "good" and "poor" learners were pooled to analyze distribution of iNOS IR among hippocampal regions. The results of this analysis are given in Figure 16 which presents the counts of IR cells expressing iNOS in different hippocampal subregions.

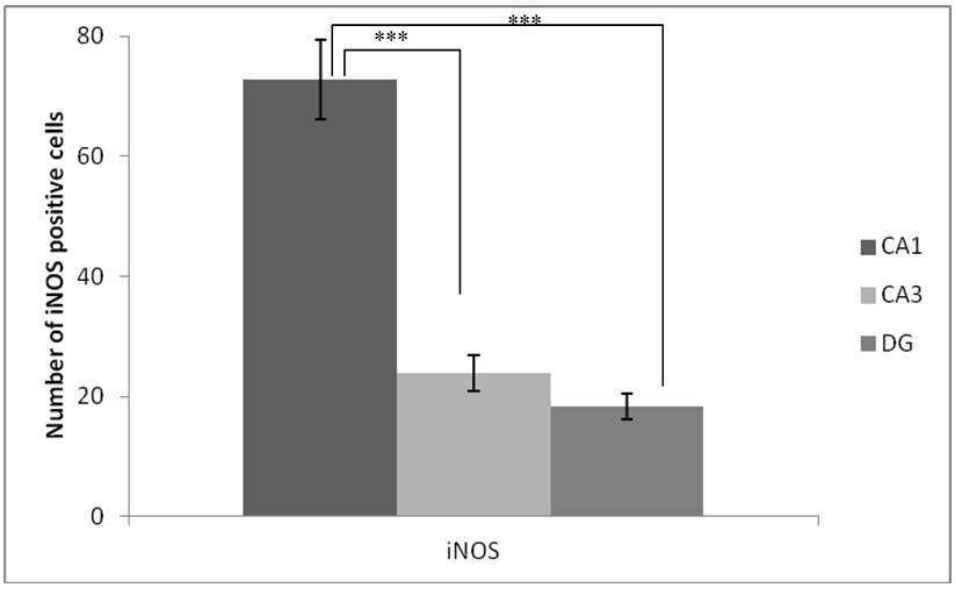


Figure 16. Mean numbers of iNOS immunoreactive cells in cornus ammoni (CA) 1 and 3 regions, and DG (dentate gyrus) region. The iNOS IR in the hilus region was very low and is not shown in the graph. Error bars denote ± SEM. Asterisk denote the level of significance: ***p<0.001.

As seen from the Figure 16, the number of iNOS positive cells in CA1 region was significantly higher than both in CA3 and DG regions (($t(14) = 9{,}427$, $p < 0{,}001$ and $t(14) = 9{,}649$, $p < 0{,}001$, respectively).

Since the observed number of iNOS positive cells in the hilus region was very low and this brings false negative result in the statistical analysis, data obtained from the hilus region was excluded from the statistical analysis and is not shown in Figure 16.

In addition to the analysis of the distribution of NOS isoforms' IRs across hippocampal regions presented above, distribution of NOS IRs was compared between experimental groups of "good" and "poor" learners for each hippocampal region independently. These data were analyzed using multivariate ANOVA (MANOVA) which revealed the multivariant main group effect significant (Wilks λ=0,297, $F_{(14,102)=}$ 6,083, $p \leq 0{,}001$) for the nNOS IR. These results are summarized below as Figure 17 and 19.

3.2.3. Comparison of nNOS immunoreactivity between good and poor learner groups

MANOVA performed on nNOS IR data showed the main group effect significant ($F_{(7;60)}$= 8,772, $p \leq 0{,}001$). According to the results of the LSD post hoc group comparisons, in good learners, region intensity of nNOS was the highest in CA3 region compared to CA1, DG, and H (p=0.007, $p \leq 0{,}001$, and p=0,017 respectively). In poor learners, the highest IR was also detected in CA3 region but significant differences were yielded between CA3 and DG, and CA3 and H regions only ($p \leq 0{,}001$, and p=0,015, respectively). In both groups, the lowest nNOS IR was found in DG with significant differences between DG and all other regions ($p \leq 0{,}05$). These results largely overlap with the analysis performed on the pooled data. The between-group comparisons (Figure 17.B) revealed significant (p=0,003) difference in nNOS IR between good and poor learners only for CA1 region with surprisingly lower nNOS in good learners compared to poor learners.

