AN ABSTRACT OF THE DISSERTATION OF

Siba Ranjan Das for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on September 23, 2010.

Title: Influence of Aging and Behavioral Experience on Expression of GluN1 Splice Variants of the NMDA Receptor in Prefrontal cortex of Mice Brain.

Abstract approved:

_____

Kathy R. Magnusson

As the aging population continues to grow the world over, age related complications become more and more apparent among the elderly population. One such complication is age associated memory impairment, which makes the elderly more dependent on caregivers early on. NMDA receptors in the brain are important for memory formation, consolidation and retrieval. Expression of NMDA receptors declines with age, which is associated with declines in memory observed during aging. Age-related changes in the protein and mRNA expression of some of the splice forms of the GluN1 (GluN1, NR1) subunit of the NMDA receptor have been seen in mice and rats. The present study was designed to determine whether individual splice forms of the GluN1 subunit of the NMDA receptor within prefrontal / frontal cortical regions contribute to memory deficits during aging and whether experience in learning tasks can influence the expression of the splice forms.

mRNA expression of 4 splice forms $GluN1_{X11}$, $GluN1_{X10}$, $GluN1_{0XX}$ and $GluN1_{1XX}$ (GluN1-1, GluN1-3, GluN1-a and GluN1-b, respectively) and mRNA for all known splice forms (GluN1-pan) were examined by *in situ* hybridization. mRNA

for the C-terminal splice forms, $GluN1_{X11}$ (GluN1-1; +C1 and +C2 cassettes) and $GluN1_{X10}$ (GluN1-3; +C1 and +C2'), showed significant declines during aging in several brain regions even though overall GluN1-pan mRNA expression was not significantly affected by aging. This work provides evidence that these splice forms are more influenced by aging than the subunit as a whole. There was an increase in the expression of $GluN1_{0XX}$ (GluN1-a; -N1 cassette) splice form in the behaviorally-experienced old mice relative to the younger groups. Old mice with the highest levels of mRNA expression for the $GluN1_{0XX}$ (GluN1-a) splice form in orbital cortex showed the best performances in spatial working and reference memory tasks, but the poorest performances in a cued, associative learning task. These results suggest that the $GluN1_{0XX}$ subunit splice variant may be important for spatial memory performance in the old animals.

Protein expression of GluN1 subunits containing C-terminal cassettes C2 or C2' were observed to decline with increasing age, regardless of experience. In middle-age animals, higher expressions of the GluN1 subunit and C2' cassette proteins were associated with good reference memory on initial search. In the aged animals, higher protein expression of GluN1 subunits containing C1 cassettes and the whole population of GluN1 subunits were found to be associated with better performance in the final phase of probe trials but this appeared to be due to perseveration or delays in applying an accurate search. These results provide support for the theory that there is heterogeneity in the effect of aging on the expression of the GluN1 subunits containing different splice cassettes. It also suggests that the GluN1 subunit might be most important for good reference memory during middle age.

The next study was undertaken to determine if the $GluN1_{0XX}$ splice form is required for good performance in reference memory tasks in young mice. Mice were injected with 5µl of either siRNA specific to $GluN1_{0XX}$, control siRNA or vehicle alone into ventro-lateral orbital regions of both sides of the brain using a stereotaxic apparatus. A fourth group of mice did not receive any injections. Five days post-injection, mice were tested for their performance in a spatial reference memory task

for 4 days using the Morris water maze. There was a 10 -19% reduction in $GluN1_{0XX}$ splice variant expression for mice after siRNA treatment in ventro-lateral orbital regions of the brain. Decline in performance in the first half of reference memory were observed in the mice receiving siRNA specific for $GluN1_{0XX}$ splice form, as compared to the mice injected with control siRNA and/or vehicle. These results suggest an important role of the $GluN1_{0XX}$ splice variant in orbital regions for spatial reference memory acquisition and/or consolidation in the early stages of memory training. These results suggest that there is a complex interaction between GluN1 splice form expression and performance of memory tasks during aging. Future studies designed to differentiate between involvement of splice variants in particular stages of memory formation would be helpful.

Influence of Aging and Behavioral Experience on Expression of GluN1 Splice Variants of the NMDA Receptor in Prefrontal Cortex of Mice Brain.

by
Siba Ranjan Das

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirement for the
degree of

Doctor of Philosophy

Presented September 23, 2010
Commencement June 2011

Doctor of Philosophy dissertation of Siba Ranjan Das presented on September 23, 2010.

APPROVED:

Major Professor, representing Molecular and Cellular Biology Program

---

Director of the Molecular and Cellular Biology Program

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Siba Ranjan Das, Author

ACKNOWLEDGEMENTS

I would like to acknowledge those individuals associated with me during my tenure as a graduate student who may have helped me directly or indirectly to complete my studies. This thesis would not have been possible without help from these wonderful individuals. It is an honor for me to thank my supervisor, Dr. Kathy Magnusson who did not loose faith in me and even inspired me all throughout, including during the lowest point in my graduate career. It is her constant encouragement and guidance that enabled me to develop my scientific skills as a researcher. I would like to thank my graduate committee members Dr. Joseph Beckman, Dr. Tory Hagen, Dr. Ling Jin and Dr. Jonathan Kaplan for taking time to make sure I qualify for the task ahead. I would also like to thank Dr. Jane Ishmael for being able to serve as a part time committee member during the preliminary examination.

I owe my deepest gratitude to my past and present lab members, including Brenna Brim, Michelle Shumaker, Xue Zhao, Ross Jensen, Rian Kelsay, Valerie Elias, and Dan Zamzow, for their assistance with experiments, friendship and encouragement any time I needed it. I am grateful Dr. Ling Jin, Dr. Jane Ishmael and Andrew Hau for providing the facilities and helping me with some cell culture experiments. I would like to show my gratitude to the Molecular and Cellular Biology Program and the Department of Biomedical Sciences at the Oregon State University for the financial support and for providing a platform for conducting research. I would like to thank my good friends Maciej, Jeff, Bill, Sam, Brendan and Kelsi for being supportive and helpful during my stint as a graduate student.

I am indebted to my parents, who have been a constant source of inspiration for me from the beginning. This thesis would not have been possible without their love, care and affection. My heartfelt thanks are due to my wife Sasmita for the sacrifices she has made during my graduate career. Her constant support has played an important role in my success in the graduate program. My sincere thanks also go to

my brother and sisters and their families for providing moral support in succeeding in the graduate program. Finally, the acknowledgements would not be complete without a special mention of our daughter, Ella who has been a special source of motivation and happiness for me.

CONTRIBUTION OF AUTHORS

Chapter I: Siba Das wrote the chapter and Kathy Magnusson corrected it.

Chapter II: Idea was conceptualized by Kathy Magnusson. Experiments were performed and manuscript prepared by Siba Das. Kathy Magnusson corrected the manuscript.

Chapter III: Idea was conceptualized by Kathy Magnusson. Siba Das performed the experiments and wrote the manuscript. Kathy Magnusson corrected the manuscript.

Chapter IV: Idea was conceptualized by Kathy Magnusson and Siba Das. Siba Das performed the experiments with the help of undergraduate students. Siba Das wrote the manuscript and Kathy Magnusson corrected the manuscript.

Chapter V: Siba Das wrote the chapter and Kathy Magnusson corrected the chapter.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF TABLES

Dedicated to my beloved Dad.

CHAPTER I

INTRODUCTION

1.1 Importance of Aging

Senescence or aging may be defined as the accumulation of changes to different functional abilities of an organism over time. Many of these changes to functional abilities in humans are detrimental to their well being, making them susceptible to different age related diseases. The aging population of the world has shifted dramatically over the last century. According to estimates by the United Nations, the aging population of the world is growing at a rate of 2.6% per year, considerably faster than the total population growth, which is increasing at 1.1% per year (United Nations, 2007). This increase in the aged population is mostly accounted for by the developed nations. In the United States, the population of people aged 65 and more were about 4.1% of the total population in the beginning of the 20th century. This age groups has increased to about 13% at present and is expected to grow to about 19.3% of the total population by the year 2030 (Federal interagency forum on aging-related statistics, 2010). The actual numbers of people over 65 years old in the year 2030 is expected to be more than double the number in the year 2000 (Federal interagency forum on aging-related statistics, 2010). This presents a significant problem as the aging population grows by giving rise to increases in the number of diseases associated with normal aging, such as heart disease, stroke, Alzheimer's disease, cancer, Parkinson's disease, dementia, memory loss, etc. Of these diseases emanating from different functional declines during aging, the decline in cognitive abilities is of significant importance as it diminishes the quality of life of aged individuals and makes them require assisted care facilities early on, resulting in a huge economic deficit on their part. Cognitive decline during aging can range from simple forgetfulness to more severe neurodegenerative dementias, including Alzheimer's disease (Kral, 1962). Of considerable importance is the normal age-associated memory impairment (Crook et al., 1990; Crook and Ferris, 1992), which is a consequence of aging although differing in severity between individuals. Humans experience decline in memory functions as early as the 5th decade of their life (Albert and Funkenstein, 1992), which is seen as one of the earlier signs of cognitive aging.

Since memory is indispensable to normal cognitive function, age-associated memory impairment can also contribute to major declines in other cognitive functions during aging (Timiras, 2003). By understanding the factors involved in normal age-associated memory impairment, it might be easier to devise therapeutics for the more severe forms of cognitive decline and improve the quality of life of the aged individuals. The current work is an attempt to understand more about the molecular mechanisms of age-associated memory impairment.

1.2 Memory

Memory is one of the important functions of the brain. It enables an individual to learn, remember and execute new ideas, either consciously or unconsciously, from the time of birth (Kandel et al., 2000). It is difficult for individuals to survive independently without a functioning memory capability. Memory may be defined as the ability to process, store and retrieve the same information (Kandel et al., 2000). Depending upon the time taken to recall, memory can be divided into three categories (Fig 1.1A): sensory memory, short-term or working memory and long-term memory (Atkinson and Siffrin, 1968). Sensory memory is very short lived, can last as much as one second and cannot be enhanced by rehearsal (Sperling, 1960; Baddeley, 1999). Short-term memory is the type of memory that is responsible for remembering information over a period of several seconds to several minutes (Atkinson and Shiffrin, 1971). Short-term memory can be greatly enhanced by trying to remember the information as meaningful words. When information in short-term memory is used for complex tasks such as learning, making calculations etc, it is called working memory (Baddeley and Hitch, 1974). As the name indicates, long-term memory is the type responsible for remembering information for several days to months or years (Baddeley, 1999). According to another classification scheme, memory can be subdivided into explicit (conscious) or declarative memory and implicit or non-declarative (unconscious) memory (Kandel et al., 2000) (Fig. 1.1B). Implicit memory is subdivided into procedural memory (remembering a procedure, e.g. how to drive a car), associative (associating two distinct processes together as if they were one, e.g.

associating movies with popcorn), non-associative memory (a form of complex memory that can not be associated; e.g. a person's knowledge on grammar) and priming (remembering a perceived object or image unconsciously and being able to perceive it easily in the near future). Explicit memory is subdivided into episodic memory (fact based memory; for example, the sun rises in the east) and semantic memory (some event in the life of a person) (Figure 1.1B).

Of the different kinds of memory discussed above, the majority of them are adversely affected by the process of aging. Short-term memory within an easy task (performing two tasks at once; for example verifying simple sentences while remembering digits) declines minimally with age (Baddeley et al., 1991), but for complex tasks (verifying complex sentences while remembering digits for example) the decline is severe (Craik et al., 1990). Working memory, which is a form of short-term memory widely declines with age (Salthouse, 2003). Different forms of long-term memory are affected differentially with increasing age. Procedural memory, which includes memory of habits, motor learning, reading, etc. hasn't shown any perceptible change with aging (Timiras, 2003). All other forms of non-declarative memory (Fig 1.1) show minor or more significant decline with increasing age (Timiras, 2003). Declarative memory declines to some extent with increasing age. One type of declarative memory that represents the ability of the organism to acquire and retain information in order to navigate properly through space, known as spatial memory, consistently declines with age (Baddeley, 1999; Penner and Barnes, 2007). The episodic part of declarative memory declines consistently with age, but the semantic part of declarative memory declines very little with increasing age (Timiras, 2003). Different types of memory are therefore differentially affected by the process of aging. In the current study, I have used spatial memory to investigate the effects of aging on memory in rodents.

1.3 Characterization of memory in animal models

Not all types of human memory can be assessed in animals because of the differences in cognitive functions (Barnes, 1987). It therefore has been difficult to

investigate all the age related declines in memory that humans encounter. Animals however, face similar and consistent problems with increasing age as humans in one kind of memory function that is critical for successful navigation in space, known as spatial memory (Barnes, 1979; Penner and Barnes, 2007; Rapp et al., 1987). Spatial learning does not require language to test and hence represents an excellent example of memory that can be tested both in human and animals.

Maze learning tasks have been extensively used and proven very useful methods for testing age-related declines in learning and memory (Barnes, 1979; Goodrick, 1968; Ingram, 1985; Lohninger et al., 2001; Morris, 1981). Several maze learning tasks such as multiple T-maze (Goodrick, 1968; Ingram, 1985; Lohninger et al., 2001), Morris water maze (Morris, 1981), Barnes maze (Barnes, 1979), eight-arm radial maze (Olton and Samuelson, 1976) have been developed by researchers to test spatial memory, primarily in rodents. Out of these different maze-learning tasks, the Morris water maze (Morris, 1981) has been most extensively used to assess age-related declines in memory (Penner and Barnes, 2007). The maze consists of a large circular tank filled with opaque water. For the spatial version of the task, an escape platform is hidden under the surface of the water (Morris, 1981), Fig. 1.2A-C). Cues to locate the platform are hung high on the wall so that they can be seen easily (Fig. 1.2A). On each trial the animal is released into the water from different locations in the tank and allowed to swim until either the escape platform is found or a certain amount of time has elapsed. This form of learning is known as place learning (Fig. 1.2A). After performing the task several times repeatedly, normal, young animals learn to find the platform and reach it more efficiently. Young animals, after several such trials, would take an almost straight path to the escape platform, whereas the old animals may still be searching and require more trials to show a straight path. Calculations are made based on the path taken and time spent in the water to reach the platform to determine how good the animal's spatial memory is. After several such place learning trials, the platform is removed and the animal is probed for the last known location of the platform for a specified period of time (Fig. 1.2B). The animals

that have learned the task and developed a spatial bias would spend a considerable time near the platform's former location, whereas those who didn't learn would spend their time elsewhere in the tank.

A variety of spatial memory functions can be assessed using the Morris water maze just by changing the task procedure. Spatial reference memory can be characterized by keeping the escape platform position in the same quadrant for the entire length of study (Magnusson, 1998). To assess the flexibility of animals to adapt to a new escape platform position after they have been trained to memorize one particular location, the platform can be removed and placed in the opposite quadrant, called a reversal task (Magnusson, 2001). Working memory can be characterized by changing the platform position for each session of the task, letting the animals find the platform during a naïve trial and analyzing if the animals remember the same location after a brief delay (Magnusson et al., 2003). Another form of memory, associative memory can also be assessed by removing the cues from the wall and marking the platform with a visible flag, so that the animals learn to associate the flag to the escape platform (Fig. 1.2C). This is the typical control for spatial reference memory in the water maze, because it is designed to test if motor ability and/or visual acuity are an issue within different treatment groups. Any difference between the animals in this control task means that the animals have different levels of motor ability, motivation, and/or visual acuity and the results of the spatial memory tasks can not be interpreted as purely memory problems (Magnusson, 2001; Magnusson et al., 2003). Spatial reference, reversal, working and associative memory tasks were used in the studies that will be presented here.

1.4 Brain regions associated with memory

Anatomically, different brain regions are responsible for different types of memory functions. Short-term or working memory is highly dependent on the prefrontal cortex of the brain (Goldman-Rakic, 1990; Kolb et al., 1983; Winocur, 1992) and the long-term memory depends mostly on the hippocampus and temporal cortex (Morris et al., 1982; Olton, 1983). The hippocampus has been identified as the

principal region for spatial long-term memory in a variety of studies using hippocampal lesions (Aggleton et al., 1986; McNaughton et al., 1989; Morris et al., 1982; Olton et al., 1978), neural recording (O'Keefe and Dostrovsky, 1971; Wilson and McNaughton, 1993) and functional brain imaging (Maguire et al., 1998; Moffat et al., 2006). These animals show severe deficits in 14-unit T-maze (Kametani et al., 1989), the Y-maze (Aggleton et al., 1986), the radial arm maze (Olton et al., 1978) and the circular platform task (McNaughton et al., 1989). However other regions also contribute to these types of memories.

The hippocampus is responsible for consolidating the less stable short-term memories into more stable long-term memories, which can then be stored in the frontal cortex (Frankland et al., 2004; Maviel et al., 2004; Takehara et al., 2003). The prefrontal cortex contributes to long-term memory by maintaining the learned information (Kessels et al., 2000). Memory retrieval tasks such as, recognition memory and picture encoding are associated with the prefrontal cortex (Grady et al., 2005; Gutchess et al., 2005). The prefrontal cortex has also been observed to play a role in certain aspects of the spatial memory. Brain lesion studies show a role for the prefrontal cortex in both spatial working memory in the radial arm maze (Kolb et al., 1983) and spatial reference memory in Morris water maze (Kolb et al., 1983; Kolb et al., 1994). Working memory and executive functioning in particular are associated with the prefrontal cortex (Divac, 1971; Goldman-Rakic, 1987).

There are regional differences (Fig. 1.3) within the prefrontal cortex as well. Lesions in the medial frontal region in rats cause mild impairments in a spatial working memory task, but severe impairments in a spatial reference memory task (Kolb et al., 1983). Lesions in the orbitofrontal regions can cause severe problems in both spatial reference and working memory tasks (Kolb et al., 1983). Both hippocampus and prefrontal cortex are therefore regions of brain important for memory functions, which is affected during aging.

Other brain regions involved in memory are the medial temporal lobe, amygdala, cerebellum, striatum and the entorhinal cortex. The medial temporal lobe

including the hippocampus is responsible for the fact based declarative memory (Corkin, 2002). The amygdala, in conjunction with the prefrontal cortex, is responsible for encoding of emotional and fear memory, which includes both associative memory and declarative types of memory (Adolphs et al., 1997; Ferry et al., 1999). Procedural memory is encoded in the cerebellum in conjunction with the striatum (Alexander and Crutcher, 1990; Saywell and Taylor, 2008). The entorhinal cortex, which acts as an interface between the hippocampus, the frontal cortex and sensory cortical regions have been shown to be important in both working and long-term memory (Frank and Brown, 2003). Of these above described regions of the brain, the prefrontal cortex and its subregions were assessed in the present study for their role in memory declines during aging.

1.5 Molecular mechanisms of glutamate-dependent memory

A great deal of effort has been made over the past century to understand the molecular mechanism underlying memory. For a long time it has been thought that the suitable place for memory to reside in the brain are in the synapses (Deutsch, 1971). Synapses (Fig. 1.4A) are the region of communication between two nerve cells, or a nerve cell and a muscle or gland cell. Communication occurs via transmitter transfer between the two cells. When released in response to a stimulus, neurotransmitters from the presynaptic neuron exert their effect on the postsynaptic membrane by binding to receptors that are specific for that transmitter (Kandel et al., 2000). Glutamate and Gamma-amino butyric acid (GABA) are two such major neurotransmitters found in the mammalian central nervous system (Kandel et al., 2000). Depending on the type of neurotransmitter (glutamate or GABA, for example) released from the presynaptic neuron, the postsynaptic neuron can either become depolarized (inside of the cell becomes more positively charged) or hyperpolarized (inside of the cell becomes less positively charged) (Kandel et al., 2000). At rest, the inside of neurons have a higher K^+, lower Na^+, and Ca^{+2} ions as compared to the outside of the cell (Kandel et al., 2000). Because of the movement of these ions, the cell can become depolarized or hyperpolarized. Whereas glutamate is an excitatory

neurotransmitter, used to depolarize the postsynaptic neuron cells, GABA is an inhibitory neurotransmitter (Kandel et al., 2000). Activity of the postsynaptic neuron depends on a balance between the excitatory and inhibitory inputs. Consolidation of some types of memory depends on several such excitatory inputs with glutamate as the neurotransmitter. Stimulation of a postsynaptic neuron by several such excitatory inputs in synchrony gives rise to an enhanced signal transmission known as long-term potentiation (LTP, Fig. 1.3B) (Cooke and Bliss, 2006). LTP has been considered as one of the major cellular mechanisms underlying learning and memory (Bliss and Collingridge, 1993). It has been established that the induction of LTP in many brain regions depends on one kind of glutamate receptor, the N-methyl-d-aspartate (NMDA) receptor (Bliss and Collingridge, 1993; Collingridge and Bliss, 1987; Malenka and Nicoll, 1999; Wigstrom and Gustafsson, 1985).

At rest, the channel of the NMDA receptor is blocked by magnesium. Glutamate, when released from the presynaptic terminal binds to glutamate receptors present in the postsynaptic membrane. Another kind of glutamate receptor, the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor, which is permeable to both Na^+ and K^+ ions, is present in the vicinity along with the NMDA receptors. Excitation by glutamate opens the AMPA receptor channel for both these ions (Fig. 1.4A), thereby depolarizing the cell membrane. If the depolarization event is sustained for several seconds (Fig. 1.4B), NMDA receptors are relieved of the magnesium block, which allows Ca^{+2} flow into the cell (Collingridge and Watkins, 1994). Ca^{+2} acts as a second messenger inside the postsynaptic neurons, which by itself and in conjunction with calmodulin, activates several kinases such as protein kinase C (PKC), protein kinase A (PKA) and calcium/calmodulin dependent kinase II (CAMKII) (Chung et al., 2004; Grant et al., 2001). The immediate effect of these kinases is the phosphorylation of AMPA receptor and insertion of more AMPA receptors, which are present in the vicinity onto the synaptic membrane (Nicoll and Malenka, 1999). This enhances postsynaptic cell depolarization to new transmitter release and represents the early phase of NMDA-dependent LTP. The hallmark of

LTP is that enhancement of post synaptic signal strength survives the decay in signal strength that initiates it. Thus the sustained effect observed after the initial trigger by transient Ca^{+2} signaling is LTP. Time limits for the initiation of LTP depends on the frequency and strength of the initial stimulation of the postsynaptic cell, and can range from several seconds to a few minutes (Roberson et al., 1996). Requirement of frequent stimulation is waived if the postsynaptic membrane is already in a depolarized state, in which case a single stimulation can invoke LTP (Gustafson et al., 1987; Wigstrom et al., 1986).

Longer-term maintenance of LTP occurs when late phase LTP is stimulated. Late phase LTP is achieved by the activated kinases, such as, PKA, CAMKII and extracellular signal-regulated kinase (ERK), which are involved in the downstream signaling cascade (Abel et al., 1997; Wang et al., 2004) leading to synthesis of new protein and activation of CREB (cAMP response element binding protein) mediated gene expression (Kandel, 2001). At the molecular level the difference between short-term and long-term memory is that long-term memory requires these extended downstream signaling pathways leading to changes in protein synthesis, which occurs with persistent LTP, whereas short-term memory requires post-translational modification of already translated or soon to be translated protein from the mRNA. Working memory also involves circuits of neurons involving NMDA receptors linked to the central executive in the prefrontal cortex (Baddeley, 1999). The circuit remains active during the entire length of time the working memory is accessed.