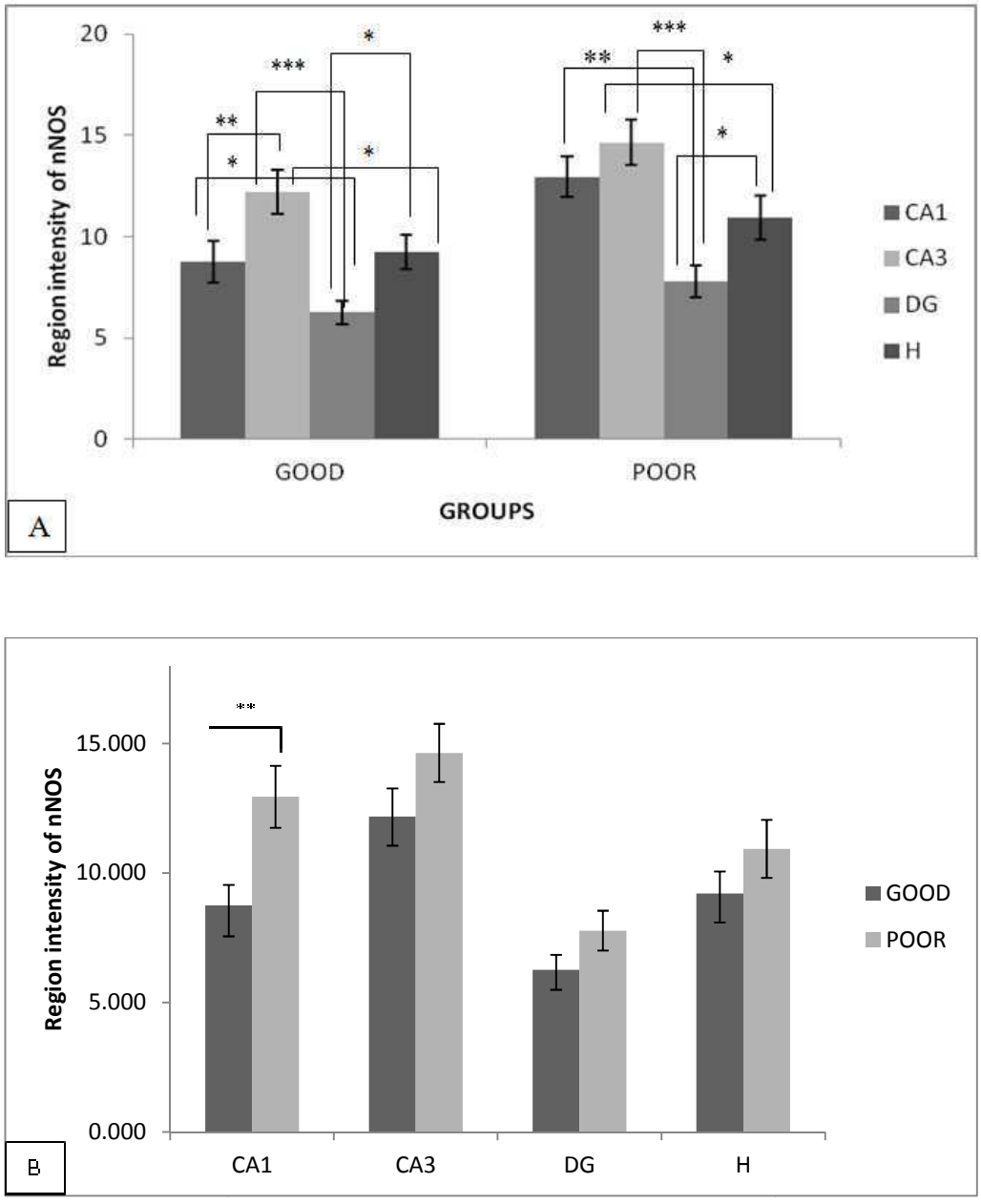


Figure 17. Comparison of nNOS immunoreactivity between CA1, CA3, DG and H hippocampal regions for good and poor learners, separately (A), and between experimental groups of "good" and "poor" learners for each hippocampal region independently (B). Error bars denote ± SEM. Asterisks denote the level of significance: * p<0.05, p<0.01 and ***p<0.001.**