From the above descriptions it is clear that NMDA receptors play an important role in the consolidation of some memories by initiating LTP. Therefore understanding their structure and function and expression during aging could help to design therapeutics against age related memory impairment.

1.6 The NMDA receptor complex

The N-methyl-d-aspartate (NMDA) receptors are a type of ionotropic glutamate receptor present throughout the brain and found abundantly in the hippocampus and frontal cortical regions (Bockers et al., 1994; Scherzer et al., 1998).

At the cellular level, these receptors are present in the postsynatpic neurons and mostly localized to the synaptic region (Kandel et al., 2000). NMDA receptors are permeable to both divalent (Ca^{+2}) and monovalent (Na^{+}, K^{+}) cations (Mayer and Westbrook, 1987). The receptor contains several binding sites, including a NMDA or glutamate binding site, a glycine binding site (glycine acts as a co-agonist) (Johnson and Ascher, 1987) and binding sites for several noncompetitive antagonists (Fig. 1.5A). To become active, the NMDA receptors require binding of both glutamate and glycine (Lynch and Guttmann, 2001). Besides NMDA, several other dicarboxylic amino acids such as L-glutamate, L-homocysteine, L-aspartate, homoquinolinate, L-homo-cysteinesulfinate, L-cysteinesulfinate, L-cysteine, and quinolinate can bind to the NMDA binding site and act as agonists (Mayer et al., 1992). Other compounds such as D-2-amino-5-phosphonopentanoic acid (AP5), D-AP7, [(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP), CGP39653, and CGS19755 can also bind to the same site and act as competitive antagonists (Dingledine et al., 1999). The glycine site can bind to several agonists, such as serine and D-cycloserine, (Hood et al., 1989) and several antagonists, such as 7-chlorokynurenate, 5,7-dichlorokynurenate and Kynurenic acid (Frankiewicz et al., 2000; Hartley et al., 1990; Khan et al., 2000). Several other non-competitive antagonists, such as (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine maleate (MK801), ketamine, phencyclidine (PCP) and 1-(1-thienyl-cyclohexyl)piperidine (TCP), bind to a site within the channel (Paoletti and Neyton, 2007; Yamakura et al., 1993). NMDA receptor function has also been modulated by binding of several other compounds to their binding sites, such as polyamines, zinc, protons, nitric oxide, alcohol and magnesium. Polyamines, such as spermine and spermidine, can either inhibit or potentiate the NMDA receptor function in a concentration dependent manner (Rock and Macdonald, 1992a; Rock and MacDonald, 1992b; Williams, 1997). At low concentrations polyamines can either increase the affinity of the receptor for glycine or increase the opening frequency of the channel without any change for glycine affinity (Rock and Macdonald, 1992a; Rock and MacDonald, 1992b). At higher concentrations, polyamines have been shown

to block the channel in a voltage dependent manner (Williams, 1997). At normal physiological concentration, zinc has been shown to act as a non-competitive antagonist inhibiting NMDA receptor function (Mayer and Westbrook, 1987). NMDA receptors are sensitive to changes in proton concentration within the physiological range (Traynelis and Cull-Candy, 1991). Slight decreases in extracellular pH can inhibit NMDA receptor function without influencing the single channel conductance (Traynelis and Cull-Candy, 1991). Nitric oxide produced by NMDA receptor activation may block the channel, initiating a negative feedback on the NMDA receptor (Fagni et al., 1995; Lei et al., 1992). At the resting membrane potential, the channels of these receptors are kept closed by the presence of magnesium, which acts as a physical block of the channel. The block is relieved only when the cell is depolarized by the influx of positive ions through other nearby channels (Mayer et al., 1984). Hence in order to conduct ions, the NMDA receptor requires three events, binding of glutamate and glycine and depolarization of the membrane, making it a complex receptor that is able to detect molecular coincidence. It also contains multiple binding sites that could be manipulated in designing therapeutics aimed at NMDA receptor functions.

1.6.1 Assembly of the NMDA receptor

The NMDA receptors are heteromeric complexes composed of a combination of subunits belonging to three distinct subunit families; GluN1 (earlier names; NMDAR1, NR1, ζ1), GluN2 (NR2) and GluN3 (Collingridge et al., 2009; Furukawa et al., 2005; Laube et al., 1998; Premkumar and Auerbach, 1997; Ulbrich and Isacoff, 2008). Eight different splice variants of the GluN1 subunit (see below), four members of the GluN2 family (GluN2A-D, formerly ε1-4 in mice) and two members of the GluN3 family (GluN3A-B) have been identified in the brain (Collingridge et al., 2009; Furukawa et al., 2005; Laube et al., 1998; Premkumar and Auerbach, 1997; Ulbrich and Isacoff, 2008). A number of studies indicate that the NMDA receptors are heteromeric complexes composed primarily of GluN1 and GluN2 subunits (Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991), although complexes of GluN1 and

GluN3 have also been reported (Cull-Candy et al., 2001). These subunits of the NMDA receptor contain an extracellular N-terminal domain, four transmembrane domains and an intracellular C-terminal domain. The second transmembrane domain forms a loop inside the membrane, which after assembly with the other subunits becomes part of the channel pore (Fig. 1.5B).

Investigations of the number of subunits required for a functional NMDA receptor have indicated either a pentameric or a tetrameric assembly of the receptor composed mostly of GluN1 and GluN2 subunits and, in certain cases, GluN3 subunits along with the previous two (Blackstone et al., 1992; Brose et al., 1993; Mayer and Armstrong, 2004; Schorge and Colquhoun, 2003). More recently, using evidence from X-ray crystallography and electron microscopy studies, it has been debated that the NMDA receptor is a tetramer composed of two GluN1 and two GluN2 subunits (Furukawa et al., 2005; Stephenson et al., 2008; Tichelaar et al., 2004; Ulbrich and Isacoff, 2008) (Fig. 1.5A). GluN3 subunit can sometimes assemble with the GluN1-N2 complex to reduce the receptor response and some other times can assemble with GluN1 subunit alone to form a glycine receptor (Cull-Candy et al., 2001). For the purpose of this study I will focus on the role of GluN1 subunit during aging.

1.6.2 The GluN1 subunit of NMDA receptor

The GluN1 subunit of the NMDA receptor is a three transmembrane domain subunit with an extracellular N-terminal and an intracellular C-terminal side (Zukin and Bennett, 1995) (Fig. 1.5B). The subunit contains the glycine binding site in the loop region between the third and fourth transmembrane domain, making it an important subunit for proper functioning of the receptor (Hirai et al., 1996). Xenopus oocytes injected with mRNA for GluN1 subunits display functional characteristics of the NMDA receptor indicating that the GluN1 subunit is both necessary and sufficient for NMDA receptor function (Meguro et al., 1992; Nakanishi et al., 1992). There is about 99% amino acid homology of the GluN1 subunit between different species (Karp et al., 1993; Le Bourdelles et al., 1994; Meguro et al., 1992; Nakanishi, 1992; Yamazaki et al., 1992). Eight different isoforms of the GluN1 subunit have been

identified in the brain (Fig. 1.6). These are generated by alternative splicing of one N-terminal (Exon 5) and two C-terminal (Exons 21 and 22) cassettes in the mRNA (Anantharam et al., 1992; Durand et al., 1992; Nkanishi et al., 1992; Sugihara et al., 1992). The C2 cassette contains a translational stop codon and, in its absence, an additional sequence with the next stop codon becomes part of the mature mRNA known as the C2' cassette (Durand et al., 1992). The N1 cassette is present on the extracellular side of the receptor and C1, C2 and C2' cassettes are present on the cytoplasmic side (Hollmann et al., 1993; Sugihara et al., 1992). I will use three subscripts to indicate the presence (1), absence (0) or either condition (X) of the N1, C1, and C2 cassettes, respectively.

Since mRNA probes have not been developed that span the N and C terminal cassettes, it has not been possible to examine each of the 8 splice variants separately. Instead studies involving splice forms of the N or C terminal cassettes alone have been reported, hence the X designation. The $GluN1_{0XX}$ (GluN1-a) splice form lacks the N1 insertion cassette, while the $GluN1_{1XX}$ (GluN1-b) splice form contains the N1 insertion cassette (Laurie and Seeburg, 1994; Lynch and Guttmann, 2001; Zukin and Bennett, 1995). In both cases, the XX indicates that the forms can contain any combination of C terminal cassettes. The designations for the C terminal splice forms are $GluN1_{X11}$ (GluN1-1: C1, C2 cassettes), $GluN1_{X01}$ (GluN1-2: C2 cassette), $GluN1_{X10}$ (GluN1-3: C1, C2' cassette) and $GluN1_{X00}$ (GluN1-4: C2' cassette) (Laurie and Seeburg, 1994; Lynch and Guttmann, 2001; Zukin and Bennett, 1995). The X in the first position indicates that these forms include both splice variants with and without the N terminal cassette.

The various cassettes of the GluN1 subunit impart different functions to neuronal cells. Splicing out of the N1 insertion cassette has been shown to reduce affinity for agonist by almost five fold (Durand et al., 1993) and decrease the current amplitude in oocytes expressing GluN1 subunits (Hollmann et al., 1993; Zheng et al., 1994). Presence of N1 insert has also been found to attenuate stimulation by polyamines, proton inhibition and zinc modulation (Durand et al., 1992; Durand et al.,

1993; Hollmann et al., 1993; Zheng et al., 1994). The C-terminal cassette, C1 is important for NMDA receptor functioning as it contains phosphorylation sites, two for PKC and one for PKA activity (Tingley et al., 1993; Tingley, 1997). The cassette appears to interact with two proteins, yatiao and neurofilament L, which contribute to clustering of receptors (Ehlers et al., 1998; Lin et al., 1998). The C-terminal is observed to be essential for NMDA-receptor dependent gene expression because of the role it has on the downstream signaling and receptor inactivation (Bradley et al., 2006). These studies suggest that changes in expression of N-terminal or C-terminal cassettes with age will have multitude of effects such as change in localization of NMDA receptors in receptor clusters, potentiation of receptor function, phosphorylation leading to alteration in cognition and behavior.

There is heterogeneity in mRNA expression of GluN1 subunit splice variants in different regions of adult rat brain. The GluN1 subunit as a whole is expressed abundantly in the hippocampus and olfactory bulb, followed by the neocortex and cerebellum, and at considerably lower levels in midbrain, hypothalamus, thalamus, basal nuclei and substantia nigra (Laurie et al., 1995). The individual splice variants also show a similar heterogeneity in their distribution pattern throughout the brain regions. The $GluN1_{0XX}$ splice variant is highly expressed in the hippocampus and olfactory bulb followed by the neocortex, and to less extent in the caudate, putamen, hypothalamus and basal nuclei (Laurie et al., 1995). However, the $GluN1_{1XX}$ splice variant, which contains the N1 cassette, is more restricted to the hippocampus, as compared to other cortical and subcortical regions (Laurie et al., 1995). Among splice variants with the C-terminal cassettes, $GluN1_{X11}$ has high mRNA expression in the hippocampus and neocortex region and to a lesser extent in other brain regions (Laurie et al., 1995). Expression of $GluN1_{X01}$ and $GluN1_{X00}$ mRNA is more or less parallel; with high levels in hippocampus and moderate to low levels in olfactory and neocortex, respectively. $GluN1_{X10}$ is present in relatively low levels in both hippocampus and neocortex (Laurie et al., 1995).

Protein levels of the subunits in adult rats also have a similar expression pattern to that described for the mRNA. Antisera derived from the N-terminal of GluN1 subunit recognizes strong expression of the GluN1 subunit in the hippocampus, followed by the cortex, striatum and thalamus, and the one derived from the C-terminal domain recognizes weaker expression levels of protein in the similar regions (Benke et al., 1995). In the hippocampus of adult rats, the N1 cassette is more expressed in the CA3 and dentate gyrus regions than the CA1 region. The C1 and C2 cassettes are highly expressed in the CA1 region as compared to the CA3 and dentate gyrus. The C2' cassette is expressed more in the dentate gyrus regions, as compared to CA1 and CA3 regions (Coultrap et al., 2005). The N-terminal cassette, N1 is present about 7% of the total GluN1 subunit in the cortex in adult rat, whereas the C-terminal cassettes, C1, C2 and C2' are present in 58%, 45% and 35% of the total GluN1 respectively (Prybylowski et al., 2000).

It is thus clear from the above studies that both the mRNA and protein expressions of the different splice variants of the GluN1 subunit are heterogeneous between different brain regions. Since the different splice cassettes of the subunit impart different functions to the NMDA receptor, this might produce populations of NMDA receptor within regions that are optimal for the function of that region. If aging differentially affects the splice variants within regions, this could alter the optimal functioning of that region. This will be explored in the studies presented in Chapters II, III and IV.

1.7 Memory, aging and NMDA receptor

NMDA receptors as a whole are implicated in cognitive functions because of their role in memory formation as described in section 1.5. Antagonists of the receptor block initiation of long-term potentiation, a cellular mechanism believed to underlie learning and memory, in both the hippocampus (Bashir et al., 1991; Bashir et al., 1994; Harris et al., 1984; Morris et al., 1986; Tsien et al., 1996) and the neocortex (Artola and Singer, 1994). Spatial memory is also inhibited by use of antagonists for the NMDA receptor (Alessandri et al., 1989; Heale and Harley, 1990; Mondadori et

al., 1989; Morris et al., 1986; Morris, 1989). Performance in spatial working memory tasks is inhibited when a delay is induced between choices (Li et al., 1997). In addition, correlations have been seen between NMDA-displaceable [^{3}H]glutamate binding and GluN1 and GluN2B subunits expression in prefrontal/frontal and hippocampal regions and reference memory performance in the Morris water maze (Davis et al., 1993; Magnusson, 1998; Magnusson, 2001; Magnusson et al., 2007).

Aging animals exhibit declines in NMDA receptor binding densities. The NMDA binding site has been shown to be more affected by aging than the other ionotropic glutamate receptors (Magnusson and Cotman, 1993a; Magnusson, 1995). A number of studies employing [^{3}H] glutamate or glutamate analog binding techniques have shown that the NMDA receptors are more susceptible to the effects of aging than any other glutamate receptors in the prefrontal cortex and in the hippocampus of the mouse brain (Magnusson and Cotman, 1993b; Magnusson, 1995; Magnusson, 1997). Similar results were also observed in rats (Kito et al., 1990; Nicolle et al., 1996; Nicolle and Baxter, 2003), dogs (Magnusson, 2000) and primates (Hof et al., 2002).

Several studies have used spatial reference memory tasks to characterize the relationship between age related declines in the NMDA receptor expression and spatial memory (Davis et al., 1993; Gage et al., 1984; Gallagher and Nicolle, 1993; Magnusson, 1997; Magnusson, 1998; Magnusson, 2001; Pelleymounter et al., 1990; Rapp et al., 1987). The decline of NMDA receptor expression in the prefrontal and hippocampal regions of the rodent brain has been shown to be associated with declines in spatial memory during aging (Pellymounter 1990; Davis 1993; Magnusson 1998; Magnusson 2001). These studies all demonstrate an important role for NMDA receptors in memory and suggest that detrimental changes to the NMDA receptor during the aging process may explain, at least in part, the memory declines that people and animals experience during the aging process. Since the focus of this study is on the GluN1 subunit of the NMDA receptor, in the next sections I will discuss the effect of aging and its consequence on the GluN1 subunit alone.

1.7.1 Aging of GluN1 splice variants in brain

In rodents, the GluN1 subunit has been observed to be vulnerable to the effects of aging (Magnusson, 2000; Magnusson et al., 2002; Magnusson et al., 2005), primarily in the frontal and occipital cortices of the neocortex and the dentate gyrus in the hippocampus, as observed by the change in mRNA expression levels in 30 month old mice as compared to the 3 month olds (Magnusson, 2000). Protein levels of the GluN1 subunit also decline with increasing age of mice in both the hippocampus and cerebral cortex regions (Magnusson et al., 2002). Analysis of the C-terminal splice variants of the GluN1 subunit shows that an overall decline in mRNA expression of the GluN1 subunit corresponding to declines in $GluN1_{X11}$ and $GluN1_{X10}$ splice variants takes place in the hippocampal and frontal cortical regions in aged, 26 month old mice as compared to the young, 3 month old animals (Magnusson et al., 2005). Expression of the whole GluN1 subunit declines in the medial and lateral frontal cortical regions of aged animals (Magnusson, 2001). The $GluN1_{X10}$ shows declines in medial and lateral frontal regions, whereas $GluN1_{X11}$ shows decline only in the deep layers of lateral frontal cortex (Magnusson et al., 2005). In the hippocampus of aged mice, $GluN1_{X10}$ is observed to have a lower expression in CA3 and dentate gyrus regions, and $GluN1_{X11}$ in only ventral dentate gyrus regions even though the whole GluN1 subunit has no change in expression, as compared to the young mice (Magnusson et al., 2005). Activity dependent increases in the surface expression of GluN1 subunit, observed in young rats is absent in the old rat hippocampus and has been observed to be associated with the decline in protein expression of the C2 cassettes (Clayton et al., 2002). These studies show that aging can affect the GluN1 subunit, but the affects on C terminal splice variants is not homogeneous.

The GluN1 subunit of NMDA receptor during aging shows heterogeneity in expression. The expression is not consistent among different studies (Magnusson, 2000; Magnusson, 2001; Magnusson et al., 2002; Magnusson et al., 2005). This will be discussed in more detail in section 1.9. The reason for this inconsistency is not

clearly understood. With the current study, I have made an attempt to explain this variability of GluN1 subunit among different studies.

1.7.2 Consequence of changes in GluN1 subunit and its splice variants on memory

The effect of aging on GluN1 expression is most evident in the frontal cortex and hippocampal regions (Magnusson, 2001). The GluN1 subunit appears to be important for the survival of new neurons in the dentate gyrus in young mice (Tashiro et al., 2006). Knockout and antagonism of the GluN1 gene in the hippocampus has been shown to block initiation of LTP (Bashir et al., 1991; Bashir et al., 1994; Harris et al., 1984; Morris et al., 1986; Tsien et al., 1996) and neocortex (Artola and Singer, 1994) and cause problems in spatial memory (Morris et al., 1986; Morris, 1989; Morris and Davis, 1994; Tsein et al., 1996). Age-related decreases in the protein expression of the GluN1 subunit within crude synaptosomes of the frontal cortex of C57BL/6 mice show a relationship to the declines in performance in a spatial reference memory task across age groups (Magnusson et al., 2007). Lower expression of the GluN1 subunit within the synaptic membrane of the hippocampus of middle-aged mice is also associated with poorer performance in the same task, though this relationship is reversed with the old mice, i.e., lower expression was associated with better memory (Zhao et al., 2009).

The effects of an age-related decline in the expression of individual splice variants of the NMDA receptor on memory have not been identified yet. The functions of the individual splice variants do suggest a role in memory performance during aging. As mentioned in section 1.6.2, studies suggest that changes in expression of N-terminal or C-terminal cassettes with age could have a multitude of effects, such as changes in localization of NMDA receptors in receptor clusters (Ehlers et al., 1998; Lin et al., 1998), changes in potentiation of receptor function (Durand et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Zheng et al., 1994) or alteration of phosphorylation (Tingley et al., 1993; Tingley et al., 1997). Any of these could lead to alterations in cognition and behavior.

1.8 Summary

Senescence is one of the major problems in the world today, because it has the ability to affect different organs of the human body in a deteriorating fashion. Memory is no exception to the effects of aging (Salthouse, 2003). The NMDA receptors, which are important for the formation and consolidation of memory, are influenced by the aging process (Magnusson and Cotman, 1993b; Magnusson, 1995; Magnusson, 1997; Magnusson, 1998). The different subunits of the NMDA receptor are affected differently by aging, indicating heterogeneity in the aging effects (Magnusson, 2000; Magnusson, 2001; Magnusson et al., 2002; Magnusson et al., 2005; Magnusson et al., 2007).

The GluN1 subunit of the NMDA receptor has shown aging changes in some studies, however, it has not been consistently affected by aging between different studies even within the same species of mice (Magnusson, 2000; Magnusson et al., 2002; Magnusson et al., 2005; Magnusson et al., 2007). This subunit shows significant declines with aging in 26-30 month old animals (Magnusson, 2000; Magnusson et al., 2002; Magnusson et al., 2005), whereas the effect of aging on them was lost in the 27 month old mice that had a behavioral learning experience (Magnusson, 2001). The difference between these studies is the behavioral learning experience, which was absent in the former studies but present in the later study. In another study aged 26 month old mice with behavioral learning experience showed decline in protein expression of GluN1 subunit in the prefrontal cortical regions (Magnusson et al., 2007). Difference between these two studies ((Magnusson, 2001) vs (Magnusson et al., 2007)) is the difference in the protocol of behavioral learning experience (12 days (Magnusson, 2001) vs 2 days (Magnusson et al., 2007)). These studies indicate a role of external factors in the effects of aging on the GluN1 subunit that could not be controlled by the genotype. Some possible explanations for this disparity among the different studies could be exposure to different environmental cues or exposure to different behavioral experience, which could have caused changes in the GluN1 subunit expression during aging. Since the GluN1 subunit contains eight different

splice variants, and these studies didn't separately analyze individual splice variants, it was also unclear about the role of the individual splice variants during aging and how they might influence the disparity among the studies. This led to the hypothesis that differential expression of individual splice variants of the GluN1 subunit is responsible for the observed changes of GluN1 subunit during aging and learning experience. In chapter II and III I have focused on differentiating mRNA (Chapter II) and protein (Chapter III) expression of these individual splice forms to better understand their fate during aging and their role in memory. I have also included 'behavioral learning experience' as another factor, versus naïve animals, to determine if it plays any role in the disparity observed in the inconsistent aging expression of GluN1 subunit in the various studies.

The results of our study in Chapter II indicated that one of the splice variants $GluN1_{0XX}$ is upregulated only in the old animals in prefrontal/frontal cortical regions, including the ventro-lateral orbital regions. Correlational analysis indicated that the splice form had a significantly positive correlation with animals' performance in reference memory. For the purpose of the publication, the correlation results were corrected for the number of comparisons, and the result yielded a corrected correlation p value of .08. We, however, were advised by a statistician that we could use the uncorrected correlations (Appendix, Table 1) to generate hypotheses for the next study. Using this data we hypothesized that a decrease in $GluN1_{0XX}$ splice form expression in the young mice will inhibit their performance in a reference memory task.

In order to establish if reducing $GluN1_{0XX}$ will inhibit memory performance in young animals I used the small interfering RNA (siRNA) technique for reducing the $GluN1_{0XX}$ expression and tested reference memory. siRNAs have been widely used in research and have shown a pretty robust transient knockdown of mRNA in cells (Elbashir 2001). siRNAs are short (about 20-25 nucleotides) double stranded RNA structures with a short nucleotide overhang on either side. When present in a cell, these small double stranded RNA structures trigger the RNA interference (RNAi)

pathway, each strand becoming part of a RNA-induced silencing complex (RISC) and process target RNA in a sequence specific manner (Bagasra and Prilliman, 2004). They are very specific in their target and have been used *in vivo* in many different tissue type including brain (Akaneya et al., 2005; Thakker et al., 2004; Thakker et al., 2005). Although *in vivo* use of this technique is limited, because of the problems in effective delivery and tissue clearance mechanism, we have shown a 10-19% knockdown in mRNA using this technique after a period of seven days. In Chapter IV, I have discussed preparation, delivery and effect of the siRNA used against the $GluN1_{0XX}$ mRNA.