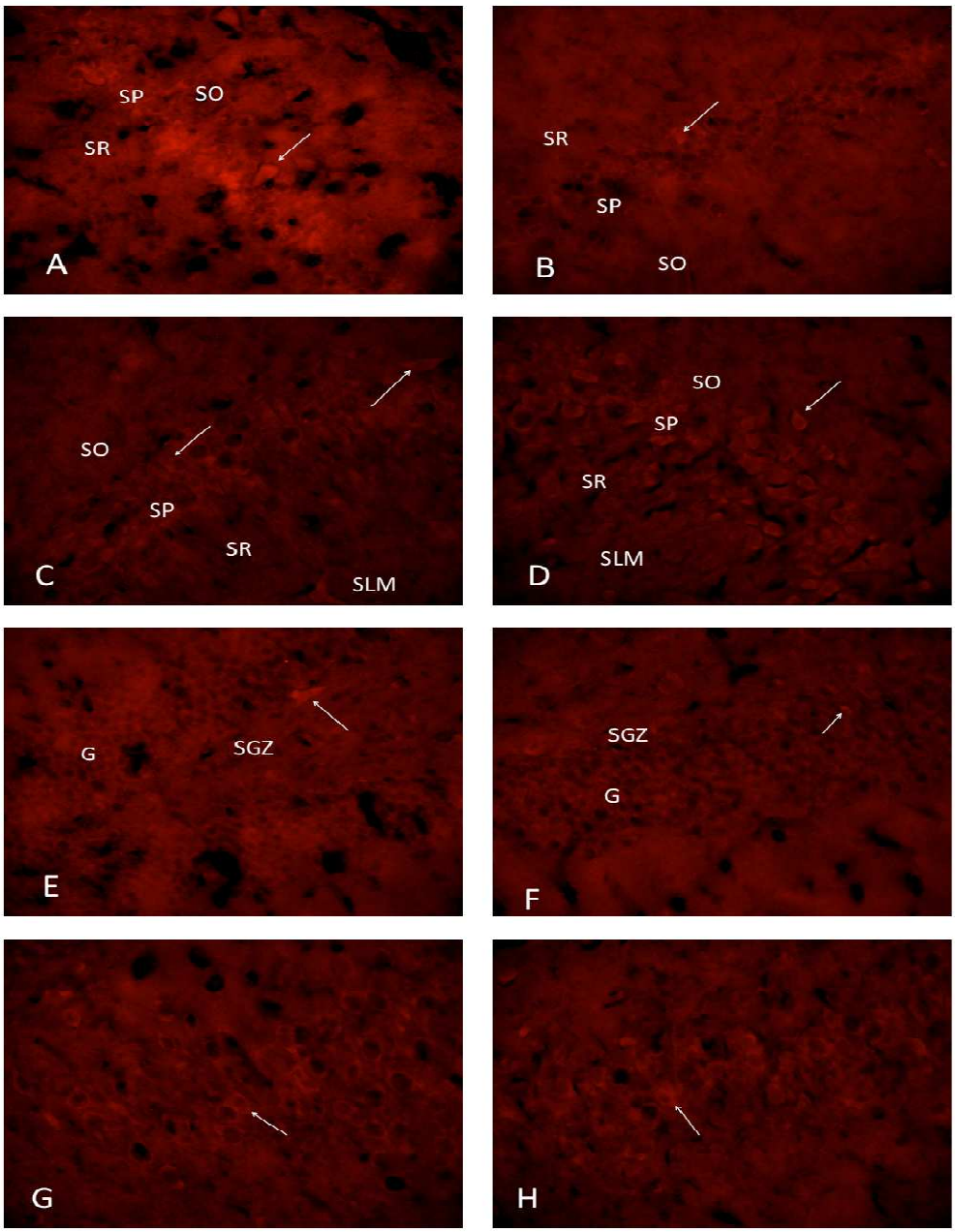


Figure 18. Photomicrographs showing nNOS immunoreactivity in good (left) and poor (right) learners for four regions of hippocampus: CA1 (A-B), CA3 (C-D), dentate gyrus (E-F), hilus (G-H). Abbreviations: SO (stratum oriens), SP (stratum pyramidale), SR (stratum radiatum), G(granular layer), SGZ (subgranular zone), SLM (stratum lacunosum moleculare). Magnification: 400X. White thin arrows indicate examples of nNOS positive neurons.

3.2.4. Comparison of eNOS immunoreactivity between good and poor learner groups

MANOVA performed on eNOS IR data showed the main group effect significant ($F(7;60)= 5,263$, $p\leq0,001$). According to the results of the LSD post hoc group comparisons, in both "good" and "poor" learners (Figure 19.A.) the eNOS IR was found significantly higher in CA3 region compared to DG and H regions ($p=0.003$ and $p\leq0,001$ respectively). There were no significant differences in eNOS IR either between CA1 and H regions or between DG and H. The post hoc group comparisons of data did not show any significant eNOS IR difference between "good" and "poor" learners in any of the regions (Figure 19.B.). In all hippocampal regions and both rat groups, the eNOS IR was significantly lower than nNOS IR.

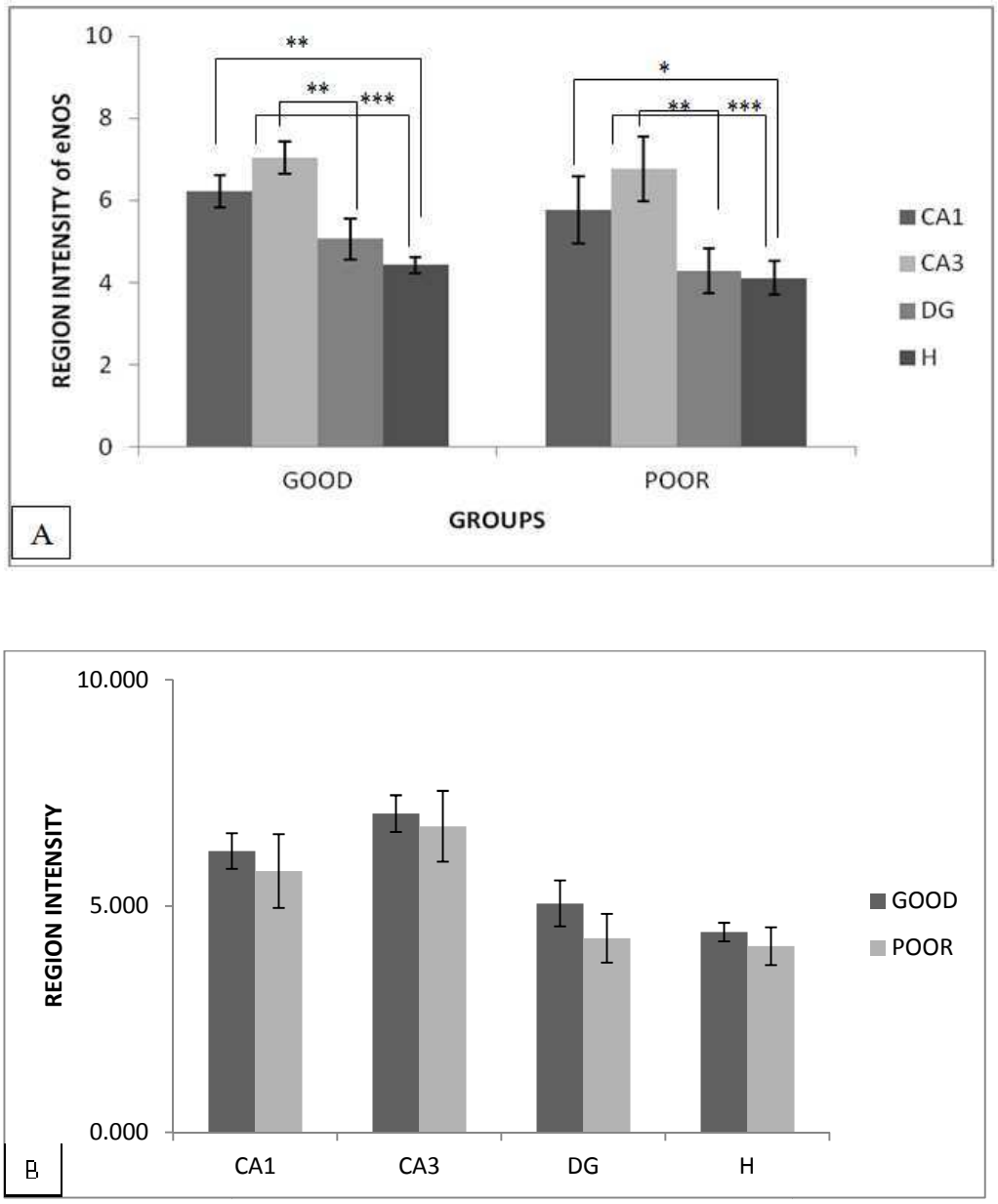


Figure 19. Comparison of eNOS immunoreactivity between CA1, CA3, DG and H hippocampal regions for good and poor learners, separately (A) and between experimental groups of "good" and "poor" learners for each hippocampal region, independently. Error bars denote ± SEM. Asterisks denote the level of significance: * p<0.05, ** p<0.01 and ***p<0.001.