The results of the above studies will delineate the changes that occur in and a role for some of the individual splice variants of the GluN1 subunit during aging. It will also indicate the role of behavioral learning experience on expression of these individual splice variants. Finally the study will also describe the consequences of knockdown of a particular splice variant on memory performance.

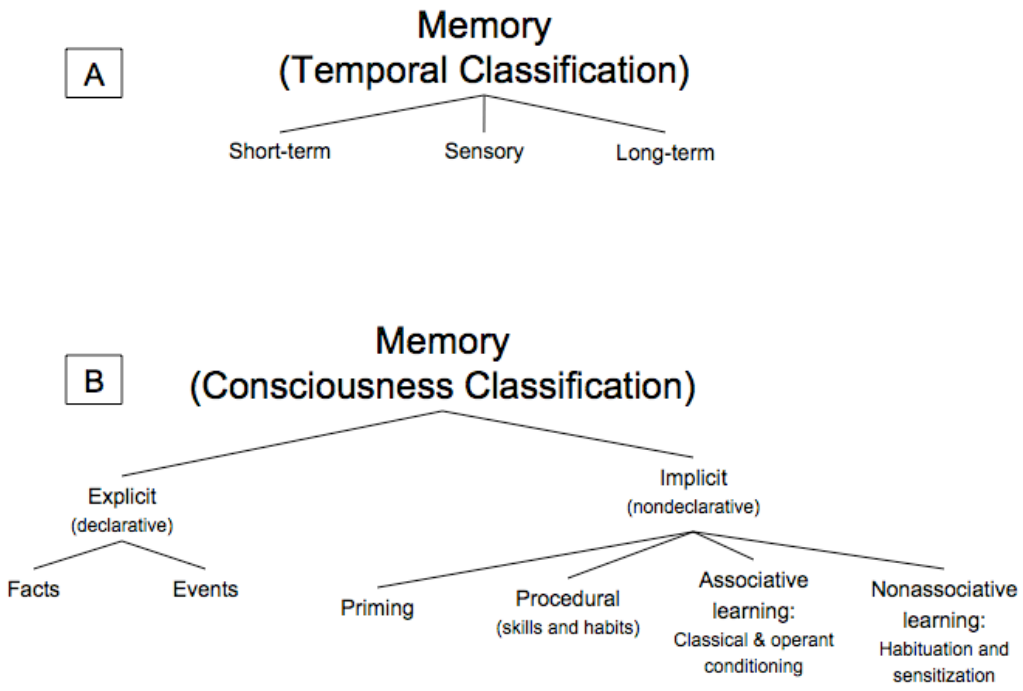

Fig. 1.1 - Schematic representation of two different classification of memory. A) Three stages of memory based on temporal classification. Adapted from Timiras (2003). B) Classification of memory based on consciousness. Adapted from Kandel et al. (2000)

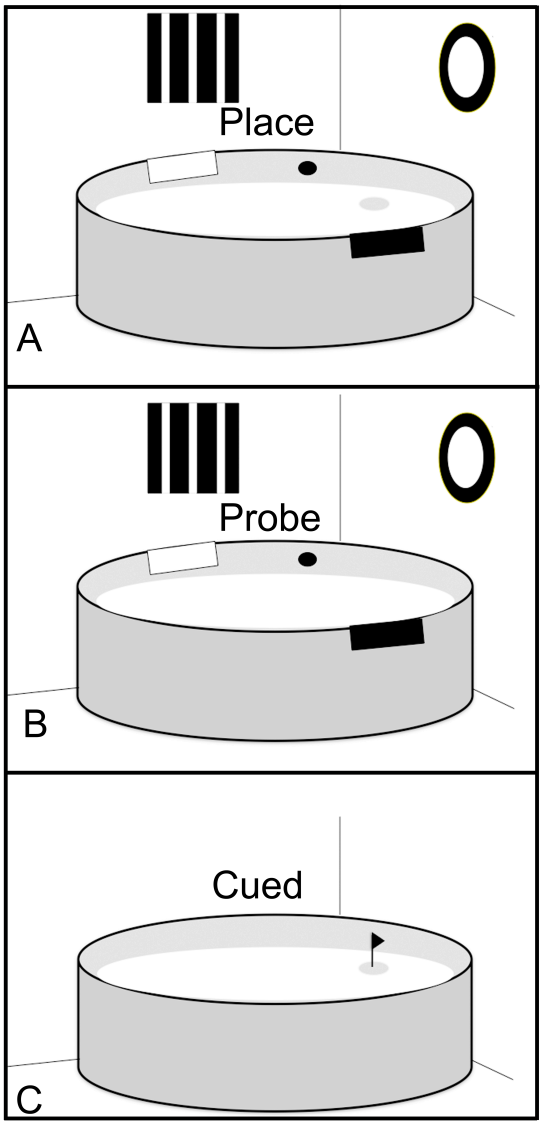

Fig. 1.2 – Schematic representation of different settings of the Morris water maze task for assessment of memory. (A) Example place trial set-up for spatial reference memory training, where the escape platform is hidden under the water and cues are hung on the wall and on the side of the tank. Animals navigate to the platform with the help of the cues. (B) Example probe trial set-up for spatial reference memory recall, designed to test the bias for a particular location. The platform is taken out of the water and animals are probed for recall of the last known location. (C) Example cued trial set-up for visual ability association memory, used as a control task to test motivation, motor performance and visual ability. Cues from the wall and the side of the tank are removed and the escape platform is marked by a visible flag.

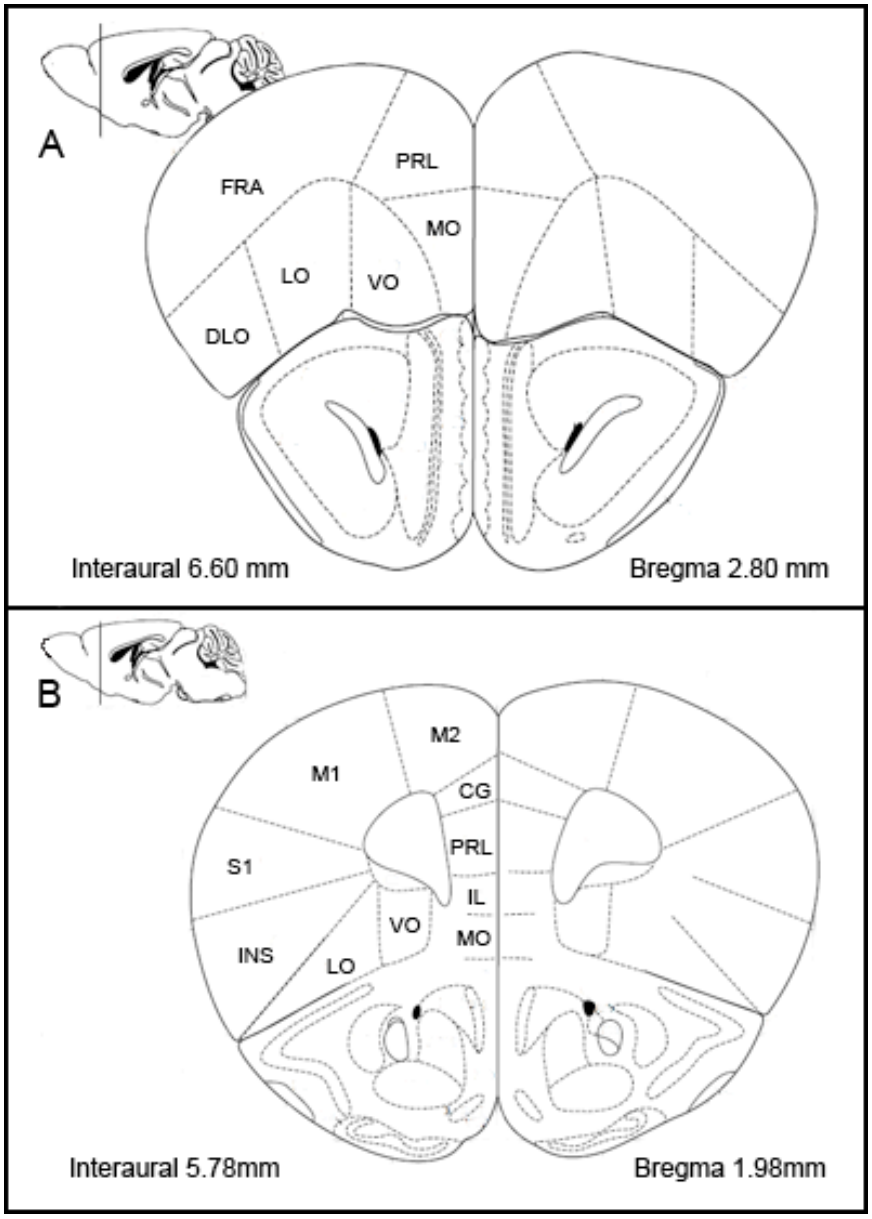

Fig. 1.3 - Schematic diagram of a prefrontal (A) and prefrontal/frontal (B) section of a mouse brain showing different sub-regions. Image adapted from Paxinos and Franklin, 2001. DLO = dorsolateral orbital; LO = lateral orbital; VO = ventral orbital; MO = medial orbital; PRL = Prelimbic cortex; FRA = Fronal Association; INS = insular cortex; CG = cingulated cortex; M2 = secondary motor cortex; M1 = primary motor cortex; S1 = primary somatosensory cortex. Image adapted from Paxinos and Franklin (2001).

Fig. 1.4 – Schematic representation of communication between two neurons and the molecular basis of NMDA receptor-dependent long-term potentiation. (A) Figure showing link between the two neurons at an axo-dendritic type of synapse, where the axon from the presynaptic neuron interacts with the dendrite from the post synaptic neuron. (B) Enlarged view of the synapse from box in A. Glutamate, which is present in vesicles near the synaptic membrane, is released from the presynaptic neuron in response to a stimulus. After release, glutamate crosses the synaptic cleft and binds to nearby AMPA and NMDA receptors in the postsynaptic neuron. In response to glutamate, AMPA receptors channels open and allow sodium ions into the cell, leading to depolarization (panel on left). NMDA receptors are closed initially because of the block by magnesium in the channel. If glycine is present in the vicinity of the synapse and the depolarization by AMPA receptor is sufficient to remove the magnesium, the NMDA receptor channels will open and conduct calcium into the post-synaptic neuron. The calcium acts as a second messenger activating kinases that are responsible for local phosphorylation of more AMPA receptors and translocating new AMPA receptors into the synaptic membrane, which initiates early phase long-term potentiation (right panel). Towards the late phase, the kinases lead to subsequent protein synthesis, further strengthening the synapse and leading to late phase long-term potentiation. Images adapted from Wang et al. (2006).

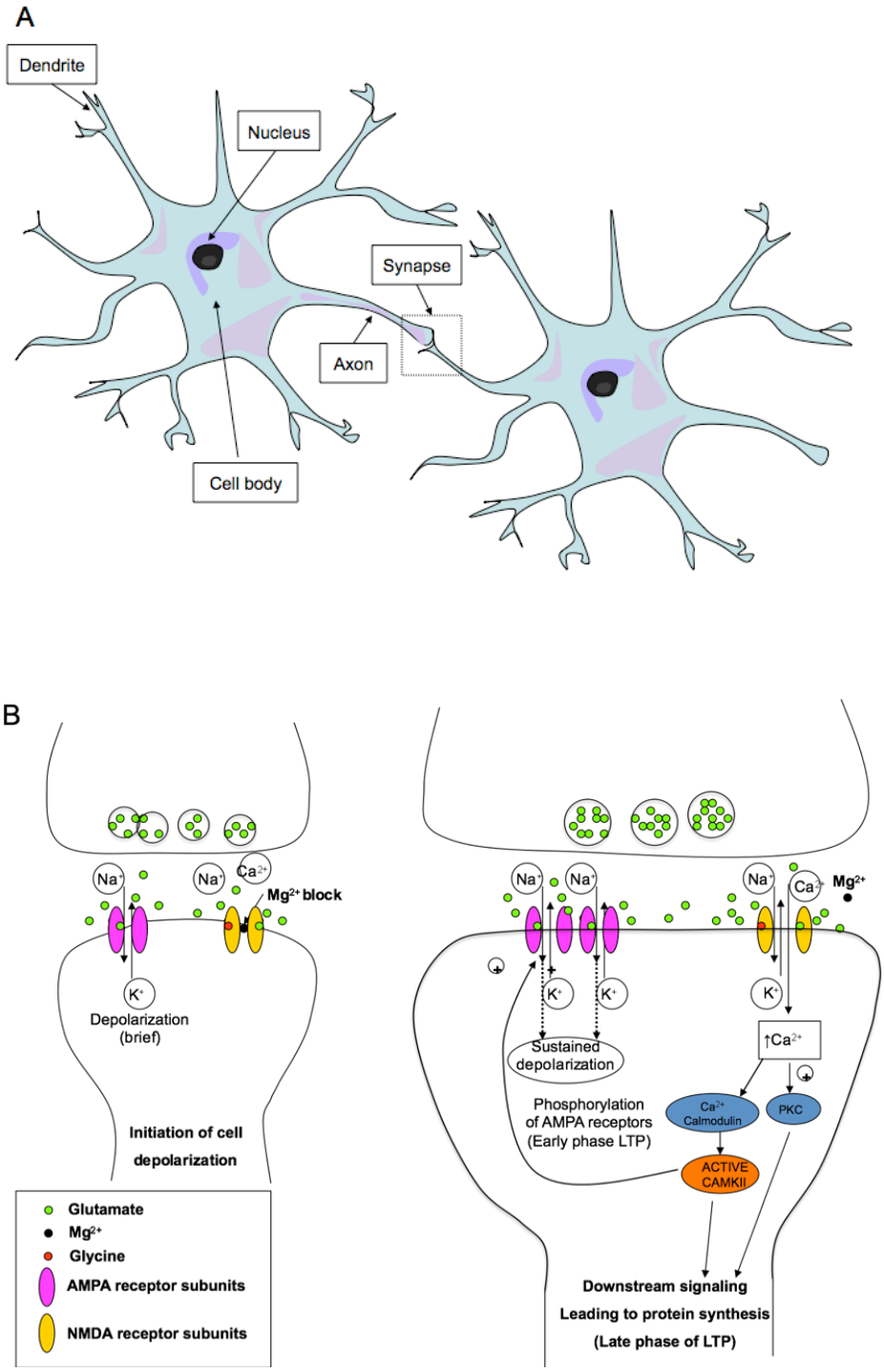
A
Dendrite
Nucleus
Synapse
Axon
Cell body
B
Na+
Na+
Ca2+
Mg2+ block
K+
Depolarization
(brief)
Initiation of cell
depolarization
Glutamate
Mg2+
Glycine
AMPA receptor subunits
NMDA receptor subunits
Na+
Na+
Na+
Ca2+
Mg2+
K+
K+
K+
Sustained
depolarization
↑Ca2+
Phosphorylation
of AMPA receptors
(Early phase LTP)
Ca2+
Calmodulin
PKC
ACTIVE
CAMKII
Downstream signaling
Leading to protein synthesis
(Late phase of LTP)

Fig. 1.4

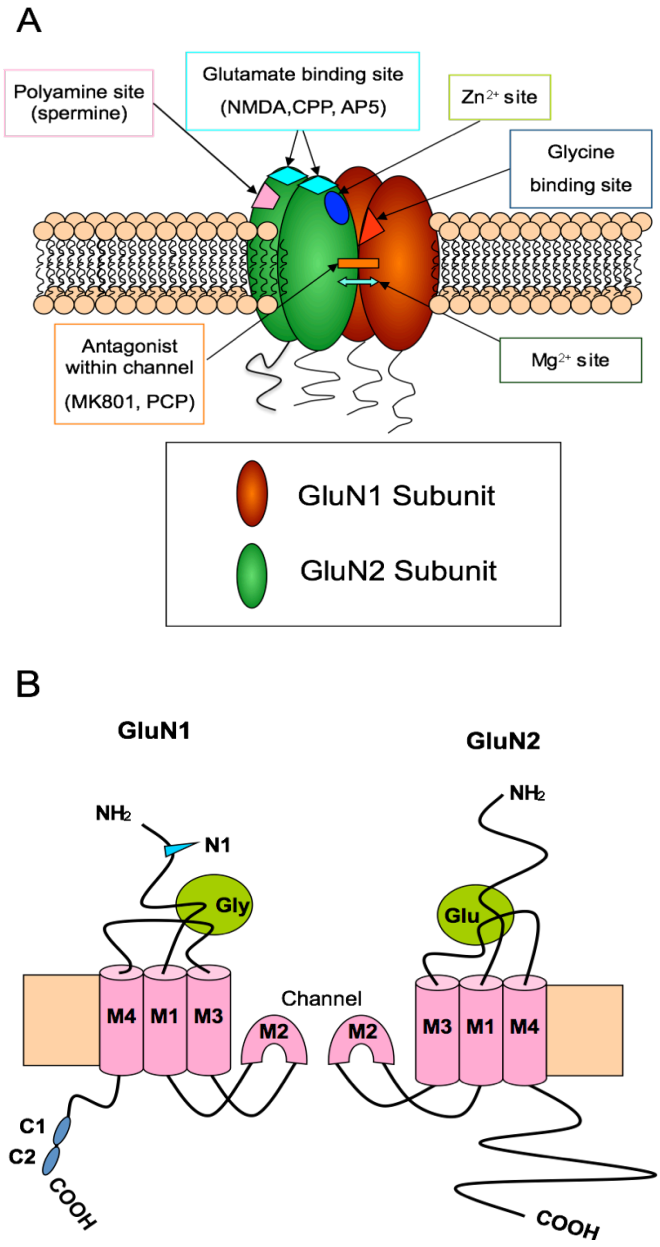

Fig. 1.5 – Schematic representation of the assembly of NMDA receptors. (A) Different binding sites on different subunits of the receptor. Adapted from Sala and Sheng (1999). (B) Each of the NMDA receptor subunits are made of 4 transmembrane domains (M1-4) with the N-terminal outside the cell and C-terminal inside the cell. The pore is formed by interaction of the loops formed by the second transmembrane domain (M2) in each subunit. In the GluN1 subunits, N1, C1, and C2 correspond to the splicing cassettes on either the N or C-terminal side of the protein. Image adapted from Liang (2004)

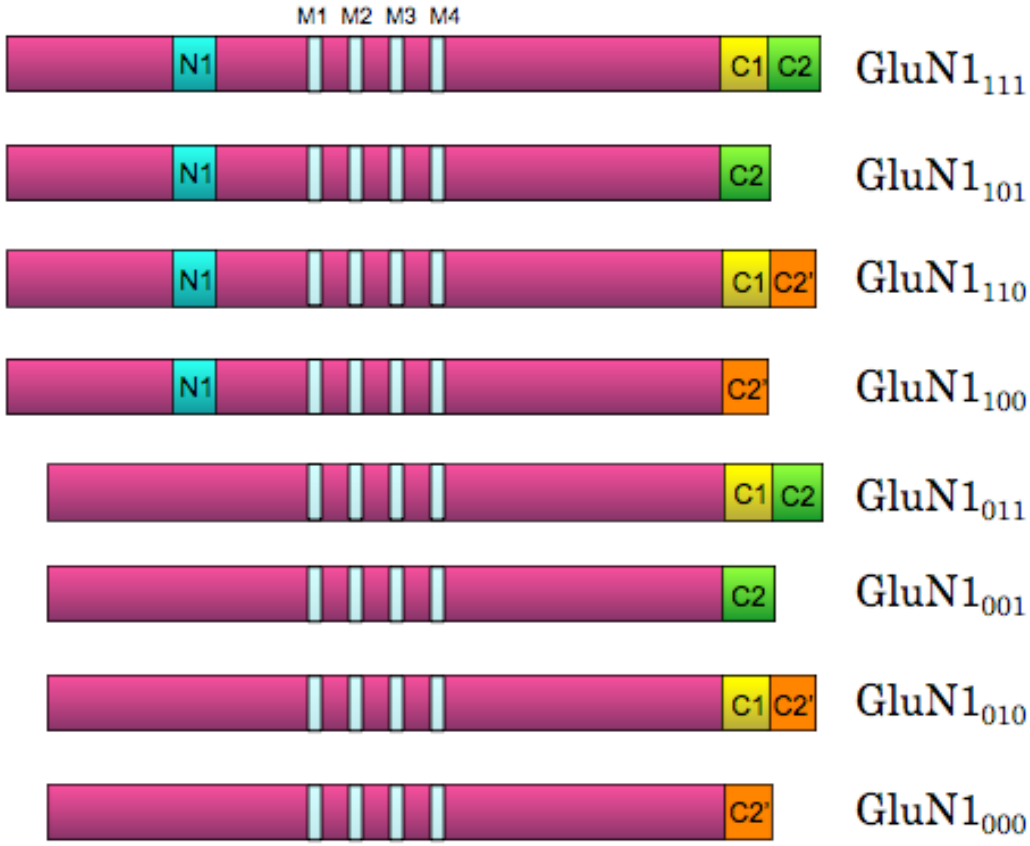

Fig. 1.6 – Schematic mRNA sequence representation of the eight different splice variants of the GluN1 subunit of the NMDA receptor. N1, C1 and C2 correspond to one N-terminal cassette and two C-terminal splicing cassettes respectively. The C2 cassette contains a stop codon and, in its absence an extended amino acid sequence becomes part of the protein called C2'. Transmembrane domains are marked by M1-4. Adapted from Zukin and Bennett (1995).

CHAPTER II

Relationship between mRNA expression of splice forms of the ζ1 (GluN1) subunit of the N-methyl-D-aspartate receptor and spatial memory in aged mice.

Siba Ranjan Das and Kathy R. Magnusson

Molecular and Cellular Biology Program & Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, US

Brain Research
Elsevier, Publishers; 201 Mission Street, 26th Floor, San Francisco, CA 94105
May, 2008 1207: 142-154.

ABSTRACT

Age-related changes in the protein and mRNA expression of some of the splice forms of the GluN1 (NR1) subunit of the NMDA receptor have been seen in mice and rats. The present study was designed to determine whether individual splice forms of the GluN1 subunit of the NMDA receptor within prefrontal / frontal cortical regions contribute to memory deficits during aging and whether experience in learning tasks can influence the expression of the splice forms. mRNA expression of 4 splice forms ($GluN1_{X11}$, $GluN1_{X10}$, $GluN1_{0XX}$ and $GluN1_{1XX}$) and mRNA for all known splice forms (GluN1-pan) were examined by *in situ* hybridization. mRNA for C-terminal splice forms, $GluN1_{X11}$ (+C1 and +C2 cassettes) and $GluN1_{X10}$ (+C1 and +C2'), showed significant declines during aging in several brain regions even though overall GluN1-pan mRNA expression was not significantly affected by aging. This suggests that these splice forms are more influenced by aging than the subunit as a whole. There was an increase in the expression of $GluN1_{0XX}$ (-N1 cassette) splice form in the behaviorally-experienced old mice relative to the younger groups. Old mice with high levels of mRNA expression for the $GluN1_{0XX}$ splice form in orbital cortex showed the best performances in the working memory task, but the poorest performances in the cued, associative learning task. These results suggest that there is a complex interaction between GluN1 splice form expression and performance of memory tasks during aging.

Classification Terms

Section: Cognitive and Behavioral Neuroscience

Key words: NMDA receptor; memory; aging; learning; splice variants; NR1

2.1 Introduction

Age associated memory decline is a phenomenon that begins early in adulthood and overlaps with other aspects of cognitive aging (Salthouse, 2003). This decline in memory is so extensive that approximately 40% of people aged 65 years of age or more can be diagnosed with some sort of age related memory impairment (Larrabee and Crook, 1994). Age associated memory decline is usually modest compared to disorders associated with dementia and lacks the pathology observed in such disorders (Crook et al., 1990), but still represents a problem for quality of life and independent living in elderly individuals. Spatial memory is one type of memory that is affected by increasing age and can be studied in animals such as rodents and primates who experience age associated memory impairments (Barnes, 1988; Gage et al., 1984; Rapp et al., 1987).

There is evidence that brain regions such as the prefrontal cortex and hippocampal formation are associated with spatial memory functions (Gallagher et al., 2003; Greenwood, 2000; Tisserand and Jolles, 2003). All of these structures show greater decline in their volume in individuals with declining memory capabilities than the individuals with stable memory over time (Persson et al., 2006). This decline in prefrontal cortex volume has been speculated to alter the functional connectivity of cortical circuits contributing to cognitive aging (O'Donnell et al., 1999). A type of glutamate receptor known as the N-methyl-D-aspartate (NMDA) receptor is abundant in these areas of the brain (Bockers et al., 1994; Scherzer et al., 1998). These receptors are important for long term potentiation, one proposed mechanism for the formation of memory by strengthening of synapses (Cotman et al., 1989; Lynch, 1998). Inhibition of this receptor by antagonists results in impaired learning and memory abilities in rodents (Alessandri et al., 1989; Mondadori et al., 1989; Morris et al., 1986). Among the glutamate receptors, NMDA receptors are more susceptible to effects of aging (Magnusson, 1997). It has been observed that NMDA receptors experience changes in gating behavior, magnesium block and response to transmitter with increasing age in rodents and primates (see review Magnusson, 1998b). In autoradiographic

experiments [^{3}H] glutamate binding to the receptor decreases with increased age, suggesting a reduction in receptor activity with increased age (Kito et al., 1990).

The NMDA receptor is composed of four or five protein subunits from three different families of proteins, the GluN1 (NR1), ε (NR2) and NR3 family in the rodents. Four members in the ε family, one member in the GluN1, with eight splice variants, and two members in NR3 families have been identified and cloned (Eriksson et al., 2002; Ikeda et al., 1992; Ishii et al., 1993; Kutsuwada et al., 1992; Matsuda et al., 2002; Meguro et al., 1992; Yamazaki et al., 1992). Expression studies in Xenopus oocytes indicate that the GluN1 subunit is sufficient for a functional channel (Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Yamazaki et al., 1992).

The GluN1 subunit contains three splicing sites, one in the N-terminal (N1 cassette) and the other two in the C-terminal (C1 and C2 cassettes) region (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Yamazaki et al., 1992). Since the C2 cassette contains the stop codon, in its absence, additional sequences become a part of the protein and is known as the C2' cassette (Zukin and Bennett, 1995). Depending on whether the N1, C1 or C2 cassettes are present or absent, they make eight different splice variants. The splice variants of NR1 (GluN1) present in the receptor complex determine a number of properties of the ion channel, such as affinity to agonist and antagonists, zinc modulation and spatio-temporal expression in the brain (Dingledine et al., 1999; Laurie and Seeburg, 1994; Prybylowski and Wolfe, 2000; Zhong et al., 1995). Due to these properties, differential effects of aging on the different splice variants could lead to important changes in the physiology and pharmacology of the NMDA receptor in the aged brain.

There is evidence that the GluN1 subunit of the NMDA receptor shows declines in mRNA and protein expression during the aging process in C57BL/6 mice (Magnusson, 2000; Magnusson et al., 2002), but some studies in the same strain of mice show no change in GluN1 during aging (Magnusson, 2001). One difference between these studies was the addition of a behavioral experience in the later study

(Magnusson, 2001). The influence that behavioral testing may have on the GluN1 subunit needs to be further explored. Calorie restriction also leads to an upregulation of GluN1 subunits in middle aged and aged mice (Magnusson, 2001). This suggests that the expression of the GluN1 subunit during aging is variable and potentially susceptible to intervention.

It has been suggested that learning experience stabilizes synaptic modification and improves NMDA receptor expression (Quinlan et al., 2004; Sun et al., 2005). Because the GluN1 subunit mRNA shows variability during aging and could possibly be influenced by learning experience, we postulated that the overall expression of GluN1 subunits during aging is a function of differential changes in individual splice forms and its stabilization is due to learning experience influencing expression of the individual splice forms. In the present study, one aim was to determine if expression of individual splice forms were differentially affected by aging in prefrontal and/or frontal regions. The other aim was to determine roles of individual splice forms in learning ability and whether they were affected by experience in learning tasks. The focus was on the splice forms that were found previously to be most affected by aging, $GluN1_{X11}$ and $GluN1_{X10}$ (Magnusson et al., 2005), and on the $GluN1_{0XX}$ (-N1 cassette) and $GluN1_{1XX}$ (+N1 cassette), which had not been assessed yet in C57BL/6 mice across ages.

2.2 Results

2.2.1 Spatial reference memory

There was a significant main effect of age on performance in the reference memory place ($F_{(2, 30)} = 7.1$, $p = 0.003$; Fig. 2.1A) and probe trials ($F_{(2, 30)} = 4.5$, $p = 0.02$; Fig. 2.1B). Twenty-six month old mice had significantly higher cumulative proximity scores than both 11 and 4-month olds in place trials (Fig. 2.1A) and higher average proximity scores than the 4-month old mice in probe trials (Fig. 2.1B) when averaged over all the respective trials. There was a significantly lower cumulative proximity value for the 4-month old mice between place trials on day 1 and day 12 ($t_{(22)} = 6.74$, $p < 0.001$, Fig. 2.1A) and between probe trials 1 and 6 ($t_{(22)} = 2.57$, $p =$

0.02, Fig. 2.1B). Both the mid-aged and old mice also had significantly lower cumulative proximity values in place trial day 12 than place trial day 1 ($t_{(22)} = 6.66$, $p < 0.001$ and $t_{(22)} = 2.6$, $p = 0.02$ respectively, Fig. 2.1A) and in probe trial 6 than probe trial 1 ($t_{(22)} = 2.43$, $p = 0.02$ and $t_{(22)} = 4.07$, $p < 0.001$ respectively, Fig. 2.1B). A learning index was calculated from the overall performances in the probe trials as described by Gallagher and coworkers (Gallagher et al., 1993). The 26-month old mice had significantly higher learning index scores than the 4-month old mice ($F_{(1, 19)} = 9.4$, $p = 0.006$, Fig. 2.1C). The young (7.23±0.28 cm/s) and mid-aged (6.71±0.28 cm/s) mice were observed to have significantly faster swim speeds than the old mice (5.09±0.14 cm/s) in the first day of training in the place trials ($p < 0.001$, not shown).

2.2.2 Spatial working memory

There was a significant main effect of age ($F_{(2, 60)} = 14.8$, $p < 0.001$) and trial type, ($F_{(1, 60)} = 6.7$, $p = 0.01$) and a significant age and trial interaction ($F_{(2, 60)} = 6.3$, $p = 0.003$), when the naïve trial (T_0) and the delay trial (T_{delay}) were considered (Fig. 2.1D). The 4-month olds had significantly lower cumulative proximities than both the 11 and 26-month old mice in T_0 trials ($p = 0.006$, Fig. 2.1D). The 26-month old mice had significantly higher cumulative proximities than both the 11 and 4-month olds overall in the T_{delay} trials ($p < 0.001$, Fig. 2.1D). To measure performance in the first test trial (T_{delay}) in comparison with the naïve trial, we analyzed the T_0/T_{delay} ratio. Greater improvements (lower proximity scores) in T_{delay} as compared to T_0 would result in higher T_0/T_{delay} ratios. Mice in the 26-month old group had significantly lower values of T_0/T_{delay} than those in the 11-month old group ($p = 0.04$, Fig. 2.1E). Swim speeds of the mid-aged mice (7.03±0.28 cm/s) were significantly faster than the old mice (5.77±0.28 cm/s) and that of the young (8.15±0.32 cm/s) mice were significantly faster than both the old and mid-aged mice in naïve trials across the trial period ($p < 0.001$, not shown).

2.2.3 Cued control task

The animals analyzed above in the reference and working memory tasks showed no significant main effect of age, $F_{(2, 30)} = 1.5$, $p = 0.23$, on cumulative

proximity scores in the cued control trials (Fig. 2.1F). Animals in all the age groups had lower cumulative proximity scores in their cued trials than the place and working memory trials in all the platform positions except the north (Fig. 2.1F). Swim speeds of the young (8.36±0.42 cm/s) and mid-aged (7.92±0.40 cm/s) animals across all the platform positions in cued tasks were significantly faster than the old (6.35±0.36 cm/s) animals ($p < 0.001$, not shown).

2.2.4 mRNA expression of GluN1 splice forms

2.2.4.1 GluN1-pan:

There were no significant main effects of behavioral experience and no significant interactions between age and behavior on GluN1-pan mRNA expression in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. A representative film image of hybridization to GluN1-pan mRNA in a young mouse is shown in Fig. 2.2A. In the deep layers of lateral orbital cortex, there was a significant increase of mRNA expression from 4 to 11-months of age ($p = 0.02$; Fig. 2.3A). No other individual regions exhibited effects of age or experience (examples in Fig. 2.3A).

2.2.4.2 $GluN1_{X11}$ splice form:

Expression patterns of $GluN1_{X11}$ splice form mRNA showed no significant main effects of behavioral experience and no significant interactions between age and behavior in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. A representative film image of hybridization to $GluN1_{X11}$ mRNA in a young mouse is shown in Fig. 2.2B. Analysis of individual brain regions showed some effects of aging on the mRNA expression of $GluN1_{X11}$ splice form. There was a significant reduction of mRNA expression from 4 to 11-months of age ($p = 0.04$) and from 4 to 26-months of age ($p = 0.004$) in superficial layers of insular cortex (Fig. 2.3B). Deep layers of insular cortex ($p = 0.04$) and lateral orbital cortex ($p = 0.03$) showed a significant decrease in mRNA expression between 4 and 26-month old animals (Fig. 2.3B). There was a significant reduction of mRNA expression between 4 (262±12 pmol labeled $^{33}P/mm^2$ tissue) and

26-months (221±15 pmol labeled $^{33}P/mm^2$ tissue) of age in superficial somatosensory cortex ($p = 0.03$). No other individual regions exhibited effects of age or experience (not shown).

2.2.4.3 $GluN1_{X10}$ splice form:

Analysis of individual brain regions indicated some significant effects of behavioral experience on the expression of $GluN1_{X10}$ splice forms, so the behavioral groups were analyzed separately (Table 2.1). It was observed that there was a significantly higher expression of $GluN1_{X10}$ mRNA ($p = 0.04$) in 11-month old behaviorally-characterized animals than the 11-month old naïve animals in superficial layers of ventral orbital cortex (Table 2.1). Effects of age on $GluN1_{X10}$ mRNA expression were not observed in the naïve animals, but were observed in the behaviorally-characterized animals in some of the brain regions (Table 2.1, Fig. 2.2C & D). A significant reduction in mRNA expression of $GluN1_{X10}$ was observed in 11 and 26-month old animals as compared to the 4-month old behaviorally-characterized animals ($p = 0.01$ for both) in the deep layers of insular cortex (Table 2.1). In the superficial layers of insular cortex, a significantly lower mRNA expression in 11-month old animals than the 4-month old behaviorally characterized animals was observed ($p = 0.03$). A difference in mRNA expression between 4 and 26-month old behaviorally-characterized animals was observed in deep layers of medial prefrontal ($p = 0.04$), primary motor ($p = 0.01$) and somatosensory cortices ($p = 0.02$) and between 11 and 26-month old animals in superficial layers of medial prefrontal cortex ($p = 0.02$).

2.2.4.4 $GluN1_{0XX}$ splice form:

Analysis of individual brain regions indicated significant or near-significant main effects of experience, so the two behavioral groups were analyzed separately (Table 2.2). A significant increase in mRNA expression was observed in 26-month old behaviorally characterized as compared to naïve animals in deep ($p = 0.02$) and superficial ($p = 0.03$) layers of insular cortex (Table 2.2). No significant differences in mRNA expression between the three age groups were observed in the naïve animals

but differences were found in the behaviorally characterized animals across age groups (Table 2.2, Fig. 2.2E, F). Significantly higher expressions of mRNA in 26-month old than the 4-month old animals were observed in deep layers of medial prefrontal ($p < 0.001$), insular ($p = 0.003$), secondary motor ($p = 0.003$), primary motor ($p = 0.01$) and somatosensory cortices ($p = 0.03$) and superficial layers of ventral orbital ($p = 0.03$), medial prefrontal ($p = 0.003$) and somatosensory cortices ($p = 0.04$). Significant increases in expression of mRNA between 11 and 26-month old animals were observed in deep layers of insular ($p < 0.001$), lateral orbital ($p = 0.03$), medial prefrontal ($p = 0.008$), secondary motor ($p = 0.001$), primary motor ($p = 0.005$) and somatosensory cortices ($p = 0.005$) and superficial layers of insular ($p = 0.02$), ventral orbital ($p = 0.02$), lateral orbital ($p = 0.04$) and somatosensory cortices ($p = 0.001$).

2.2.4.5 $GluN1_{1XX}$ splice form:

There were no significant main effects of behavioral experience and no interactions between age and behavior on $GluN1_{1XX}$ mRNA expression in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. In the individual brain regions, there was a significant reduction of mRNA expression from 4 (246±12 pmol labeled $^{33}P/mm^2$ tissue) to 11-months (204±11 pmol labeled $^{33}P/mm^2$ tissue) of age ($p = 0.02$) in superficial layers of insular cortex when collapsed across experience groups. There were no other regions that showed significant difference between age groups (not shown).

2.2.5 Correlations

We performed correlations between mRNA densities in the insular and orbital regions and learning index for reference memory and T_0/T_{delay} ratio for working memory using individual old animals alone in order to determine if the reference and working memory performance correlated with the mRNA densities for GluN1 splice forms in different brain regions.

2.2.5.1 Reference memory:

Learning index scores as a measure of reference memory performance, did not correlate significantly with the mRNA expression of the whole GluN1-pan or the

individual splice forms in the old animals at a significance level of .05 (not shown). We observed a trend for a negative correlation (r = -.76, corrected p = .08) between $GluN1_{0XX}$ mRNA densities in superficial layers of lateral orbital cortex and learning index scores (Fig. 2.4A). Higher densities were associated with better performances in the reference memory task.

2.2.5.2 Working memory:

The T_0/T_{delay} ratios, which represents working memory performance, were significantly positively correlated with mRNA expression of $GluN1_{0XX}$ splice variant in the deep layers of ventral orbital cortex (r = .82, corrected p = .04; Fig. 2.4B). Higher T_0/T_{delay} values indicate better working memory performance; so positive correlations indicated high $GluN1_{0XX}$ mRNA densities associated with better performance. No other significant correlations were found (not shown).

2.2.5.3 Cued control task:

We performed correlations of cued trial performance with mRNA expression of different splice forms. The results indicated positive correlations for only $GluN1_{0XX}$ mRNA expression with cued trial performance in deep and superficial layers of ventral orbital cortex and lateral orbital cortex (Fig. 2.4C, r range = .93 to .86, corrected p range = .003-.01). A correlation of T_0/T_{delay} ratio with cued trials indicated a positive relationship between measurements (Fig. 2.4D). However, poor performance (a higher score) in cued trial performance in this case was associated with better performance (higher score) in working memory tasks and vice versa.

2.3 Discussion

This study provides new evidence for a differential effect of behavioral experience on the expression of different N- and C-terminal splice forms of the GluN1 subunit during aging and evidence for a role of the $GluN1_{0XX}$ splice form within orbitofrontal brain regions in working memory and associative memory performances in aged mice. The oldest mice showed significant deficits in both spatial reference and spatial working memory ability as compared to the young and middle-aged mice.

There was little effect of aging on expression of mRNA that is found in all known splice variants, but mRNA for two of the C-terminal splice forms, $GluN1_{X11}$ and $GluN1_{X10}$, showed significant declines during aging in several brain regions. Behavioral experience was associated with an up-regulation of the $GluN1_{0XX}$ mRNA in prefrontal and frontal cortex regions in old mice. Within the old mice high $GluN1_{0XX}$ was associated with good working memory performance, but poor associative memory performance.

2.3.1 Reference and Working Memory Performance

Using the Morris water maze to test the spatial reference memory performance, we observed that there were signs of reduction in learning ability between the ages of 11 and 26-months as evident by the place learning trials. Similar age-related differences were observed in our previous studies (Magnusson, 1998a; Magnusson, 2001). There was evidence of improved learning across trials in all ages of mice indicating that learning had occurred across trials. The learning index scores used were a continuous, graded measure of the severity of age-related memory impairments of mice in the probe trial task as described originally by Gallagher and coworkers (Gallagher et al., 1993). Both the probe trial proximity measures and leaning index scores indicated that the old animals did worse than the young when the platform was not present.

In the cued task, all the mice were able to improve their performances except the three mice removed from the study (see experimental procedure; data analysis). Following this removal all of the groups performed similarly in their cued tasks throughout the trials. This indicated that motor control or motivation were not issues for the mice used in the study. The learning curves for place and probe trials looked different than for cued trials indicating that mice performed differently when they could see the platform.

Swim speed differences were observed between different ages of mice in each of the water maze tasks used. Therefore, the traditional measure of latency was not used. Path length was also not considered a good option with these mice, because

older ones tend to float when initially placed in the tank (unpublished observation). Cumulative and average proximity measures used in this study were corrected for ideal path cumulative proximity by using individual swim speed for that trial. This measure thus is less affected by swim speed than latency and more reflective of the bias for the platform than path length (Gallagher et al., 1993). With these measures, we observed similar age related changes in both place and probe trials. The probe trial measurements were resistant to the differences in swim speed because the trial time was same for all probe trials. The use of the ratio for working memory also reduced swim speed influences by being a within individual measurement. In addition, despite differences in swim speed, the cued control task showed no aging differences.

In the working memory tasks, we detected a decline in performance in older animals as compared to the young in both the naïve and delayed working memory trials. Better performance of the young, compared to older mice in the naïve trials suggests that they learned something about the platform location or had a better search strategy than the older mice. Lack of improvement in the delayed working memory trials was observed only in the case of 26-month old mice. Similar results were obtained in our previous experiment with C57BL/6 mice where middle-aged mice performed well in the delayed working memory trials but the old mice were impaired (Magnusson et al., 2003). To study working memory in rats using the water maze, several investigators have considered using multiple trials within one session to represent working memory (Galea et al., 2000; Kikusui et al., 1999; Lehmann et al., 2000), whereas several others have used only the first trial after a naïve swim (Frick et al., 1995; Morris et al., 1986; Steele and Morris, 1999). Frick and coworkers have shown that out of the four trials in working memory tasks, trials 1 and 2 were associated with working memory and trials 4 and 5 were more associated with reference memory (Frick et al., 1995). In our previous experiment we used trial 4 as the delayed trial, but it was not clear if the mice were using working memory for that trial (Magnusson et al., 2003). In the present study we used the second trial (next trial after naïve swim) as the delayed trial (10 minutes delay after naïve swim) and found

similar results in both middle-aged and old mice.

Cumulative proximity measurements from the delayed working memory trial or from the naïve trial only gives an absolute value per trial, not relative between trials. However, the ratio between the naïve and delayed trial gives a comparison between the two trials and better explains their working memory performance relative to each other (Inman-Wood et al., 2000). A high ratio indicates improved performance in the delayed trial as compared to the naïve trial and vice versa. The use of this ratio showed that the old mice performed worse than the middle-aged mice in working memory trials.

2.3.2 mRNA Expression of GluN1 splice forms

There have been different results seen for the expression pattern of GluN1-pan in the frontal cortex region. Our lab has observed significant effects of aging on GluN1-pan protein expression (Magnusson et al., 2002) and mRNA in frontal cortex (Magnusson, 2000; Magnusson et al., 2005), yet in another study, there was no effect of aging on the GluN1-pan mRNA expression (Magnusson, 2001). This suggests that some factor(s) produce variability in the effects of aging on GluN1-pan. GluN1-pan mRNA expression in the present study only showed declines between young and middle-aged mice in one region, lateral orbital cortex. There were significant reductions of $GluN1_{X11}$ mRNA with increased age in insular, lateral orbital and superficial somatosensory regions. In the present study, $GluN1_{X10}$ mRNA was affected by aging in insular, medial prefrontal and primary motor areas, but only in the behaviorally-experienced animals. In our previous study, $GluN1_{X11}$ and $GluN1_{X10}$ were affected by aging when GluN1-pan showed significant declines with aging (Magnusson et al., 2005). The present results suggest that $GluN1_{X11}$ and $GluN1_{X10}$ mRNA expression were affected before there were significant signs of aging overall in GluN1-pan mRNA expression. In addition, they showed that behavioral experience was associated with a significant age-related decline in $GluN1_{X10}$ mRNA expression that wasn't seen in the naive mice.

The $GluN1_{X11}$ and $GluN1_{X10}$ splice forms both contain the C1 cassette, which

is abundant during development, present at about 50% of the total GluN1 subunit, and present in slightly higher amounts in adult stages, at about 58% in the rat cortex (Prybylowski and Wolfe, 2000). This cassette is important for NMDA receptor functioning as it contains phosphorylation sites, two for PKC and one for PKA activity (Tingley et al., 1997). In culture, C1 cassettes target GluN1 subunits to the receptor rich domains (Ehlers et al., 1995; Ehlers et al., 1998). The cassette appears to interact with two proteins, yatiao and neurofilament L, which contribute to clustering of receptors (Ehlers et al., 1998; Lin et al., 1998). These studies (Ehlers et al., 1995; Ehlers et al., 1998; Lin et al., 1998; Tingley et al., 1997) suggest that decreases in C1, as observed in aged mice, could lead to decreased localization of NMDA receptors in receptor clusters and less potentiation of receptor function due to decreased phosphorylation. Our preliminary results on protein expression of different cassettes from these same animals indicated that decline in both C1 and C2 cassette expressions occurred during aging (unpublished observation).

Expression of $GluN1_{0XX}$ mRNA in the naïve animals was not affected by the process of aging. In the behaviorally experienced animals however, $GluN1_{0XX}$ mRNA expression was significantly increased in aged animals in most of the brain regions analyzed. The $GluN1_{0XX}$ splice form doesn't contain the insertion cassette N1. Splicing out of the N1 insertion cassette has been shown to reduce affinity for agonist by almost five fold (Durand et al., 1993) and decrease the current amplitude in oocytes expressing GluN1 subunits (Hollmann et al., 1993; Zheng et al., 1994). Presence of N1 insert has also been found to attenuate stimulation by polyamines, proton inhibition and zinc modulation (Durand et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Zheng et al., 1994). In rat brain, $GluN1_{0XX}$ splice forms have been found to be the most abundant form of splice variants and are expressed all throughout the brain (Laurie and Seeburg, 1994; Laurie et al., 1995). A higher expression of $GluN1_{0XX}$ mRNA in the 26-month old animals indicated that there was a higher amount of receptor protein without the N1 insert. This might lead to a significantly lower response of the NMDA receptor population to the transmitter glutamate but a

higher response to other modulators (Durand et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Zheng et al., 1994). $GluN1_{1XX}$ mRNA expression showed no significant effects of aging. It is not clear why there wasn't a corresponding decrease in the $GluN1_{1XX}$ splice form containing the N1 cassette.