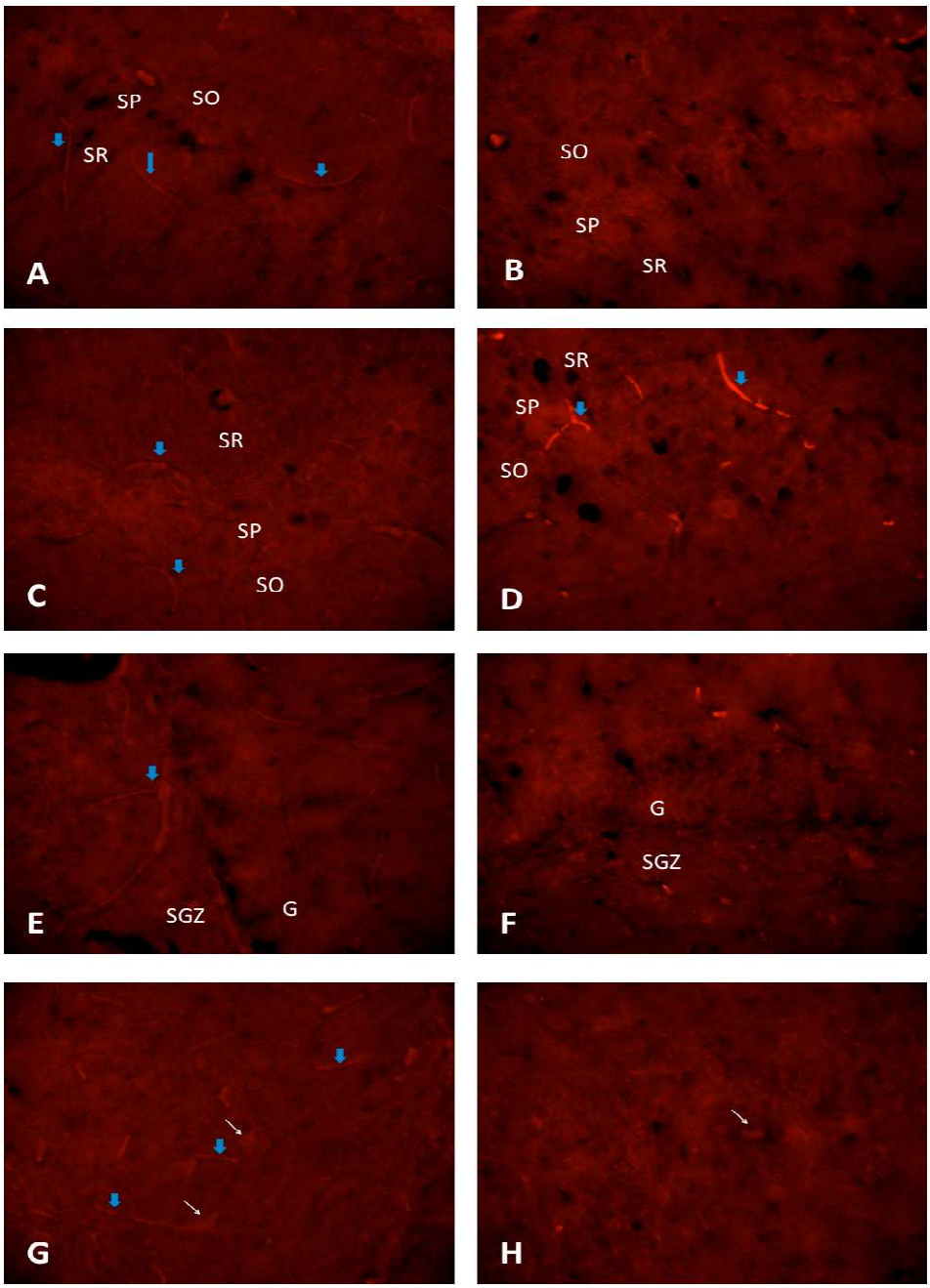


Figure 20. Photomicrographs showing eNOS immunoreactivity in good (left) and poor (right) learners for four regions of hippocampus: CA1 (A-B), CA3 (C-D), dentate gyrus (E-F), hilus (G-H). Abbreviations: SO (stratum oriens), SP (stratum pyramidale), SR (stratum radiatum), G (granular layer), SGZ (subgranular zone). Magnification: 400X. White thin arrows indicate examples of eNOS positive neurons, blue thick arrows indicate examples of eNOS stained vessels.

3.2.5. Comparison of the counts of iNOS immunoreactive cells in different hippocampal regions between good and poor learner groups

The general trend observed in whole population analysis (Fig.16) was preserved in both, good and poor learner groups with the mean number of iNOS positive cells in CA1 region significantly higher compared to CA3 and DG regions ($t(8) = 5{,}765$, $p < 0{,}001$, and $t(8) = 6{,}396$, $p < 0{,}001$, respectively, in good learners, and $t(8) = 9{,}914$, $p < 0{,}001$) and $t(8) = 7{,}975$, $p < 0{,}001$, in poor learners) with no difference between CA3 and DG regions (please see Figure 21.A). As indicated in Section 3.2.2., hilus region was not included into analysis due to lack of iNOS positive cells in this region in all groups. The data analyzed with one-way ANOVA did not show any significant iNOS immunoreactivity difference between "good" and "poor" learners in any regions (For CA1 region $F_{(1;14)} = 2{,}402$; $p=0{,}145$, for CA3 region $F_{(1;14)} = 2{,}674$; $p=0{,}126$, for DG region $F_{(1;14)} = 3{,}100$; $p=0{,}102$). (Fig.21.B.).

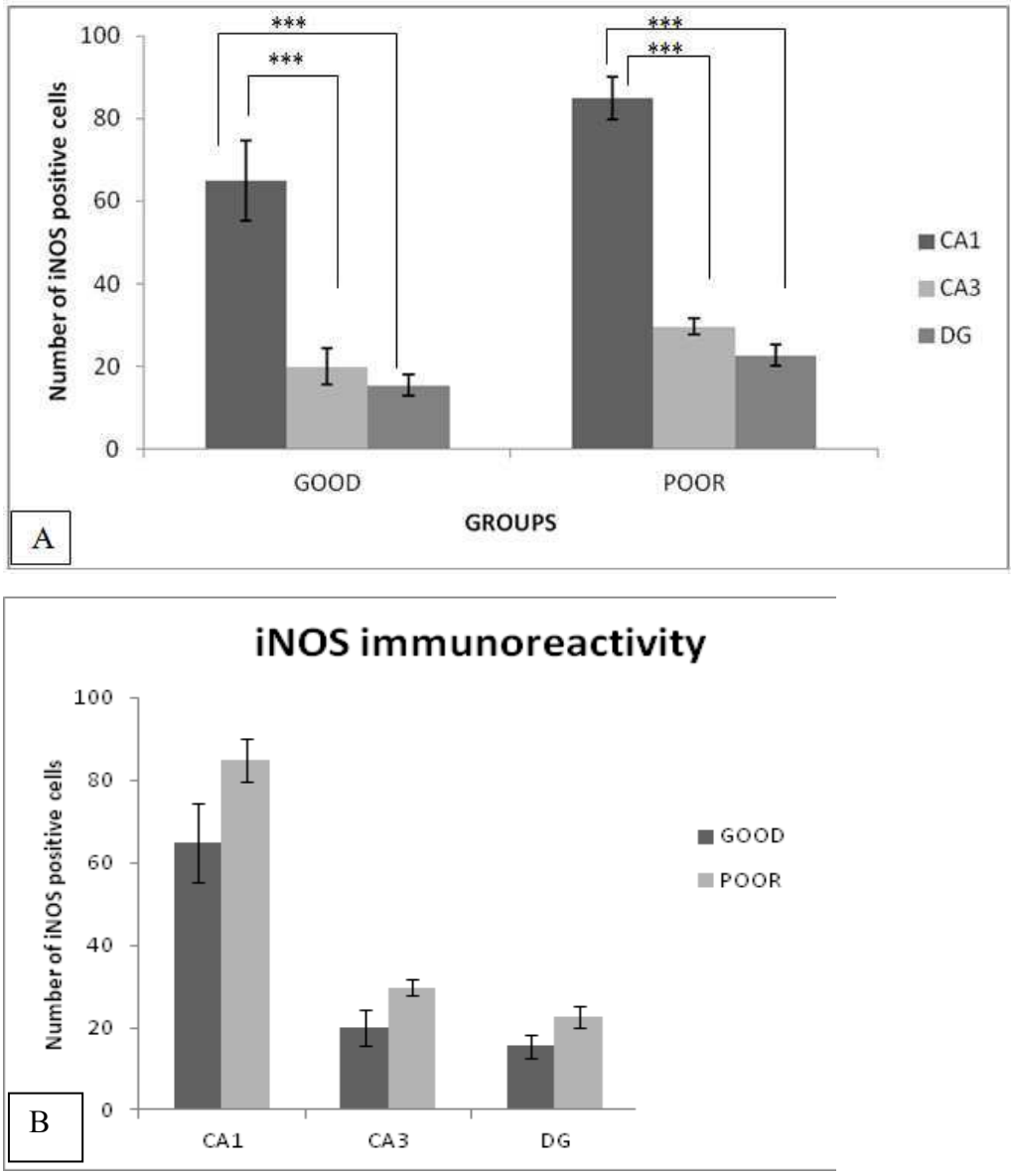


Figure 21. Comparison of the numbers of iNOS immunoreactive cells between CA1, CA3 and DG hippocampal regions for good and poor learner groups, independently (A), and between experimental groups of "good" and "poor" learners for each hippocampal region, separately (B). Error bars denote ± SEM. Asterisks denote the level of significance: ***p<0.001.