2.3.3 Correlations

Correlational analyses of reference and working memory performance with mRNA expression of various splice forms were assessed in the old animals alone. The orbital and insular cortex regions were used for the correlational analysis because of previous evidence of their involvement in reference and working memory tasks (Bermudez-Rattoni et al., 1991; Kolb et al., 1983; Nyberg et al., 1995; Paradiso et al., 1997).

No significant correlations between mRNA expression of any of the splice forms and learning index (measure of reference memory performance) were observed in any of the brain regions analyzed. A near-significant relationship however, was observed between high $GluN1_{0XX}$ mRNA density in lateral orbital cortex and good performance in the spatial reference memory task. Reference memory, involving recall of stored information, has been found to be associated with orbital frontal cortex (Nyberg et al., 1995; Paradiso et al., 1997).

Higher expression of the $GluN1_{0XX}$ splice form in ventral orbital cortex was associated with better working memory performance. This finding fits with human studies in which higher activity in the orbital region was observed in older individuals performing working memory tasks in a functional MRI study (Cook et al., 2007). Since there was an association of poor performance in cued trials with good performance in working memory tasks, the correlation of cued trials and mRNA expression of $GluN1_{0XX}$ in prefrontal orbital cortex was not unexpected. Dependency of associative learning on orbital frontal cortex has been shown by an inability of rats to access cues with orbitofrontal cortex damage (Gallagher et al., 1999). It is, however, not clear why working memory and associative learning in the cued task should be negatively correlated.

In summary, there were heterogeneous temporal and regional effects of aging on the different splice forms of the NMDA receptor complex. Aging of the GluN1-pan subunit of the NMDA receptor seemed to be contributed by the combinatorial effects of aging on $GluN1_{0XX}$, $GluN1_{X11}$ and $GluN1_{X10}$ splice forms. Up-regulation of $GluN1_{0XX}$ splice form message, influenced by behavioral experience, seemed to have benefited working memory ability in aged animals. Associative learning was negatively affected by the same increases. Overall these results indicate that GluN1 pan mRNA expression is regulated by a combination of complex changes in the different splice forms.

2.4 Experimental Procedure

2.4.1 Animals

A total of 72 C57BL/6 mice (National Institute on Aging, NIH) from three different age groups (four, ten and twenty-six months of age) were used for the study. They were fed *ad libitum* and housed in cages under 12hr light and 12hr dark cycle. The animals were divided into two behavioral groups; naïve and behaviorally-characterized, containing twelve animals from each age group. Animals in the behaviorally-characterized group were subjected to learning experience with the use of the Morris water maze as discussed below. The animals in the naïve group were housed for the same amount of time as the behaviorally-characterized animals. After the behavioral testing, all animals were euthanized with exposure to CO_2 and decapitated. The brains were then harvested, frozen rapidly with dry ice and stored at -80 °C until further processing.

2.4.2 Behavioral Testing

Spatial reference and working memory and cued control task ability were tested using the Morris Water Maze. A 4-foot diameter metal tank was covered with white contact paper and filled with water that was made opaque white with non-toxic paint. A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth. The cues were placed high on walls of both the room and the tank. There were seven different

platform positions located at five different distances from the tank wall. Trials were video taped using a camera placed above the center of the tank on the ceiling of the room. Paths of the trials were analyzed by using the "SMART" video tracking system (San Diego Instruments, San Diego, CA). There were different entry points for each trial and the mice were placed in the tank facing the wall.

2.4.2.1 Pretraining:

Pretraining was done during the 2 days prior to reference memory training and consisted of each mouse swimming for 60 s in the tank without the platform and then being trained to remain on the platform for 30 s each day. This platform position was different from the one used for reference memory testing.

2.4.2.2 Spatial reference memory:

On days 3 through 14, mice underwent reference memory trials. The task consisted of 2-3 place trials per day for 12 days (Gallagher et al., 1993) and probe trials every alternate day. The platform was kept in the same quadrant for each place trials. Place trials consisted of 60 s in the water searching for the platform, 30 s on the platform and 60 s of cage rest. If a mouse failed to find the platform within the designated 60 s time, it was led to the platform by the experimenter. Assessment of the animal's ability to show a bias for the platform location was done by a probe trial every other day in place of every sixth place trial (Gallagher et al., 1993). During the probe trial the platform was removed and the mouse was allowed to search in the water for 30 s.

2.4.2.3 Spatial working memory:

On Days 15 through 22, mice were tested in a spatial working memory task (Magnusson et al., 2003). The task consisted of two sessions per day for 8 days. There was a two-day break between sessions eight and nine. The platform positions were changed between each session. Each session consisted of 4 trials. The naïve trial started by placing a mouse into an entry point facing the tank and allowing it to search the platform for a maximum of 60 s (T_0) after which the mouse was allowed to remain on the platform for 30 s (first test trial) followed by cage rest for 10 minutes (delay

period). In the second trial it was placed in the water at a different entry point from the naïve trial and allowed to search the platform for a maximum of 60 s (T_{delay}). The mouse was again allowed to stay on the platform for 30 s and allowed to rest in the cage for 60 s. The mouse was placed into the water 2 more times at 2 different entry points and allowed to find the platform for 60 s (T_2 and T_3). They spent 30 s on the platform and rested in the cage for 60 s between trials. They were then placed into their cages until the next session. If the mouse failed to find the platform within the designated 60 s for any of the trials, it was led to the platform by the experimenter. The entry points within one session were randomly assigned for each trial, with no entry points repeated within a session. Working memory was assessed between T_0 and T_{delay}. The extra sessions were performed based on previous findings that mice need additional trials to show improvement between trials (Magnusson et al., 2003).

2.4.2.4 Cued control task:

Cued trials were designed to test motivation, visual acuity, and physical ability for the task. On day 23, mice underwent 6 cued trials. The platform was kept submerged but was marked by a 20.3 cm support with a flag. For each cued trial, the platform was changed to a different position and the mouse was placed into the tank facing the wall at one of the entry points and was allowed to search for the platform for 60 s. All mice were tested at one platform position before the platform was moved to a new position.

2.4.3 Brain Sectioning

The frozen brain from each animal was cut mid-saggitally into two halves. One half was used for sectioning for *in situ* hybridization. Use of the left and right half of the brain was varied between individuals. Slides used for brain tissue sections were coated with 0.5% gelatin solution and air-dried before hand. Coronal brain sections of 12 μm thickness, representing animals from each age and behavioral group were placed on each slide and kept frozen at -80 °C until used. Slides were divided into 12 cutting groups, each group containing animals from each age and behavioral group.

Position of animal brain sections on slides were changed randomly between each cutting groups to control for the variability while washing during *in situ* hybridization

2.4.4 In situ hybridization

Oligonucleotides used for the *in situ* hybridization were commercially prepared (Macromolecular Resources, Colorado State University, Fort Collins, CO). The sequences used were:

$GluN1_{0XX}$, AACTGCAGCACCTTCTCTGCCTTGGACTCCCGTTCCTCCA;

$GluN1_{1XX}$, GCGCTTGTTGTCATAGGACAGTTGGTCGAGGTTTTCATAG;

$GluN1_{X11}$, TCCACCCCCGGTGCTCGTGTCTTTGGAGGACCTACGTCTC;

$GluN1_{X10}$, GATATCAGTGGGATGGTACTGCGTGTCTTTGGAGGACCTA; and

GluN1-pan, GCACAGCGGGCCTGGTTCTGGGTTGCGCGAGCGCGACCAC-CTCGC (Laurie and Seeburg, 1994).

Oligonucleotides were labeled with ^{33}P-dATP (Perkin Elmer, Waltham, MA) of specific activity: 3103 to 3238 Ci/mM using terminal deoxyribonucleotidyl transferase (Invitrogen Corp., Carlsbad, CA) and purified in Microspin G-25 columns (Amersham Bioscience, Piscataway, NJ). The specific activities for the labeled oligonucleotides were calculated to be 40 to 140 dpm/fmol of $GluN1_{0XX}$ probe, 85 to 139 dpm/fmol of $GluN1_{1XX}$ probe, 87 to 155 dpm/fmol of $GluN1_{X11}$ probe; 81 to 140 dpm/fmol of $GluN1_{X10}$ probe, and 105 to 130 dpm/fmol of GluN1-pan probe, depending on the labeling experiment.

In situ hybridization was performed as described by Watanabe and coworkers (Watanabe et al., 1993) and previous study in our lab (Magnusson et al., 2005). Briefly, each solution step was performed with gentle rotation on a rotating table except for the fixation and hybridization steps. Slides with sections were thawed, air-dried, fixed in 4% paraformaldehyde-PBS, pH 7.2 (25 °C) for 15 min, placed in 2 mg/ml glycine in PBS, pH 7.2 (25 °C) for 20 min, and placed in 0.25% acetic anhydride-0.1M triethanolamine, pH 8.0 (25 °C) for 10 min. Slides were placed in

coplin jars (25 °C) for 2 hr in a prehybridization solution consisting of 50% formamide, 0.1M Tris-HCl, pH 7.5, 4X SSC (1X SSC = 150mM NaCl and 15mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2% sarkosyl, and 250 µg/ml salmon testes DNA. Slides were then successively washed for 5 min each in 2X SSC, 70 and 100% ethanol, and air-dried for 15 min. Hybridization was performed by placing 150 µl of prehybridization solution containing 10% dextran sulfate and 0.33pmoles of ^{33}P-labeled oligonucleotide probe onto the slides, covering the slides with parafilm, and incubating them for 18 hr in a 42 °C oven, humidified with 5X SSC. After incubation, coverslips were removed; slides were rinsed for 40 min in 2X SSC and 0.1% sarkosyl (25°C) and for 2×40 min in 0.1X SSC and 0.1% sarkosyl (55 °C) and air-dried. Nonspecific hybridization was determined by addition of 50-fold excess non-radiolabelled oligonucleotide to the hybridization solution on some slides. Slides were exposed to Kodak Biomax films for 3-8 days depending on the splice form along with slide containing ^{14}C standards. Brain and standard images were captured using a Macintosh G4 computer with a Powerlook 2100 XL scanner (UMAX, Taiwan) and NIH Image software. Quantitative densitometry was performed on the images from four sections for total hybridization and two sections for nonspecific hybridization from each animal with the use of NIH Image software. The different prefrontal and frontal cortex brain regions analyzed for mRNA expression were deep (cortical layers IV-VI) and superficial (cortical layers II-III) layers of ventral orbital cortex, lateral orbital cortex, medial prefrontal cortex (areas containing cingulate cortex, infralimbic cortex and prelimbic cortex), insular cortex (areas containing both granular and agranular insular cortex), secondary motor cortex, primary motor cortex and the somatosensory cortex (areas containing both primary and secondary somatosensory cortex). The sections ranged from 0.38 to 1.94 mm rostral to bregma (Paxinos and Franklin, 2001). Specific signal was determined by subtracting nonspecific hybridization from total hybridization. Nonspecific hybridization of ^{33}P labeled with mRNAs of different splice forms ranged from 16 to

53% of total hybridization. The ^{14}C standards were used to convert optical density to fmol of labeled ^{33}P-dATP/mm^2 tissue (Eakin et al., 1994).

2.4.5 Data analysis

Data for behavioral testing were analyzed as described earlier (Magnusson et al., 2003). Cumulative proximity was used to measure performance in the place, working memory and cued trials. Cumulative proximity was obtained from the Smart system according to the method of Gallagher and coworkers (Gallagher et al., 1993), and was manually corrected for start position. Briefly, the animal's distance from the platform, or proximity measure, was measured by the computer every 0.2 seconds for the duration of the animal's swim. These proximity measures were then added together to give a cumulative proximity. The proximity measures were corrected for start position by calculating the cumulative proximity for the ideal path, based on swim speed and starting point and subtracting this from the cumulative proximity measurement from the tracking system. Average proximity to the platform was used to assess performance in the probe trials (Gallagher et al., 1993). The data was collected similar to the cumulative proximity measure, but after correcting for starting point, the proximity measures were averaged over the 30s trial (Gallagher et al., 1993). Learning index scores were calculated from the probe trial data according to Gallagher and coworkers (Gallagher et al., 1993). The mean average proximity measurements for the young mice in the first probe trial (probe trial 1) were divided by the mean measurements for the young mice in each separate probe trial in order to obtain a multiplier for each probe trial. The multipliers obtained were as follows: 1.00, 1.32, 1.25, 1.66, 1.70, 1.35 for probe trials 1-6, respectively. For each mouse, the average proximity scores for each trial were multiplied by the respective multipliers for each trial and the products were summed to obtain a learning index score for that mouse. For both cumulative and average proximity and learning index scores, higher values represented poorer learning ability and lower values indicated better learning performance. Proximity measures were used to assess performance in these studies because they are less influenced by swim speed differences than more traditional

measures such as latency to reach the platform (Gallagher et al., 1993; Magnusson, 1998a). The proximity measures are also more sensitive to some of the alternative strategies that animals can use to find the platform that may not involve place learning (Gallagher et al., 1993). The learning index score provides similar information to traditional measurements of time spent in the correct quadrant, but has the added advantage of providing a single value that can represent the spatial bias in multiple probe trials and also reflect the learning curve by being weighted to reward those animals who acquire the task faster (Gallagher et al., 1993).

Working memory data were measured by cumulative proximity scores corrected for the start position as described above. In order to assess how performance improved between T_0 and T_{delay}, a ratio (T_0/T_{delay}) was calculated for each session, averaged across sessions and used for correlations with mRNA. This method of ratio gives a high score to mice exhibiting better performance.

Cued trials were used as a control for non-spatial memory influences and subsequent inclusion in the study. Three 26-month old animals were removed from the study due to their performance in cued trials 2-6 being higher than the highest score in the young (Magnusson, 1998a; Magnusson, 2001; Magnusson et al., 2003). All the behavioral data presented reflected omission of these three animals.

Data from image analysis were normalized to the average of 4-month old behaviorally-characterized mice to reduce variability between films and assays (Magnusson, 1997; Magnusson et al., 2005; Ontl et al., 2004). Normalization factors were obtained by dividing the overall averages of a subset of the brain regions that were analyzable within all the young behaviorally-characterized animals by the averages for the young behaviorally-characterized animals within each assay group and were then multiplied by all animals' values within the assay.

Age-related differences in performance in working and reference memory and cued tasks were analyzed separately by repeated measures ANOVA and two-way ANOVA followed by Fisher's protected post-hoc analysis using Statview software

(SAS Institute Inc., Cary, NC). Age-related differences were analyzed separately for each brain region by two-way ANOVA followed by Fisher's protected post-hoc analysis. When there were no significant differences between the naïve and behaviorally-characterized groups, the values were averaged across these two treatments.

Pearson correlation coefficients between mRNA densities in insular and orbital regions and working (T_0/T_{delay}) and reference (learning index score) memory measurements in old mice alone were obtained to determine how the working and reference memory performance were related to different densities of mRNAs in different brain regions. Only the insular and orbital regions were selected for correlational analysis because of their established role in reference and working memory tasks (Bermudez-Rattoni et al., 1991; Kolb et al., 1983). To correct for the number of comparisons, a recently developed method, p_ACT version 1.0 (Conneely and Boehnke, 2007) was used and run using R statistics software version 2.6.1 (R Development Core Team, 2007) with the package *mvtnorm* version 0.8-1 (Genz et al., 2007). This method of adjustment adjusts for p-values of different correlation tests sequentially and is based on a procedure described previously (Holm, 1979). The correction was applied by setting the value of alpha to .05 and comparing mRNA expression of the six brain regions with individual tests of memory performance.

Acknowledgements

We sincerely acknowledge Dr. Karen Conneely for helping us with corrections for the multiple correlation tests. This work was supported by NIH grant AG16322 to K.R.M. & P20RR16454-02 to BRIN.

Fig. 2.1 - Performance of mice in behavioral trials. Figures above show performance in 12 days of place trials (A), 6 days of probe trials (on even place trial days; B) and learning index scores from probe trial data (C) for the reference memory tasks; averaged naïve (T_0) and delayed (T_{delay}) trials (D) and the ratio, T_0/T_{delay} (E) for working memory tasks and cued control trials at the 6 different platform positions (F) for the 4, 11 and 26-month old mice. * $p< 0.05$ for differences from 4-month old animals for the same trials, # $p< 0.05$ for differences from 11-month old animals for the same trial(s), † $p< 0.05$ for differences from overall performance in naïve trials (T_0) within same age group. (A, B, D & E) Significant differences were seen with the averages across all trials.

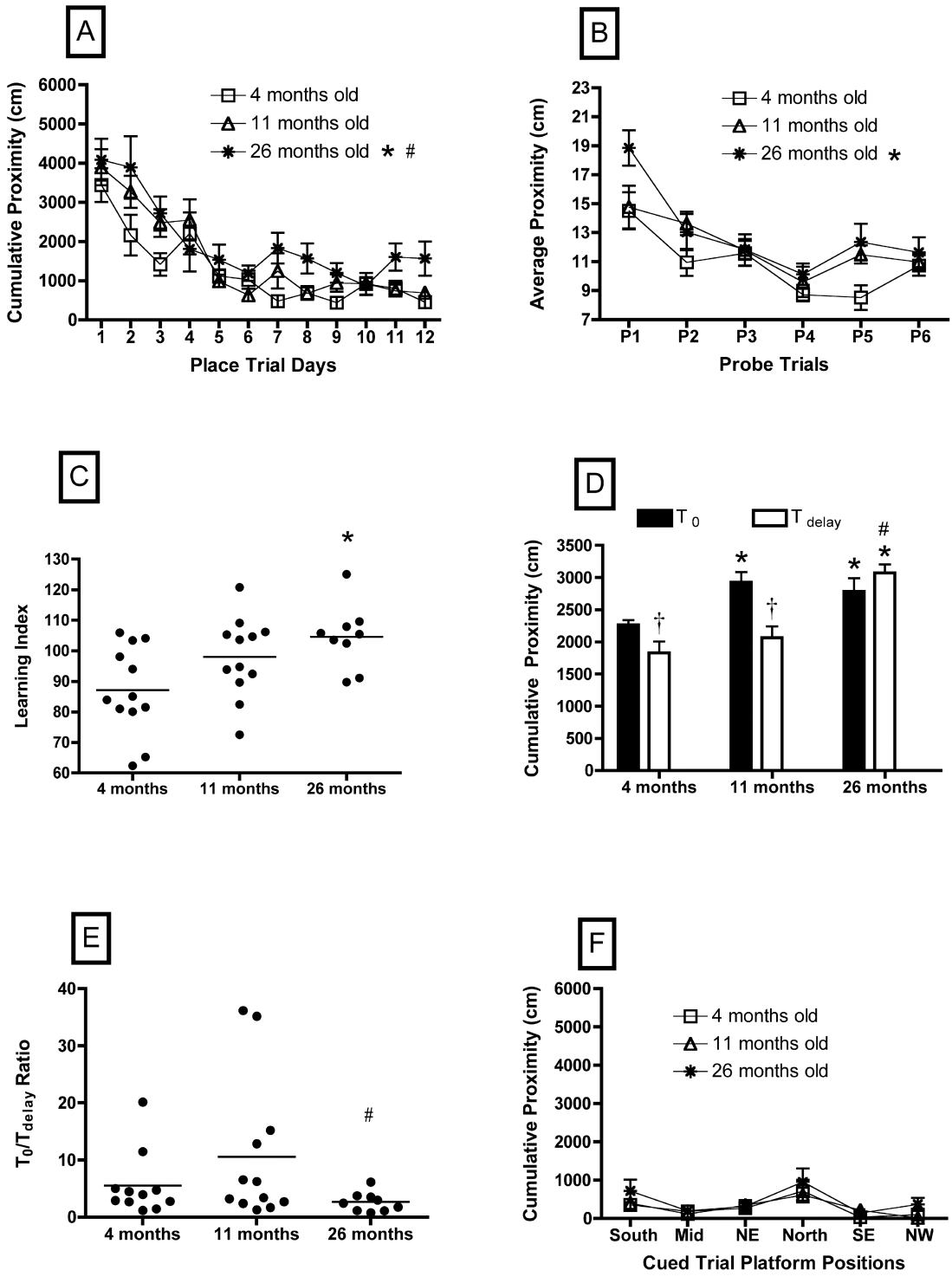
A
Cumulative Proximity (cm)
6000
5000
4000
3000
2000
1000
0
4 months old
11 months old
26 months old * #
1 2 3 4 5 6 7 8 9 10 11 12
Place Trial Days
B
Average Proximity (cm)
23
21
19
17
15
13
11
9
7
4 months old
11 months old
26 months old *
P1 P2 P3 P4 P5 P6
Probe Trials
C
Learning Index
130
120
110
100
90
80
70
60
*
4 months
11 months
26 months
D
T 0
T delay
Cumulative Proximity (cm)
3500
3000
2500
2000
1500
1000
500
0
†
†
*
*
*
#
4 months
11 months
26 months
E
T0/Tdelay Ratio
40
30
20
10
0
#
4 months
11 months
26 months
F
Cumulative Proximity (cm)
6000
5000
4000
3000
2000
1000
0
4 months old
11 months old
26 months old
South Mid NE North SE NW
Cued Trial Platform Positions

Fig. 2.1

Fig. 2.2- Representative film images of mRNA hybridization of different splice forms of behaviorally characterized animals. In situ hybridization images from a young mouse for GluN1-pan (A) and $GluN1_{X11}$ (B). $GluN1_{X10}$ mRNA showed higher density of hybridization in some areas in young behaviorally-characterized animal brain (C) than in the old animals from the same behavioral group (D). $GluN1_{0XX}$ mRNA showed lower densities of hybridization in many regions of young animal brains (E) than in the old animals (F). Representative in situ image from a young mouse for $GluN1_{1XX}$ (G). Diagrams adapted from Paxinos and Franklin (2001) showing analyzed brain regions of the prefrontal and frontal cortex of mice used in this study (H & I). Even numbers indicate superficial layers (II and III) and odd numbers indicate deep layers (IV-VI) of prefrontal and frontal cortical regions of mouse brain. 1, 2 ventral orbital cortex; 3, 4 lateral orbital cortex; 5, 6 medial prefrontal cortex; 7, 8 secondary motor cortex; 9, 10 primary motor cortex; 11, 12 somatosensory cortex and 13, 14 insular cortex. Standard images and the equivalent pmol of labeled $^{33}P/mm^2$ tissue are shown to the right of each brain image.