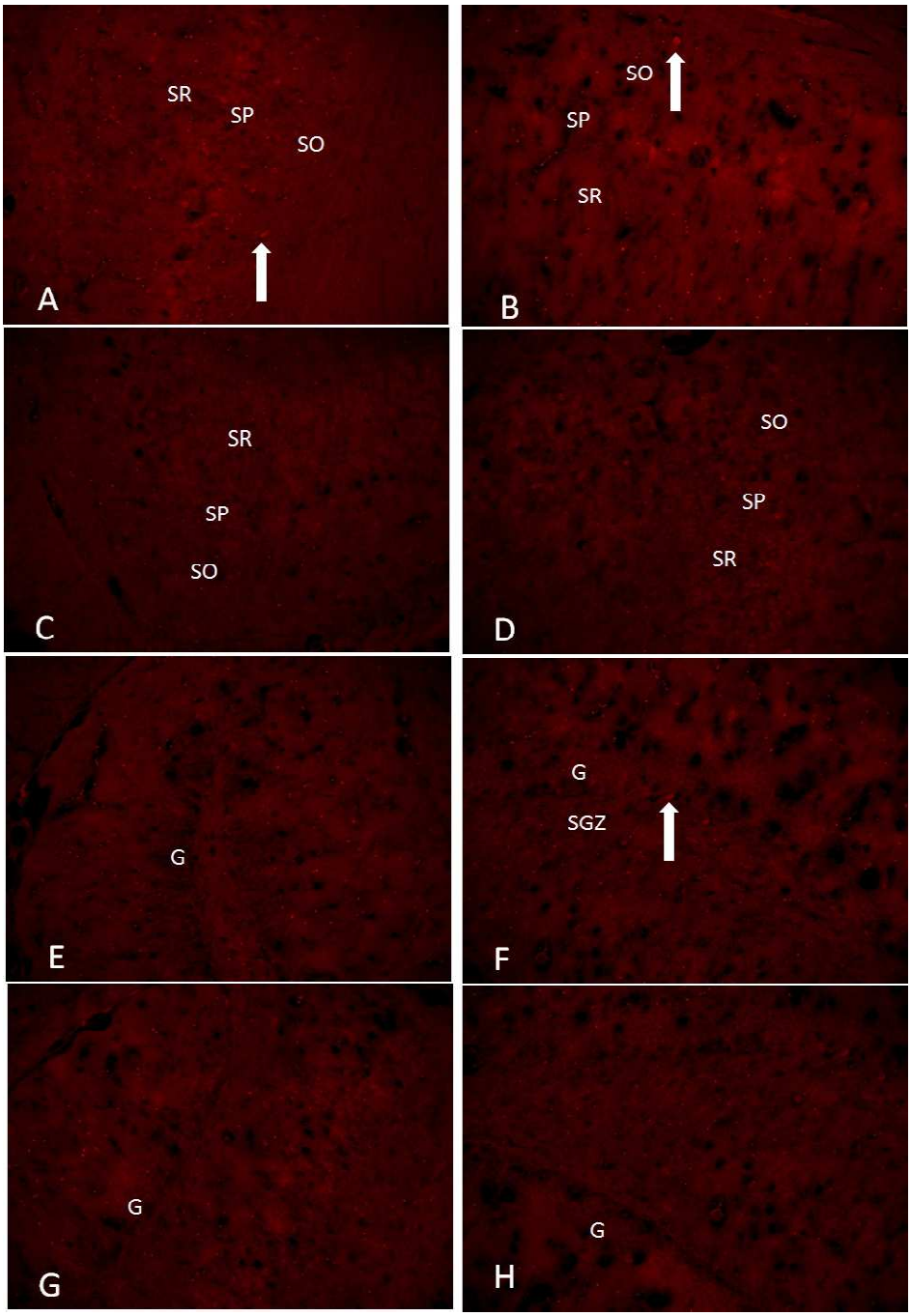


Figure 22. Photomicrographs showing iNOS immunoreactivity in good (left) and poor (right) learners for four regions of hippocampus: CA1 (A-B), CA3 (C-D), dentate gyrus (E-F), hilus (G-H). Abbreviations: SO (stratum oriens), SP (stratum pyramidale), SR (stratum radiatum), G (granular layer), SGZ (subgranular zone). Magnification: 200X. Arrows indicate examples of iNOS positive neurons.

CHAPTER 4

DISCUSSION

As mentioned in Section 1.4.5, all three isoforms of NOS display different distribution patterns from each other. Especially, differential distribution of both nNOS protein and nNOS mRNA along dorsoventral axis was reported (Jinno *et al*., 1999; Blackshaw *et al*., 2003). Therefore, sampling of sections throughout the brain may affect results in the studies on nNOS expression. In the present study, area of interest was determined according to reports implicating specific role of dorsal hippocampus in spatial memory (Fanselow *et al*., 2010). Additionally, as shown by some previous studies, the nNOS IR was stronger in dorsal than in ventral hippocampus (Jinno *et al*., 1999).

In this study, overall hippocampal levels of nNOS assessed semi-quatitatively by NOS intensity scores were significantly higher than those of eNOS and iNOS. The level of n- and eNOS was higher in CA1 compared to DG/hilus areas, but lower than that in CA3 region. The expression of iNOS was the highest in CA1 and the lowest in hilus region. nNOS IR was significantly higher in "poor" than in "good" learners but only in CA1 region. No significant between-group differences were found in eNOS expression. iNOS expression was higher in "poor" learners but it did not reach the required significance level. These results are partially inconsistent with the results obtained recently in our laboratory by Gökçek-Saraç and colleagues (2012), who examined the correlation between rats' performance in the partially baited RAM task and

hippocampal levels of three NOS isoforms as assessed by a quantitative Western Blot analysis. They found a positive correlation between animals learning skills and hippocampal nNOS level and a negative correlation with e and iNOS levels with relatively high overall iNOS expression. However, it should be noted that in these two studies different quantification methods were applied and when in the latter study homogenates from the whole hippocampus were used for the biochemical assays, in the present study, the NOS expression was examined specifically in dorsal hippocampus. Briefly speaking, results of nNOS expression obtained from WB assay from whole hippocampus may not exactly correspond to the results of IHC assay restricted to the dorsal hippocampus. Also in the literature, there are discrepancies related to the expression levels and the distribution of NOS isoforms among different regions of the brain and their localization in different cell types, therefore, our immunoreactivity results should be interpreted carefully.

Unlike in the present study, where the constitutive NOS (n/eNOS) signals were significantly stronger in CA3 area compared to the other regions in some other studies, the number of cNOS-IR cells were found to be higher in CA1 than CA2 and CA3 (Rodrigo *et al*., 1994, Jinno *et al*., 1999; Blackshaw *et al*., 2003). Also in the study performed by Dinerman and colleagues (1994), strong eNOS IR was found in CA1 pyramidal layer and in DG granular layer with weaker eNOS-IR in CA3 region. However again it is difficult to compare studies where more quantitative measures such as counting IR cells were used, with studies as our, where semi-quantitative measures based on the relative signal intensity were taken.

However, the lower overall level of nNOS in good learners compared to poor learners found in the present study was contradictory to the findings of several previous studies demonstrating importance of nNOS signaling in mnemonic processes (Chapman *et al*., 1992; Son *et al*., 1996; Susswein *et al*., 2004).

Interestingly, in the present study, the difference in nNOS signal intensity between good and poor learners regions was significant for the CA1 region but not for any other hippocampal region.