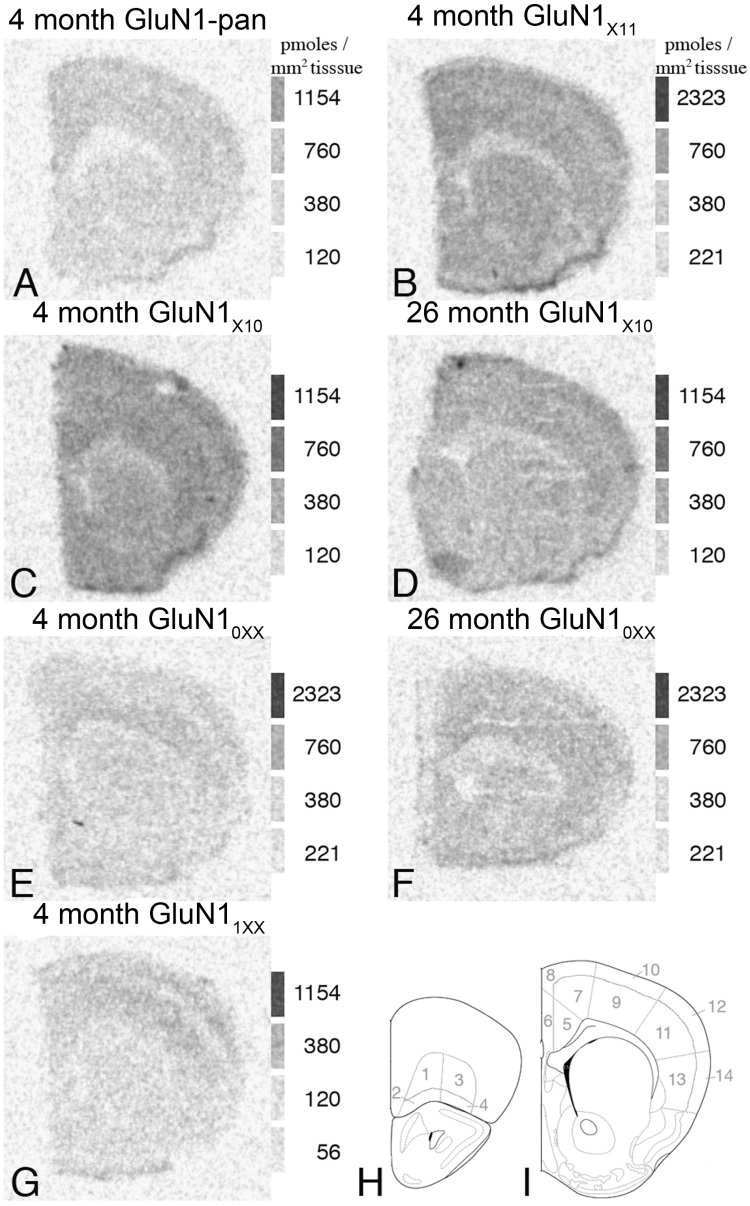
4 month GluN1-pan
pmoles / mm² tisssue
1154
760
380
120
A
4 month GluN1X11
pmoles / mm² tisssue
2323
760
380
221
B
4 month GluN1X10
1154
760
380
120
C
26 month GluN1X10
1154
760
380
120
D
4 month GluN10XX
2323
760
380
221
E
26 month GluN10XX
2323
760
380
221
F
4 month GluN11XX
1154
380
120
56
G
H
1
2
3
4
I
5
6
7
8
9
10
11
12
13
14

Fig. 2.2

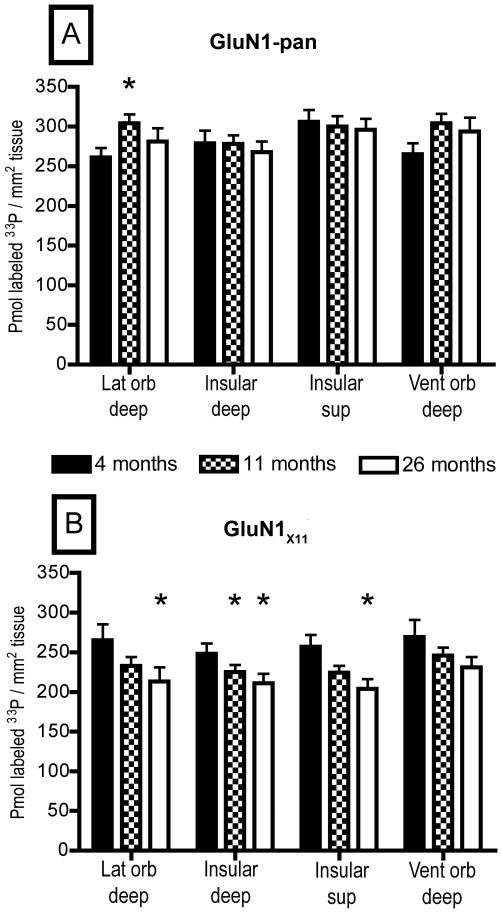

Fig. 2.3 – mRNA densities of GluN1-pan and GluN1$_{X11}$. Graphs showing densities of mRNA expression of GluN1-pan (A) and GluN1$_{X11}$ splice form (B) in prefrontal cortical regions of mice brain. * $p < 0.05$ for difference in expression of mRNA from 4-months old animals. Data indicate mean±SE. Lat orb, lateral orbital cortex; vent orb, ventral orbital cortex; sup, superficial cortical layers II-III; deep, cortical layers IV-VI.

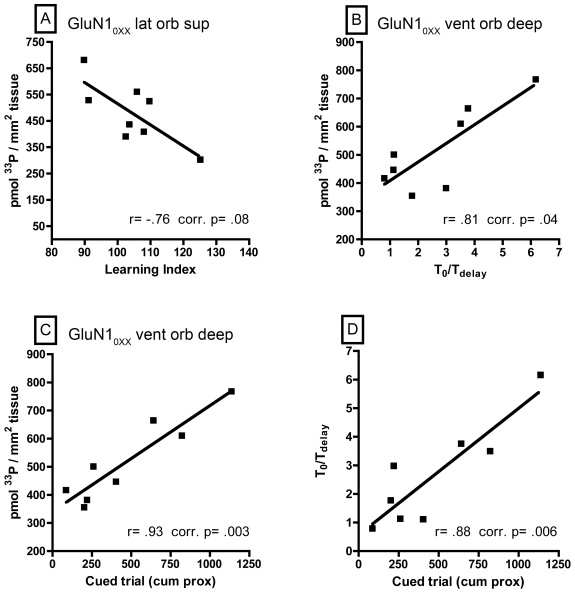

Fig. 2.4 – Graphs showing correlation analysis of mRNA for the $GluN1_{0XX}$ splice form in orbital cortices of old mice and performance in a reference memory task, as shown by learning index scores (A); in a working memory task, as shown by the ratio of T_0/T_{delay} (B); and in the cued control task, as shown by averaged cumulative proximity (cum prox; C). D) Graph showing correlation analysis between performance in the working memory task and cued trials. Please note that high learning index scores and cumulative proximities are associated with poor performance in reference and cued trials, respectively. High T_0/T_{delay} ratios indicate good performance. Lat orb, lateral orbital cortex; vent orb, ventral orbital cortex; T_0, initial naïve trial; T_{delay}, first test trial with 10 minute delay from naïve trial; corr p, corrected p-value (p_ACT statistical method).

Table 2.1: mRNA expression of $GluN1_{X10}$ in different brain regions of the prefrontal and frontal cortex in naïve and behaviorally-characterized groups in different age groups of mice. Data indicates mean±SEM.

Cortical regions		Naïve			Behaviorally-characterized		
		4-months	11-months	26-months	4-months	11-months	26-months
Insular cortex	-deep	261±26	228±7	241±18	274±16	229±7*	220±15*
	-superficial	255±22	225±14	232±16	260±22	211±12*	228±13
Ventral orbital cortex	-deep	227±26	218±23	222±24	282±40	274±16	198±29
	-superficial	234±28	224±24	212±25	283±38	286±18†	220±25
Lateral orbital cortex	-deep	222±35	217±15	203±19	273±35	253±14	213±20
	-superficial	218±23	206±17	203±19	248±38	249±19	206±16
Medial prefrontal cortex	-deep	250±23	235±8	229±22	253±21	244±8	205±15*
	-superficial	242±20	229±15	236±22	237±20	259±8	236±22#
Secondary motor cortex	-deep	238±24	234±11	237±20	252±20	226±5	219±10
	-superficial	76±16	255±16	252±24	259±7	259±17	243±8
Primary motor cortex	-deep	236±24	220±8	226±15	241±12	222±7	201±10*
	-superficial	260±25	253±17	242±15	254±24	224±11	212±12
Somatosensory cortex	-deep	248±24	219±7	238±23	253±15	224±7	212±11*
	-superficial	267±25	241±11	251±25	267±20	245±8	235±11

mRNA expression is measured in pmoles of labeled $^{33}P/mm^2$ of tissue.
* $p < 0.05$ for differences from 4-month old behaviorally-characterized animals
# $p < 0.05$ for differences from 11-month old behaviorally-characterized animals
† $p < 0.05$ for differences from the naïve animals in same age group
deep = cortical layers IV-VI
superficial = cortical layers II-III

Table 2.2: mRNA expression of $GluN1_{0XX}$ in different brain regions of the prefrontal and frontal cortex in both naïve and behaviorally-characterized groups in different age groups of mice. Data indicates mean±SEM.

Cortical regions		Naïve			Behaviorally-characterized		
		4-months	11-months	26-months	4-months	11-months	26-months
Insular cortex	-deep	414±34	417±30	417±32	439±14	423±13	521±26* # †
	-superficial	402±17	414±32	438±24	462±25	421±23	508±19 # †
Ventral orbital cortex	-deep	437±34	460±32	465±46	440±28	417±16	557±61
	-superficial	445±29	462±33	485±43	446±22	439±11	557±43* #
Lateral orbital cortex	-deep	398±28	443±31	430±47	431±27	399±19	516±63#
	-superficial	393±21	432±29	434±43	410±18	406±20	502±53#
Medial prefrontal cortex	-deep	390±43	398±22	428±34	394±22	421±11	498±21* #
	-superficial	387±32	406±27	446±35	417±18	449±16	498±11*
Secondary motor cortex	-deep	378±37	383±23	425±36	401±18	394±14	486±19* #
	-superficial	441±32	431±32	465±24	461±23	455±28	521±69
Primary motor cortex	-deep	366±33	381±22	426±42	414±18	404±14	490±27* #
	-superficial	415±20	451±35	455±35	465±16	429±23	523±57
Somatosensory cortex	-deep	393±38	397±32	392±39	432±23	409±13	501±28* #
	-superficial	429±18	448±34	458±28	488±22	447±15	552±23* #

mRNA expression is measured in pmoles of labeled $^{33}P/mm^2$ of tissue.
* $p < 0.05$ for differences from 4-month old behaviorally-characterized animals
# $p < 0.05$ for differences from 11-month old behaviorally-characterized animals
† $p < 0.05$ for differences from the naïve animals in same age group
deep = cortical layers IV-VI
superficial = cortical layers II-III

CHAPTER III

Expression of splice cassettes of NMDA receptor GluN1 subunits and memory performance in prefrontal cortex of mice during aging

Siba Ranjan Das and Kathy R. Magnusson

Molecular and Cellular Biology Program & Dept. of Biomedical Science, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA

Submitted for publication to:
Behavioral Brain Research
Elsevier, Publishers; 201 Mission Street, 26th Floor, San Francisco, CA 94105

ABSTRACT

Age-related decline in memory has been associated with changes in mRNA and protein expression of different NMDA receptor subunits. The NMDA receptor GluN1 subunit appears to be necessary and sufficient for receptor function. There is evidence that the mRNA expressions of some splice forms of the subunit are influenced by behavioral testing experience in old mice. The present study explored the relationships between behavioral testing experience and protein expression of different GluN1 subunit isoforms in the prefrontal/frontal cortex of the brain during aging. Aged C57BL/6 mice with behavioral testing experience showed declines in performance in both spatial working and reference memory tasks. Protein expression of GluN1 subunits containing C-terminal cassettes C2 or C2' were observed to decline with increasing age, regardless of experience. In middle-age animals, higher expressions of the GluN1 subunit and C2' cassette proteins were associated with good reference memory on initial search. In the aged animals, higher protein expression of GluN1 subunits containing C1 cassettes and the whole population of GluN1 subunits were found to be associated with better performance in the final phase of probe trials but this appeared to be due to perseveration or delays in applying an accurate search. These results indicate that there was heterogeneity in the effect of aging on the expression of the GluN1 subunits containing different splice cassettes. It also suggests that the GluN1 subunit might be most important for good reference memory during middle age.

Key words: NMDA; aging; NR1; Zeta1; GluN1; splice form, splice variant; cassette; water maze; learning index; memory; cumulative proximity

3.1 Introduction

Aging is one of the main factors associated with deficits in memory functions in various organisms. A type of memory characterized by the interaction of environment and organisms, spatial memory, has been shown to decline with increasing age in humans (Kirasic and Bernicki, 1990; Moore et al., 1984). Rodents experience similar declines in spatial memory abilities as humans and have been used as a model for age related memory decline (Barnes, 1988; Gage et al., 1984; Rapp et al., 1987). One type of excitatory glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor has been shown to be important for learning and memory, including spatial memory (see reviews: Magnusson, 1998b; Magnusson et al., 2010). These receptors are abundantly expressed in the frontal cortex and hippocampus (Jarvis et al., 1987; Kohama and Urbanski, 1997), regions responsible for encoding and retrieval of memory (see reviews: Fletcher and Henson, 2001; Martin and Clark, 2007). Antagonists of NMDA receptors inhibit memory performance (Alessandri et al., 1989; Mondadori et al., 1989; Morris, 1989; Morris et al., 1986) and block initiation of long-term potentiation (Bashir et al., 1991; Harris et al., 1984; Morris, 1989; Morris et al., 1986), indicating their importance in learning and memory. Aged individuals exhibit declines in NMDA receptor expression in prefrontal/frontal cortical regions (Kito et al., 1990; Magnusson, 1997; Tamaru et al., 1991). Declines in NMDA receptor expression in these regions that are observed during aging have been correlated with declines in memory ability (Magnusson, 1998a; Magnusson, 2001).

NMDA receptors are excitatory receptors on neurons, which form heteromeric channels composed of two GluN1 (earlier names: NMDAR1, NR1, GluN1) subunits and two other subunits, either from the GluN2 family (GluN2A-D, formerly ε1-4 in mice) or from the GluN3 family (GluN3A-B) (Collingridge et al., 2009; Furukawa et al., 2005; Laube et al., 1998; Premkumar and Auerbach, 1997; Ulbrich and Isacoff, 2008). Eight different splice variants of the GluN1 subunit have been identified in the brain. These are generated by alternative splicing of one N-terminal (Exon 5) and two C-terminal (Exons 21 and 22) cassettes in the mRNA (Anantharam et al., 1992;

Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992) (Fig. 1.6). The C2 cassette contains a translational stop codon and, in its absence, an additional sequence with the next stop codon becomes part of the mature mRNA known as the C2' cassette (Durand et al., 1992). The N1 cassette is present on the extracellular side of the receptor and C1, C2 and C2' cassettes are present on the cytoplasmic side (Hollmann et al., 1993; Sugihara et al., 1992). These cassettes have been shown to be involved in various functional aspects of the NMDA channel, such as zinc modulation, altering affinity for agonists and antagonists, and spatio-temporal expression in the brain (Durand et al., 1993; Hollmann et al., 1993; Traynelis et al., 1995).

Previous studies in our laboratory have shown that expression of some of the NMDA receptor subunits decline during aging in C57BL/6 mice (Magnusson, 2000; Magnusson et al., 2002). The GluN1 subunit has been shown to decline during aging (Magnusson et al., 2002) and to be associated with age-related memory decline in rodents (Magnusson et al., 2007). In other studies, however, no significant decline in GluN1 subunit expression with aging in the same strain of mice was observed (Magnusson, 2001; Ontl et al., 2004; Zhao et al., 2009). We have hypothesized that this is the result of heterogeneity in the effects of aging and/or behavioral testing experience on different splice variants. In C57BL/6 mice, mRNA of the $GluN1_{X11}$ splice form (containing both C1 and C2 cassettes) of the GluN1 subunit is more susceptible to aging changes than other C-terminal splice forms (Das and Magnusson, 2008; Magnusson et al., 2005). Old animals that underwent behavioral testing also show increased mRNA expression of splice forms lacking the N1 cassette ($GluN1_{0XX}$) and an exacerbation of the effects of aging on mRNA expression of $GluN1_{X10}$, one of the splice forms lacking the C2 cassette in prefrontal/frontal cortex (Das and Magnusson, 2008). In the present paper, we have focused on examining the effects of aging and experience in a behavioral task on protein expression of the different splice variants of the GluN1 subunit. Antibodies were not available for differentiating each of the GluN1 splice variants, so the investigation was confined to using antibodies raised against each of the four splice cassettes, N1, C1, C2, C2' and all GluN1 splice

variant proteins (GluN1). These were the same animals whose mRNA changes were reported previously (Das and Magnusson, 2008).

3.2 Materials and Methods

3.2.1 Animals

A total of 72 male C57BL/6 mice (National Institute on Aging, NIH) from three different age groups (four, eleven and twenty-six months of age) were used for the study. They were fed *ad libitum* and housed under 12 hr light and 12 hr dark cycle. The animals were randomly divided into two behavioral groups; naïve and behaviorally tested. Initially there were twelve animals in each age/behavioral group. Animals in the behaviorally tested group were subjected to a learning experience with the use of the Morris water maze during the 12 hr light cycle, as discussed below. The animals in the naïve group were housed for the same amount of time as the behaviorally tested animals. After the behavioral testing, all animals were euthanized with exposure to CO_2 and decapitated. The brains were then harvested, frozen rapidly with dry ice and stored at -80 °C until further processing.

3.2.2 Behavioral testing

Spatial reference memory, working memory and cued control task abilities were tested using the Morris Water Maze. A 1.2 m diameter metal tank was covered with white contact paper and filled with water that was made opaque white with non-toxic paint. A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth. The cues were placed high on the walls of both the room and the tank. There were seven different platform positions available at five different distances from the tank wall. Trials were video taped using a camera placed above the center of the tank on the ceiling of the room. Paths of the trials were analyzed by using the “SMART” video tracking system (San Diego Instruments, San Diego, CA, USA). There were different entry points for each trial and the mice were placed in the tank facing the wall.

3.2.2.1 Pretraining:

Pretraining was performed 2 days prior to reference memory training and consisted of each mouse swimming for 60 seconds in the tank without the platform. After all the mice were done with the swimming training, a platform was placed in a location not used for memory testing and the mice were trained to remain on the platform for 30 seconds. This procedure was also performed on the second day of pretraining.

3.2.2.2 Spatial reference memory:

On days 3 through 14, mice underwent reference memory testing. The task consisted of 3 place trials per day for 12 days with one additional probe trial every alternate day (Gallagher et al., 1993). The platform was kept in the same quadrant (NW) for each place trial and the start positions were randomly assigned (SE, NE and SW). Place trials consisted of 60 seconds maximum in the water searching for the platform, 30 seconds on the platform and 60 seconds of cage rest. If a mouse failed to find the platform within the designated 60 seconds, it was led to the platform by the experimenter. Assessment of the animal's ability to show a bias for the platform location was done by a probe trial as the third trial every other day (Gallagher et al., 1993). During the probe trial the platform was removed and the mice were allowed to search in the water for 30 seconds from a randomly assigned start position. Probe trials were followed by an additional place trial that was not analyzed.

3.2.2.3 Spatial working memory:

On days 15 through 24, mice were tested in a spatial working memory task (Magnusson et al., 2003). The task consisted of two sessions per day for 8 days. There was a two-day break between sessions eight and nine. The platform positions were changed between each session. Each session consisted of 4 trials. The first trial was a naïve trial (T_0) started by placing a mouse into an entry point and allowing it to search for the new platform position for a maximum of 60 seconds, after which the mouse was allowed to remain on the platform for 30 seconds, followed by cage rest for 10 minutes (delay period). In the second trial (T_{delay}) the mouse was placed in the water at

a different entry point from the naïve trial and allowed to search for the platform for a maximum of 60 seconds. The mouse was again allowed to stay on the platform for 30 seconds and allowed to rest in the cage for 60 seconds. The mouse was placed into the water 2 more times at 2 different entry points and allowed to find the platform for 60 seconds. They spent 30 seconds on the platform and rested in the cage for 60 seconds between trials. Mice were then placed into their cages until the next session, which started about 3 hrs from the beginning of the first session. If the mouse failed to find the platform within the designated 60 seconds for any of the trials, it was led to the platform by the experimenter. The entry points within one session were randomly assigned for each trial. Working memory was assessed between T_0 and T_{delay}. The extra sessions were performed based on previous findings that mice need additional trials to show improvement between trials (Magnusson et al., 2003).

3.2.2.4 Cued control task:

Cued trials were designed to test motivation, visual acuity, and physical ability for the task. On day 25, mice underwent 6 cued trials. The platform was kept submerged but was marked by a 20.3 cm support with a flag. For each cued trial, the platform was changed to a different position and the mouse was placed into the tank facing the wall at one of the entry points and was allowed to search for the platform for 60 seconds. All mice were tested at one platform position before the platform was moved to a new position.

3.2.3 Tissue subfractionation and protein isolation

Brains were hemisected along the plane of the longitudinal fissure. One half was dissected to obtain prefrontal/frontal cortices. Biochemical fractionation of the dissected tissue was performed as previously described (Dunah and Standaert, 2001) with a few modifications. Briefly, the tissue was homogenized in TE buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) plus 320 mM sucrose with the help of a Dounce homogenizer. The resulting homogenate was centrifuged at 1000 X g for 3 minutes using a Savant μSpeedFuge SFR13K refrigerated centrifuge with RSR20 rotor (Thermo Fisher Scientific, Waltham, MA, USA) and the pellet (P1) was

discarded. The supernatant (S1) was centrifuged at 9000 × g for 11 minutes using the same centrifuge to produce the crude synaptosome pellet, P2. The P2 was resuspended in TE buffer and sonicated. Protein determinations were made with Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.4 Western blotting

Sodium Dodecyl Sulfate - Poly Acrylamide Gel Electrophoresis (7.5%) was used for Western blotting and was performed as described previously (Magnusson et al., 2002). Each gel contained four different μg loads, viz. 1.5, 3, 6 and 12 μg/well, of standards obtained from crude synaptosomes prepared from combined caudal cortices from all the naïve young animals. Protein samples from different age/treatment groups were loaded on each gel to the left of standards and analyzed in triplicate. Gels were allowed to run for 2 hours at 125V. Proteins were transferred to Immobilon-FL Poly Vinylidene Flouride membranes (Millipore, Billerica, MA, USA) for 90 minutes at 100V at 4°C. Membranes were blocked for 1 hr at room temperature with shaking in 1:1 dilution of LI-COR Odyssey buffer (LI-COR Biosciences, Lincoln, NE, USA) and tris buffered saline (TBS). Membranes were then incubated overnight in one of the following primary antibodies (NMDAR1, Zymed Laboratories, San Francisco, CA, USA; N1, C2 and C2', Novus Biologicals, Littleton, CO, USA; C1, Sigma Aldrich, St. Louis, MO, USA) prepared in 1:1 dilution of LI-COR Odyssey buffer and TBS. After rinsing for four times, five minutes each, in TBS + 0.1% Tween20, membranes were incubated in fluorescence-based secondary antibody (Alexa Fluor 680, LI-COR Biosciences; IR Dye 800, Rockland Immunochemicals, Gilbertsville, PA, USA) for one hour. Bands were viewed by scanning in the LI-COR Odyssey imager.

3.2.5 Data analysis

Data for reference and working memory tests were analyzed as described earlier with a few modifications (Das and Magnusson, 2008). Briefly, the distance of the animal from the platform (proximity to platform) was measured every 0.2 seconds by the computer for the whole duration of the trial. These proximities were added together to generate a cumulative proximity for the trial. Correction for start position

was performed using a macro in Excel software (Microsoft Corp., Seattle, WA, USA). A cumulative proximity measurement for the ideal path using the start position, average swim speed and platform position was calculated with the help of this macro. This cumulative proximity measure for the ideal path was subtracted from the cumulative proximity score for the whole track to obtain the corrected cumulative proximity scores for the place trials in reference memory tasks, all trials in working memory tasks and the cued control tasks. For the probe trials of the reference memory tasks, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score. Analysis of probe trials was done in two ways; first by calculating corrected cumulative proximity using data from full 30-second probe trials and then by dividing each probe trial into three intervals of 10 seconds each and calculating uncorrected cumulative proximities of each of the three intervals. Cumulative proximity measurements for the three intervals were not corrected because the second and third starting positions in the intervals were dependent on the animals' search pattern and correction for start position did not seem appropriate. The cumulative proximity measurements were averaged across the 30s or 10s trials to give average proximity. Learning index was then calculated from the average proximity measurements generated by the two different analyses as described earlier (Gallagher et al., 1993). Briefly, a multiplier for each probe was obtained by dividing the mean average proximity measure of the young naïve animals from a given probe trial day by that of the first probe trial day. This factor was then multiplied by each animals average proximity measure for that day. The resulting values for each individual animal were added across the probe trials to obtain learning index scores. The ratios of naïve to delayed trials were calculated from their respective corrected cumulative proximity measurements in the working memory tasks.

Protein blots were analyzed using Li-Cor Odyssey software version 1.1. Integrated intensity measures were recorded with the help of the software using median background subtraction method. A standard curve was obtained using a linear

fit with Prism software version 4.0 (GraphPad Software Inc., La Jolla, CA, USA) from the observed integrated intensity values for known loads of caudal cortex. Sample values were interpolated from the standard curve. Statistical analyses for both behavioral trials and protein expression were done with analysis of variance (ANOVA) followed by Fisher's protected least significant difference using Statview software version 5.0.1 (SAS Institute, Cary, NC, USA). If no significant effect of behavioral testing was observed on protein expression of the individual splice cassettes, results from the two behavioral groups were averaged together for further analysis.

Pearson's correlation coefficients were calculated to assess the relationship between different protein expressions of different cassettes of the GluN1 subunit and reference (learning index) and working memory (T_0/T_{delay}) separately in 4, 11 and 26 month old mice. To correct for the number of comparisons, a recently developed method, p_ACT version 1.0 (Conneely and Boehnke, 2007) was used and run using R statistics software version 2.6.1 (RDevelopmentCoreTeam, 2007) with the package mvtnorm version 0.8.1 (Genz et al., 2007). This method adjusts p-values of different correlation tests sequentially and is based on a procedure described previously (Holm, 1979). The correction was applied by comparing the protein expression of each different cassette with all the tests of memory performance separately for each age group.

3.3 Results

3.3.1 Spatial memory characterization

A significant main effect of age was observed in the place trials of the reference memory task ($F_{(2,30)} = 7.1$, $p = .003$). Twenty-six month old mice had significantly higher cumulative proximity scores averaged across all trials than both younger ages (Fig. 3.1A). There was a significant main effect of age on the learning index scores of the full 30-second probe trials averaged across all probe trials ($F_{(2,30)} = 4.95$, $p = .01$). Both the eleven month old ($p = .05$; Fig. 3.1B) and twenty-six month

old ($p = .004$; Fig. 3.1B) mice had significantly higher learning index scores as compared to the 4 month olds in the full 30-second probe trials.

For analyzing persistence and flexibility, probe trials were divided into 3 intervals of 10 seconds each and analyzed separately. A significant main effect of age ($p = .001$) was observed only in the first 10-second interval of probe trials but not on the second or third 10-second intervals (Fig. 3.1B). Learning index scores in the four month olds were significantly lower than those of the eleven ($p = .003$, Fig. 3.1B) and twenty-six ($p < .001$) month olds in the first 10-second interval of probe trials. There was a significant main effect of age on the learning index scores in probe trials collapsed across the different intervals ($F_{(2,95)} = 4.84$, $p = .01$) and a significant main effect of intervals collapsed across different ages ($F_{(2,95)} = 8.25$, $p < .001$). There was no significant interaction between age and intervals, but since the comparisons between individual intervals was a part of our experimental plan, performance of each age group between the intervals was analyzed individually. Four month old mice did not have a significant change in learning index scores between first and second interval, but had significantly higher scores in the third as compared to the second probe trial interval (Fig. 3.1B, $p = .025$). Learning index scores in the 11-month old animals were significantly lower in the second interval as compared to the first ($p = .04$, Fig. 3.1B) and third ($p = .03$, Fig. 3.1B) intervals. The twenty-six month olds had significantly lower learning index scores in the second interval ($p = .04$, Fig. 3.1B) as compared to the first, but did not change significantly in the third interval ($p = .08$, Fig. 3.1B).

A significant main effect of age was observed in both the naïve and delayed trials of working memory tasks ($F_{(2,63)} = 11.36$, $p < .001$). The 11 and 26 month old mice had significantly higher cumulative proximity scores than the 4 month olds in naïve trials of working memory tasks (Fig. 3.1C). In the delayed trials of working memory tasks, the 26 month old mice had significantly higher cumulative proximity scores than both the 4 and 11 month old mice (Fig. 3.1C). Significantly lower cumulative proximity scores in the delayed trials, as compared to the naïve trial, in the

working memory task were observed in 4 and 11 month old mice, but not in the 26 month olds (Fig. 3.1C). There was a larger ratio of the naïve over delayed trials in 11 month old mice as compared to the 26 month olds (Fig. 3.1D).

The cued control task was designed to test motivation, motor ability and visual acuity among animals used in the study. Cumulative proximity scores of three 26 month old mice in the cued trials were two standard deviations higher than the mean of the animals included in behavioral testing and so were excluded from all the analysis reported in this study (not shown). Remaining mice showed no significant difference in the cumulative proximity scores in the cued trials between the different age groups of animals included in the study (Fig. 3.1E). Performance of mice in all the platforms except for the north was lower than the cumulative proximity scores observed during both reference and working memory trials (Fig. 3.1E).

The young and middle-aged mice were observed to have significantly faster swim speeds than the old mice in the place trials ($p < 0.001$, Table 3.1) of the reference memory task. In the naïve trials for working memory, swim speeds of the 11 month old mice were significantly faster than the 26 month old mice and that of the 4 month old mice were significantly faster than both 11 and 26 month old mice in naïve trials across the sessions ($p < .001$, Table 3.1). Swim speeds of the 4 and 11 month old mice across all the platform positions in cued tasks were significantly faster ($p < .001$, Table 3.1) than the 26 month olds.

3.3.2 Protein expression

3.3.2.1 GluN1 subunits, N1 and C1 cassettes:

Analysis of expression of a protein sequence that is common to all the splice variants (GluN1) showed no main effect of behavioral testing experience ($F_{(1,62)} = .001$, $p = .98$; Fig. 3.2A). There was also no effect of age on the protein expression of GluN1 when the data were collapsed across the two behavioral groups ($F_{(2,65)} = .7$, $p = .49$; Fig. 3.2A). No significant main effect of behavioral treatment was observed on the protein expression of the N1 cassette ($F_{(1,62)} = 1.0$, $p = .32$; Fig. 3.2B), which is present on the extracellular side of the plasma membrane, or the C1 cassette ($F_{(1,62)} =$

1.1, p = .29; Fig. 3.2C), which is present on the intracellular side of the plasma membrane. There was no significant main effect of age on protein expression of N1 ($F_{(2,65)}$ = 1.4, p = .26; Fig. 3.2B) or C1 ($F_{(2,65)}$ = 1.7, p = .19; Fig. 3.2C) cassettes when the data were collapsed across the two behavioral groups.

3.3.2.2 C2 and C2' cassettes:

There was no significant main effect of behavioral testing experience on protein expression of the intracellular C2 ($F_{(1,62)}$ = 0.02, p = .88; Fig. 3.2D) or C2' cassettes ($F_{(1,62)}$ = 1.7, p = .19; Fig. 3.2E). A significant main effect of age was observed on the GluN1 subunits containing C2 ($F_{(2,65)}$ = 3.8, p = .03; Fig. 3.2D) and C2' ($F_{(2,65)}$ = 4.8, p = .01; Fig. 3.2E) cassettes. A reduced expression of GluN1 subunits containing C2 (p = .008, Fig. 3.2D) or C2' (p = .003, Fig. 3.2E) cassettes was observed in 26 month old mice as compared to the 4 month olds when the data were collapsed across behaviorally tested animals.

3.3.3 Relationships between protein expression and reference memory

Pearson's correlation coefficients were calculated for the learning index scores of the full 30-second probe intervals and the three 10-second intervals of probe trials for reference memory and the T_0/T_{delay} ratios for working memory with the protein expression of the different cassettes of the GluN1 subunit in different age groups of mice. Significant negative relationships were observed in the 26 month old animals between performance in the third interval of the probe trials of reference memory and protein expression of GluN1 subunit as a whole (Fig. 3.3C, Table 3.2) and GluN1 subunits containing the C1 cassette (Fig. 3.3F, Table 3.2). Since higher learning index scores indicate poorer performance, the presence of a negative relationship indicated an association of good performance in the third interval with higher levels of expression of all GluN1 subunits and subunits containing C1 cassettes. There was no such significant relationship in the 4 (Fig. 3.3A) and 11 (Fig. 3.3B) month old animals between all GluN1 subunits (Fig. 3.3A, B) or cassettes (not shown) and learning index in the third interval. The 26 month old mice showed no significant relationship between all GluN1 subunits (not shown) and the C1 cassettes (Fig. 3.3D-E) and

learning index in the first (Fig. 3.3D) or second interval (Fig. 3.3E) of the probe trials. The correlation coefficient for the second interval, although not significant, was positive (Fig. 3.3C) whereas the significant correlation in the third interval was negative (Fig. 3.3F).

The 11 month old animals showed significant negative relationships between the learning index for the first 10-second interval of the probe trials and protein expression of all GluN1 subunits (Fig. 3.4B, Table 3.2) and the GluN1 subunits containing the C2' cassette (Fig. 3.4E, Table 3.2). The four (Fig. 3.4A, D) and twenty-six (Fig. 3.4C, F) month old animals did not show any significant linear relationship between protein expression and performance in the first interval of the probe trials. No significant relationship between the working memory ratio (T_0/T_{delay}) and expression of splice cassettes was observed in any of the age groups.

3.4 Discussion

This study provides evidence for a heterogeneous effect of aging on the expression of the N and C-terminal cassettes of the GluN1 subunit of the NMDA receptor in the prefrontal/frontal cortex. Protein expressions of two C-terminal cassettes, C2 and C2', of the GluN1 subunit of NMDA receptors were observed to be affected negatively by aging. All GluN1 subunits and GluN1 subunits containing N1 or C1 cassettes, did not appear to be influenced by aging or behavioral testing experience. Deficits in reference and working memory abilities were observed in the old compared to the young and middle-aged mice. High expressions of all GluN1 subunits as a group and those containing the C1 cassettes were associated with good performance in the last phase of the probe trials for reference memory in the old mice. High expression of all GluN1 subunits and those containing C2' cassettes were associated with good performance in the initial phase of probe trials for reference memory in middle-aged animals, but this relationship was not maintained in the aged mice.

3.4.1 Age-related changes in memory

Old mice in the present study were observed to have memory deficits in both the reference and working memory tasks as compared with the young and/or the middle-age mice. This was also observed in our previous studies (Magnusson, 2001; Magnusson et al., 2003; Magnusson et al., 2007). In the place trials of the reference memory tasks, 26 month old mice were observed to have higher cumulative proximity scores than both the four and eleven month olds, suggesting poorer spatial reference memory in older mice, as compared to the young ones. Similar observations were reported in our earlier studies involving 12 days of reference memory tasks in C57BL/6 mice (Magnusson, 1998a; Magnusson, 2001; Magnusson et al., 2007). Other labs working on cognitive decline of mice have observed a similar trend in old mice performing reference memory tasks (Frick and Fernandez, 2003; Harburger et al., 2007). The probe trials, which are designed to illustrate the developed spatial bias, also indicated a decline in reference memory by 26 months of age. This result was observed in both the direct average proximity measurements (not shown) and the graded learning index scores. During the working memory tasks, older animals were observed to perform worse, as compared to the young animals, both in the naïve and delayed trials. Better performance of the young, as compared to older mice, in the naïve trials suggests that they may have had a better search strategy than the older mice. Absence of improvement in the delayed trials from the naïve trials indicated a deficit in working memory in 26 month old mice. Similar results were obtained in our previous experiment with C57BL/6 mice where young and middle-aged mice performed well in the delayed working memory trials but the old mice were impaired (Magnusson et al., 2003).

Our lab has observed a progression of behaviors in mice during probe trials, consisting of an initial phase of approaching the platform location, followed by repeated search on or around the former platform location and then a widening of search for the platform in other parts of the tank (unpublished observation). Similar observations were made by Maei and coworkers, who described a peak in search

accuracy in mice by 10-15 seconds during a probe trial and decline thereafter (Maei et al., 2009). They reasoned the decline as an inclination of mice to search elsewhere when the platform is absent in the original location. In an effort to separate these different phases and to determine whether perseverance in mice can be attributed to NMDA receptors, we divided the full 30-second probe trials into three intervals of 10 seconds each. Our data from the young mice suggests that the first phase included the initial approach to the learned platform position, the second phase may have reflected persistence and the third phase may have indicated a loss of perseverance in young mice.

Relationships between different ages in learning index scores of the first 10-second interval of the probe trials were similar to the full probe trial showing higher scores in the old mice than the younger ones. Twenty-six month old mice in the first 10-second interval spent more time away from the platform compared to the young mice, suggesting that there was more difficulty remembering the platform location for the old than the young. The old mice did spend significantly more time near the platform location during the second 10-second interval, as compared to the first 10-second interval. This could indicate some persistence of memory in the second interval or may be related to the slower swim speed impacting the first interval more than the second. The young and middle-aged mice showed a loss in persistence by changing the strategy to search for the platform at different locations as evidenced by a significant increase in learning index scores in the third 10-second interval as compared to the second 10-second interval. Dean and coworkers have described a deficit in memory ability of aged mice and attributed it to increased perseveration in old mice (Dean et al., 1981). Prefrontal cortex has been identified as a brain region responsible for increases in perseveration in humans, primates and other rodents (Clarke et al., 2004; Hampshire et al., 2008; Head et al., 2009; Schwabe et al., 2004). Although the old mice did not show a significant worsening of performance in the third versus the second 10-second interval, there was not strong evidence of performance perseveration in the aged mice as a group in this study.

In the cued task, all the mice were able to improve their performances except the three old mice that were subsequently removed from the study. Following this removal, all of the groups performed similarly in their cued tasks throughout the trials. This indicated that motor control, vision and/or motivation were not issues for the mice used in the study. Swim speed differences between the old mice and younger mice were seen in all tasks, including the cued task. The lack of significant differences between different ages in cued trial performance suggest that using the proximity measures and correcting for start position helped to diminish the influence of swim speed differences on the performance measures. The learning curves for place and probe trials looked different than for cued trials indicating that mice performed differently when they could see the platform. Thus, inability of mice to reach the target was not due to the lack of motivation to find the escape platform and difficulty in their motor or visual ability to differentiate between various spatial cues (for detailed results see Das and Magnusson, 2008).

3.4.2 Age-related changes in GluN1 subunit protein expression

The whole population of GluN1 subunits in the present study did not show any change in protein expression pattern with age or behavioral testing experience. Protein expression of the GluN1 subunit in some of our other studies also showed no effects of the aging process (Ontl et al., 2004; Zhao et al., 2009). Major differences between the current study and other studies showing changes in GluN1 subunit expression with age (Magnusson et al., 2002) are the ages of the oldest mouse group (30 mo. in Magnusson et al. (2002) vs. 26 months in the present study) and use of different methods for normalization for loading in the protein gels (µg protein loaded in Magnusson et al. (2007) vs. GAPDH in the same lane in the current study). The N-terminal cassette, N1, and C-terminal cassette, C1, also did not show any change in protein expression with age in the current study, although they had trends for declining expression with increasing age.

GluN1 splice variants containing C-terminal cassettes C2 or C2' in the current study showed declines with increasing age across the two behavioral groups. Similar

decline in protein expression of NR1 subunits containing C2 cassette, but not the C2' cassette, was observed in hippocampus of 24 month old rats as compared to the 6 month olds (Clayton and Browning, 2001). The C-terminal tail of the GluN1 subunit of the NMDA receptor has been suggested to be important for several functions including retention and export from the endoplasmic reticulum (Mu et al., 2003; Scott et al., 2001; Standley et al., 2000), assembly and trafficking (Mu et al., 2003; Standley et al., 2000), long-term stabilization of synapses and spines (Alvarez et al., 2007) and phosphorylation of the subunit by PKA and PKC (Durand et al., 1992; Tingley et al., 1997). The C2' cassette contains a signal responsible for export of the GluN1 subunit from the endoplasmic reticulum (Mu et al., 2003; Standley et al., 2000). Maintenance of normal spine density and stabilization of synapses in rat pyramidal neurons depends on the expression of C2 cassettes (Alvarez et al., 2007). Potentiation due to phosphorylation of GluN1 subunits of NMDA receptors depends on the presence or absence of C1 (Tingley et al., 1997) or C2 cassettes (Durand et al., 1992). Clustering of receptors depends on the interaction of proteins yotiao and neurofilament L with C1 cassette (Ehlers et al., 1998; Lin et al., 1998). Splicing out of the C2 cassette results in seven-fold increase of potentiation of NMDA receptors by PKC (Durand et al., 1992). Thus, reduction in expression of the GluN1 subunits containing the C2 cassette may result in altered phosphorylation state of the receptor due to the loss of phosphorylation sites. It may also result in instability of the synapses dominated by the NMDA receptor in the prefrontal/frontal cortex. These studies suggest that the C-terminal tail of the NMDA receptor subunits, specifically expression levels of the C2 and C2' cassettes, are important regulators of the GluN1 subunit of the NMDA receptor. Changes in expression of these cassettes during aging could alter plasticity and morphology of neurons.

Since the whole population of GluN1 subunits did not show any change in expression with age and the presence of C2' cassette on a GluN1 subunit excludes the presence of C2 cassette and vice versa, an opposite trend in expression was expected between the GluN1 subunits expressing C2 and C2' cassettes. GluN1 subunits

containing C2 or C2' cassettes, however, were both observed to show reduced expression with increasing age. One possible explanation for this could be due to change or damage to the C-terminal cassettes in the aged brain that does not result in removal of the whole subunit protein.

The mRNA expression of different splice forms of the GluN1 subunit in the same animals was reported previously (Das and Magnusson, 2008). mRNA expression of the $GluN1_{X11}$ (containing C1 and C2 cassettes) splice form showed overall declines with age and mRNA of $GluN1_{X10}$ (contains C1 and C2' cassettes) splice form showed declines with age only in behaviorally-characterized animals (Das and Magnusson, 2008). The protein expression declines in C2 and C2' cassettes thus may be due to mRNA changes. In addition, there was a trend for decline in the C1 protein expression in aged mice in this study. Absence of any difference between the behaviorally characterized and naïve animals in the current study could be due to the fact that the mRNAs for GluN1 splice forms were measured in individual prefrontal/frontal cortical regions, whereas the proteins were analyzed in crude synaptosomes from whole prefrontal/frontal cortex. In addition, the major effect of behavioral testing on mRNA expression was in the $GluN1_{0XX}$ splice form. In the present study, however, we could not measure the specific expression of a splice variant lacking the N1 cassette because of the lack of availability of a primary antibody to identify it. Overall, these results support a differential effect of aging on both mRNA and protein expression of different cassettes of the GluN1 subunit of the NMDA receptor.

3.4.3 Relationships between memory and GluN1 subunit protein expression

Significant relationships were found between GluN1 subunit expression and performance within intervals of probe trials for reference memory, but there were no significant relationships between either performance in a working memory task or in full probe trials for reference memory and protein expression of the GluN1 subunit splice cassettes or all GluN1 subunits in any age groups of mice. When individual 10-second probe trial intervals were analyzed, higher expression of all GluN1 subunits and GluN1 subunits containing C1 cassettes ($GluN1_{X10}$ and $GluN1_{X11}$) was observed

to be associated with better performance in the third interval of the probe trials in old animals. This is the only age group that showed a significant relationship in this final interval. In examining the significant correlations in the final interval alone, it appeared that the old mice with high protein expression of GluN1 subunits as a whole and those with the C1 cassette in the prefrontal/frontal cortex showed more perseverance that those with lower expression levels. However, examination of the aged mice within all three intervals showed that the mice with higher expression of all GluN1 subunit and C1 cassette proteins were the worst performers within the second interval, the interval in which the aged mice, on average, performed the best. This suggests that high expression of GluN1 subunits and C1 cassettes may have been associated with a delay in applying an accurate search strategy. The aged mice with lower expression levels of the GluN1 subunit and C1 cassettes appeared to show a loss of perseveration between the second and third interval. This suggests that the GluN1 subunit within the prefrontal/frontal cortex of older individuals may contribute to a less than optimal search strategy when the platform is absent, either through perseveration or a delay in accurate searching. The old animals with lower amounts of all GluN1 subunits and GluN1 subunits containing C1 cassette appeared to show a loss in perseveration by the third interval as compared to the second interval of the probe trial.

A positive relationship was observed in the middle-aged animals between performance in the first interval of the probe trial and higher protein expressions of all GluN1 subunits and subunits containing the C2' cassette ($GluN1_{X10}$ and $GluN1_{X00}$). This relationship was not maintained in other age groups. This suggests that the GluN1 subunit in the prefrontal/frontal cortex played a role in enhancing accuracy of the initial approach to the platform. This role appeared to be most important in the middle-aged mice. These correlational results suggest that the NMDA receptors in aged individuals did not function the same as in the middle-aged.

In conclusion, this study provides evidence for heterogeneity in expression of the GluN1 subunits with different splice cassettes during aging. Expression of C-

terminal cassettes C2 and C2' were reduced with increasing age, but were not affected by behavioral testing experience. Other splice cassettes showed no significant effect of aging. NMDA receptors in the prefrontal/frontal cortex of old mice appeared to be important for performance in the final phase of searching in a spatial reference memory task, but may have contributed to a suboptimal search strategy. In contrast, it appeared that middle-aged mice benefited more from the GluN1 subunit for good reference memory than both younger and older mice, suggesting that there is a change in the importance or role of NMDA receptors across aging.

Acknowledgements

Funding sources: This research was supported in part by NIH grants AG016322 (KRM) & P20RR16454-02 (Idea Network of Biomedical Research Excellence, Idaho).

Fig. 3.1 – Memory performance of mice in different behavioral tasks. Graphs above show cumulative proximity scores of mice averaged across 12 days of place trials (A) and learning index scores calculated from the full 30s and three 10s intervals of probe trials (B) for the reference memory tasks; cumulative proximity scores for naïve (T_0) and delayed (T_{delay}) trials (C) and the ratio T_0/T_{delay} (D) averaged across all working memory sessions, and cued control trials at the 6 different platform positions (E) for the 4, 11 and 26 month old mice. * $p < .05$ for differences from 4-month old animals for the same trials, # $p < .05$ for differences from 11-month old animals for the same trial(s), ¢ $p < .05$ for difference from the first interval of probe trial in the same age group, § $p < .05$ for difference from the second interval of probe trial in the same age group, † $p < .05$ for differences from overall performance in naïve trials (T_0) within same age group. A-C, E) Data indicate mean±SEM. Statistical analysis performed with ANOVA and Fisher's LSD post-hoc tests.