Although the differences between good and poor learners in eNOS IR were yielded insignificant, in all hippocampal regions studies, there was a general trend towards higher eNOS expression in good learners compared to poor learners. Actually, the higher eNOS levels if correlated with increased NO production should facilitate the learning-dependent neuroplasticity by promoting vasodilation and thus increased blood supply to the active brain regions (Boo *et al*., 2003).

In the current study, the number of iNOS positive cells in CA1 region was significantly higher than both in CA3 and DG regions but overall iNOS expression in hippocampus estimated via iNOS positive cell numbers was very low as expected from healthy and young animals (Adachi *et al*., 2011).When comparing iNOS IR across different hippocampal subregions (except hilus) between good and poor learners, despite the lack of a significant between- group differences, a general trend towards lower iNOS IR in good learners as compared to poor learners was observed.

In summary, the present results manifested regional differences in the hippocampal expression of the three NOS isoforms with their higher expression in cornu ammonis than in the dentate gyrus. The relative expression of the constitutive NOS isoforms (neural and endothelial) was the highest in CA3 region while that of iNOS in CA1 region. Among three NOS isoforms, nNOS showed the highest overall expression. The expression of eNOS was approximately half that of nNOS while iNOS expression was so low that it was impossible to process the data using imageJ software. The comparison of NOS levels between good and poor learners revealed a significant difference only

for nNOS and only in CA1 hippocampal region. The nNOS expression was significantly lower in good learners compared to poor learners, the result contradictory to most of the previous reports positively correlating nNOS expression with the learning skills. The general trend in overall levels of eNOS was towards its higher expression in good learners while the general trend in overall levels of iNOS was towards its higher expression in poor learners. The latter results argue against strong relation between NOS isoforms and spatial learning capacity in young Wistar rats.

The fact that our results are discrepant from some previous results by other authors inevitably brings about a discussion related to IHC method that was applied in the present study. There might be some methodological issues affecting our results and they will be considered below. First of all, in our study, postfixation time was determined as 12-24 hrs based on previous NOS IHC studies (Holtz *et al*., 2001). Since our procedure was directly adapted from the previous work by Kajitani and colleagues (Kajitani *et al*. 1995), we omitted optimization for postfixation step and applied immersion postfixation with 4% PFA for 12 hrs. In the previous work of Buwalda (Buwalda et al., 1995), it was also stated that aldehyde fixation doesn't affect nNOS immunostaining. Nevertheless, it is possible that in the present study, too long incubation in PFA during postfixation blocked epitope sites on antigens available for antibody binding and weakened the signal. Gonzalez-Hernandez and colleagues (Gonzalez-Hernandez et al., 1996), investigated the effect of fixation and other parameters related with IHC staining on NOS isoforms immunoreactivity and NADPH-diaphorase staining in adult rat brain tissue. Experiment was designed for three perfusion volumes (150-200 ml, 300-400 ml or 500 ml), and three different incubation times for postfixation (0, 4-6 hrs and 14-16 hrs). 4% PFA was used as a fixative. IHC assays for these different conditions were performed by using antibodies against nNOS, eNOS and iNOS. It was observed that nNOS immunostaining was affected from postfixation time and

volume of fixative used. Protocol 150-200 ml/0-6 hrs and Protocol 300-500 ml/0-6 hrs provided adequate labeling for clear identification of nNOS positive neurons and these conditions were named as "optimal labeling." However, Protocol 300-500 ml/14-16hrs substantially reduced intensity of neuronal labeling even if number of neurons remained unaltered. Investigation of eNOS staining for the same parameters gave similar results encouraging effect of postfixation on IHC signal quality and revealed that in the tissues treated either with Protocol 150-200 ml/0-6 hrs or Protocol 300-500 ml/ 0-6 hrs endothelial cells known to express eNOS are optimal. It is also possible that injury of endothelial lining due to mechanical stress brought by perfusion reduced antigeneity of the tissue for eNOS (Stanarius *et al*., 1997; Fritschy *et al*., 2008).

CHAPTER 5

CONCLUSION

- The results of spatial learning task performed on partially baited 12-arm maze showed that there is an individual variation within a random population of young and healthy rats to reach acquisition criteria with "poor learners" executing significantly more both reference and working memory errors.

- There are significant differences of distributions of n-, e-, iNOS proteins among hippocampal subregions of CA1, CA3, dentate gyrus (DG) and hilus.

- The nNOS immunoactivity was significantly higher in CA region compared to DG, with the highest level observed in CA3 area.

- Also eNOS IR was significantly higher in CA region compared to DG with the highest expression in CA3 area.

- In all hippocampal regions and both rat groups, the eNOS IR was significantly lower than nNOS IR.

- Generally, the iNOS IR was very low with the highest number of iNOS positive cells recorded in CA1 region .

- The comparison of NOS isoforms IRs in the hippocampi belonging to poor and good learners revealed a significantly higher nNOS IR in CA1 of poor learners compared to good learners. No other difference has been found.

- Results obtained in the present study suggest lack of correlation between hippocampal expression of NOS isoforms and learning skills in laboratory Wistar rats.

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