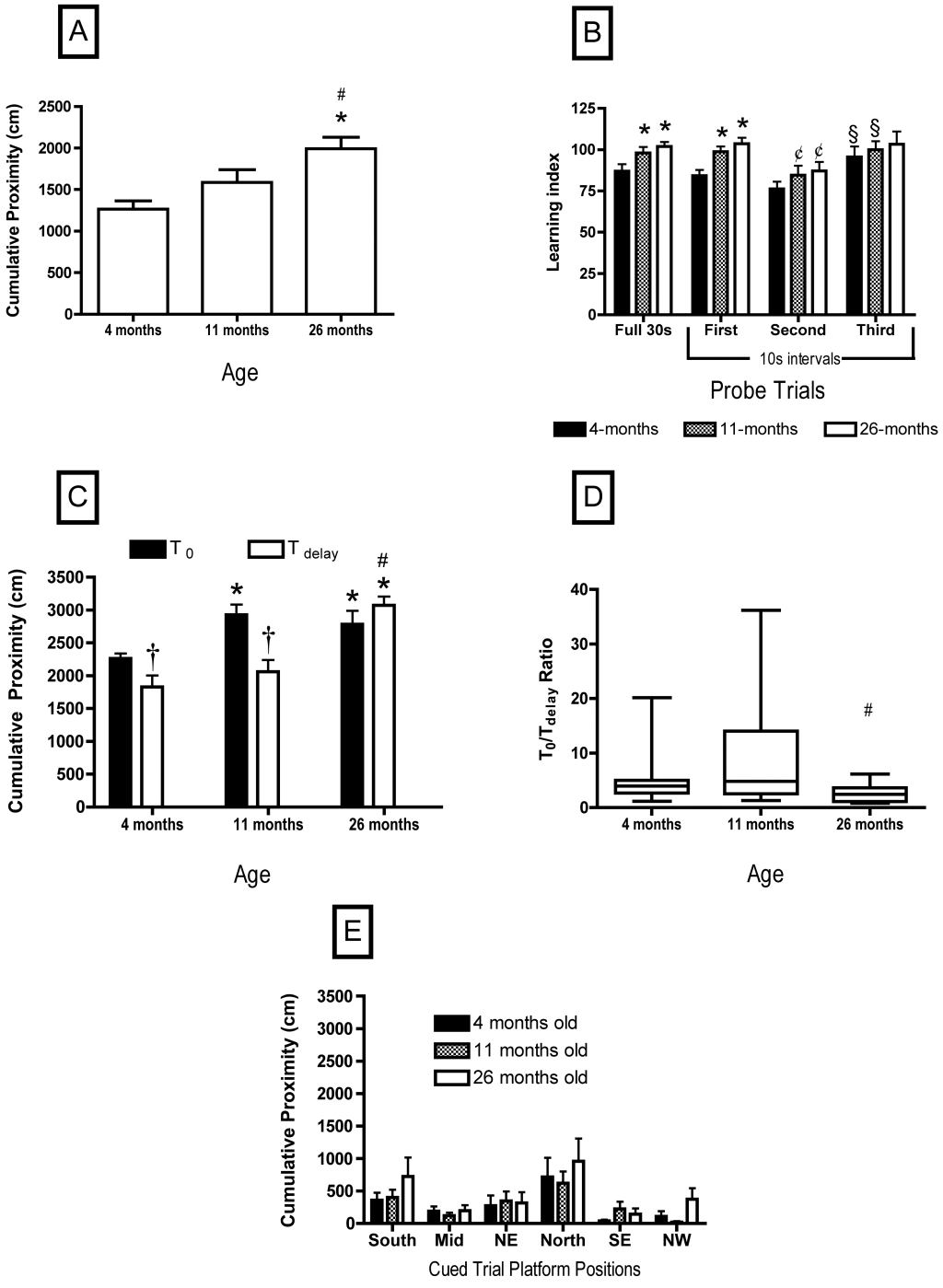
A
Cumulative Proximity (cm)
2500
2000
1500
1000
500
0
#
*
4 months
11 months
26 months
Age
B
Learning index
125
100
75
50
25
0
*
*
*
*
¢
¢
§
§
Full 30s
First
Second
Third
10s intervals
Probe Trials
4-months
11-months
26-months
C
T 0
T delay
Cumulative Proximity (cm)
3500
3000
2500
2000
1500
1000
500
0
†
†
*
*
*
#
4 months
11 months
26 months
Age
D
T0/Tdelay Ratio
40
30
20
10
0
#
4 months
11 months
26 months
Age
E
Cumulative Proximity (cm)
3500
3000
2500
2000
1500
1000
500
0
4 months old
11 months old
26 months old
South
Mid
NE
North
SE
NW
Cued Trial Platform Positions

Fig. 3.1

Fig. 3.2 – Protein expression of all NMDA receptor GluN1 subunits and the GluN1 subunits containing the N or C-terminal cassettes. A-E) Graphs showing protein expression of all GluN1 splice variants together (A) and GluN1 subunits containing N-terminal cassette N1 (B) or C-terminal cassettes C1 (C), C2 (D) or C2' (E) in prefrontal/frontal cortical regions of mouse brain. (F) Representative images of the blots used for quantification of the above-mentioned cassettes and all GluN1 subunits. GAPDH was used as a loading control and data were normalized with the amount of GAPDH expressed in each lane. * $p < .05$ for difference in expression of protein from 4-months old animals when

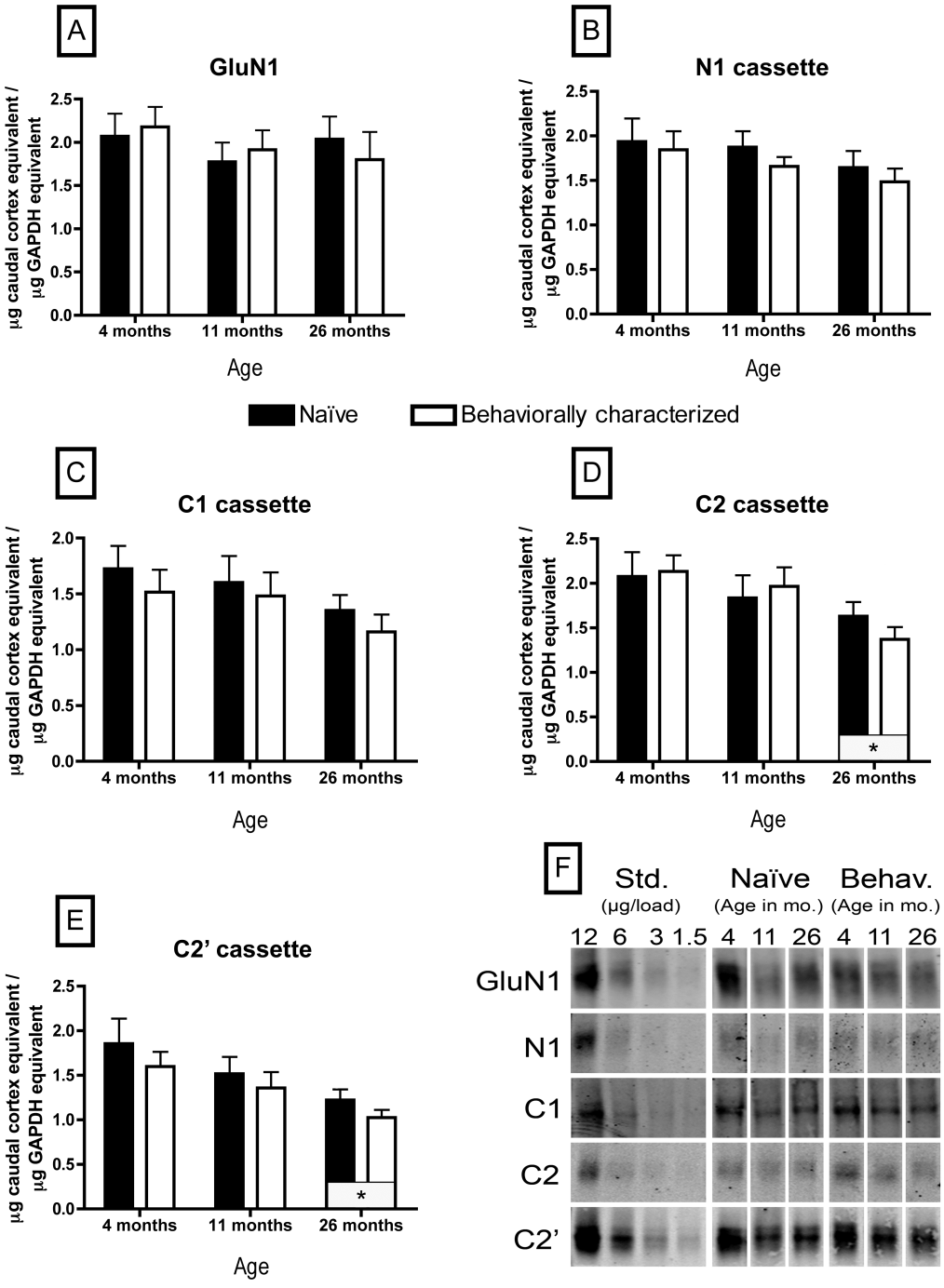
A
GluN1
µg caudal cortex equivalent / µg GAPDH equivalent
2.5
2.0
1.5
1.0
0.5
0.0
4 months
11 months
26 months
Age
B
N1 cassette
µg caudal cortex equivalent / µg GAPDH equivalent
2.5
2.0
1.5
1.0
0.5
0.0
4 months
11 months
26 months
Age
Naïve
Behaviorally characterized
C
C1 cassette
µg caudal cortex equivalent / µg GAPDH equivalent
2.0
1.5
1.0
0.5
0.0
4 months
11 months
26 months
Age
D
C2 cassette
µg caudal cortex equivalent / µg GAPDH equivalent
2.5
2.0
1.5
1.0
0.5
0.0
*
4 months
11 months
26 months
Age
E
C2' cassette
µg caudal cortex equivalent / µg GAPDH equivalent
2.5
2.0
1.5
1.0
0.5
0.0
*
4 months
11 months
26 months
Age
F
Std.
(µg/load)
Naïve
(Age in mo.)
Behav.
(Age in mo.)
12 6 3 1.5 4 11 26 4 11 26
GluN1
N1
C1
C2
C2'

Fig. 3.2

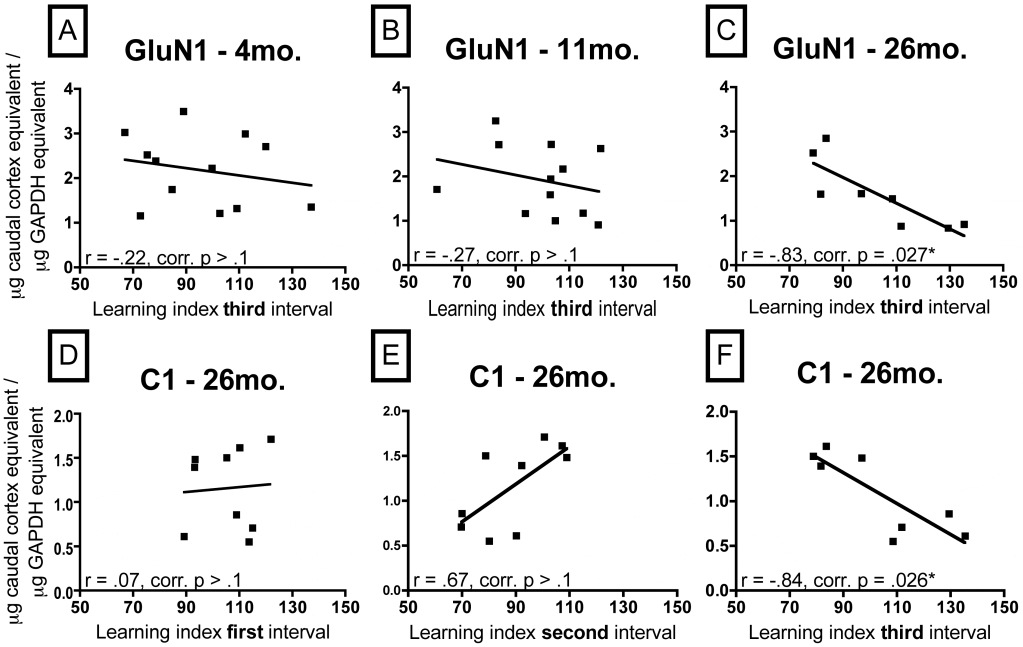

Fig. 3.3 – Relationship between learning index from probe trial intervals and protein expression of all GluN1 splice variants and the GluN1 subunits containing C1 cassettes. Graphs show relationships between protein expression of all GluN1 subunits (A-C) in prefrontal cortex of 4 (A), 11 (B) and 26 month (C) old animals and learning index in the third 10-second interval across probe trials or between protein expression of C1 cassettes (D-F) in prefrontal cortex of 26 month old mice and learning index in the first (D), second (E) or the third (F) 10-second interval of the probe trials in a reference memory task. The p-values were corrected by p_ACT statistical method (corr. p).

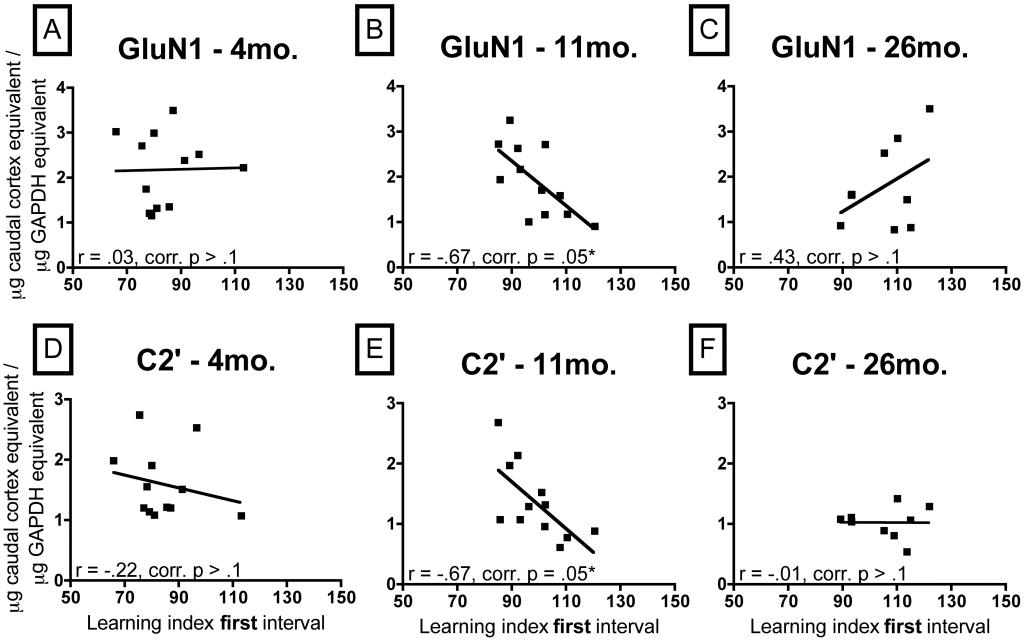

Fig. 3.4 – Relationship between learning index from the first 10-second interval in probe trials and protein expression of all GluN1 splice variants (A-C) and the GluN1 subunits containing C2' cassettes (D-F). Graphs showing relationship between protein expression of all GluN1 subunits (A-C) or C2' cassettes (D-F) in prefrontal cortex of 4 (A, D), 11 (B, E) and 26 (C, F) month old mice and learning index in the first 10-second interval of the probe trials in a reference memory task. The p-values were corrected by p_ACT statistical method (corr. p).

Table 3.1 - Swim speeds of mice of different age groups in different behavioral tasks.

Behavioral task	Age		
	4mo. (cm/s)	11mo. (cm/s)	26mo. (cm/s)
Reference memory (Place trials)	7.23±0.28	6.71±0.28	5.09±0.14*†
Working memory (Naïve trials)	8.15±0.32	7.03±0.28*	5.77±0.28*†
Cued task (All trials)	8.36±0.42	7.92±0.40	6.35±0.36*†

mo., months of age, * $p < .05$ for difference from the 4 month old animals. † $p < .05$ for differences from the 11 month old animals. Data indicate mean±SEM.

Table 3.2 - Pearson's correlation coefficients of the relationship between the performance of each age group of mice in different measures of reference and working memory tasks and expression of the whole GluN1 subunit and GluN1 subunits containing different N and C terminal cassettes.

	GluN1			N1			C1			C2			C2'		
	4mo.	11mo.	26mo.	4mo.	11mo.	26mo.	4mo.	11mo.	26mo.	4mo.	11mo.	26mo.	4mo.	11mo.	26mo.
Reference memory - Probe trials															
Full 30s	-.09	-.27	-.44	.30	-.31	-.51	-.06	-.16	-.56	.15	-.11	.25	.02	-.36	-.05
1st 10s interval	.03	-.67*	.43	.29	-.36	.50	.004	-.49	.07	-.35	-.36	.25	-.22	-.67*	-.01
2nd 10s interval	-.003	.14	.57	.15	-.23	.06	.18	.06	.67	.42	.05	.19	.20	-.13	.64
3rd 10s interval	-.22	-.27	.83*	.23	-.09	-.63	-.28	-.10	-.84*	.12	-.05	.20	-.03	-.14	-.32
Working memory															
T_0/T_{delay}	.015	-.20	.37	.45	.02	.47	-.10	-.05	.04	-.31	-.50	-.21	-.29	-.38	-.16

* corrected $p \leq .05$ for Pearson's correlation coefficient. mo., months of age, T_0, naïve trial, T_{delay}, delayed trial

CHAPTER IV

Reduction of GluN1$_{0XX}$ (1-a) splice variants of NMDA receptor impair spatial reference memory in mice

Siba R. Das and Kathy R. Magnusson

Molecular and Cellular Biology Program & Dept. of Biomedical Science, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA

Manuscript in preparation

ABSTRACT

GluN1 subunits of the NMDA receptor shows age-related changes in expression pattern, some of which correlate with spatial memory performance in mice. Aged C57BL/6 mice show an age-related increase in mRNA expression of one of the splice forms of the GluN1 subunit, $GluN1_{0XX}$ (GluN1-a). This increase in expression is associated with good performance in reference and working memory tasks. The present study was undertaken to determine if the $GluN1_{0XX}$ splice form is required for good performance in reference memory tasks in young mice. Mice were injected with either siRNA specific for $GluN1_{0XX}$, control siRNA or vehicle alone bilaterally into ventro-lateral orbital regions using a stereotaxic apparatus. A fourth group of mice did not receive any injections. Five days post-injection, mice were tested for their performance in spatial reference and associative memory tasks over 4 days using the Morris water maze. There was a 10-19% reduction in $GluN1_{0XX}$ splice variant expression for mice after siRNA treatment in ventro-lateral orbital regions of the brain. Declines in performance within the first half of reference memory testing were seen in the mice receiving siRNA against the $GluN1_{0XX}$ splice form, as compared to the mice injected with control siRNA and/or vehicle. These results suggest an important role of the $GluN1_{0XX}$ splice variant in orbital regions for early acquisition and/or consolidation of spatial reference memory.

4.1. Introduction

The aging process has been shown to cause functional declines in many different processes (Salthouse, 2003). Memory is one of the functions that is affected early in the aging process. One type of memory, spatial memory, which allows organisms to orient and interact within three-dimensional space, is particularly affected by the aging process (Gallagher et al., 2003; Penner and Barnes, 2007). Brain regions that are important for acquisition, consolidation and retrieval of spatial memory include the prefrontal cortex and hippocampus (Gallagher et al., 2003; Greenwood, 2000; Tisserand and Jolles, 2003). Animals, such as rodents and primates, have been used to model different aspects of spatial memory involving both the hippocampus and prefrontal cortex (Barnes, 1988; Gage et al., 1984; Rapp et al., 1987). The prefrontal cortex has also been shown to be important for flexibility of learning within different memory tasks (De Bruin, 1994; Li et al., 1997). The aging process hampers performance in reversal tasks, which are used to assess flexibility (Barense et al., 2002; Leite-Almeida et al., 2009).

Prefrontal cortex and hippocampus have a high concentration of N-methyl-d-aspartate (NMDA) receptors, a type of glutamate receptor involved in learning and memory (Bockers et al., 1994; Scherzer et al., 1998). NMDA receptors are particularly involved in spatial memory (Morris and Davis, 1994). The NMDA receptor also is involved in the flexibility of animals to adapt a new platform location during reversal tasks (Nicolle and Baxter, 2003). NMDA receptors are heteromeric tetramers composed of a combination of three families of subunits, GluN1, GluN2 and GluN3 (Zukin 1995). There exist eight splice variants in the GluN1 family subunit due to the presence of one N-terminal and two C-terminal cassettes (Zukin and Bennett, 1995). These eight splice variants are heterogenic in their expression pattern both during development (Laurie 1995) and aging (Magnusson, 2005; Das, 2008).

Evidence shows that NMDA receptor expression declines with increasing age in both the hippocampus and prefrontal cortex (Kito et al., 1990; Magnusson, 1997; Magnusson et al., 2010). The GluN1 subunit of the NMDA receptor has been shown

to decline in expression of both protein and mRNA during aging in the prefrontal cortex of C57BL/6 mice (Magnusson, 2000; Magnusson et al., 2002). The individual splice forms of this subunit are heterogenous with respect to changes in their expression pattern during aging. Our earlier studies have shown that the mRNA expression of $GluN1_{X11}$ (GluN1-1) and $GluN1_{X10}$ (GluN1-3) splice forms of the GluN1 subunit in the prefrontal/frontal cortex and hippocampus decline during aging (Magnusson et al., 2005), but there is an increase in $GluN1_{0XX}$ (GluN1-a) splice form mRNA in response to behavioral testing experience in the prefrontal cortex of old mice (Das and Magnusson, 2008). We also have observed trends for associations of higher expression of $GluN1_{X10}$ and $GluN1_{0XX}$ mRNA within the orbital cortex with better performance in reference memory tasks in aged mice (Das and Magnusson, 2008). It is however, not known how important these splice forms are individually to memory. It is also not known how much influence the GluN1 subunits of the NMDA receptor have on observed flexibility in animals. The present paper explored the decreased expression of the individual splice form $GluN1_{0XX}$ with the use of *in vivo* siRNA and determined whether it played a role in spatial reference memory in young C57Bl/6 mice, which have the advantage of not having all of the confounds of other changes that can occur during aging.

4.2. Methods

4.2.1 Animals

A total of 48 male three-month-old C57BL/6 mice (The Jackson Laboratory, Maine) were used for the study. They were fed *ad libitum* and housed in cages under 12 hr light and 12 hr dark cycle. The animals were randomly divided into four treatment groups of twelve animals; siRNA against $GluN1_{0XX}$, control siRNA, vehicle and no treatment. After the behavioral testing on animals was performed, all animals were euthanized with exposure to CO_2 and decapitated. The brains were then harvested, frozen rapidly with dry ice and stored at -80 °C until further processing.

4.2.2 Injection solutions

A custom designed $GluN1_{0XX}$ siRNA and predesigned control siRNA (Catalog # 4404021) were purchased from Applied Biosystems. $GluN1_{0XX}$ siRNA was designed so that it did not interfere with any of the other GluN1 splice variants. The sequence used for $GluN1_{0XX}$ siRNA was; sense: 5'-CGUGA-GUCCAAGGCAGAGAtt-3' and antisense: 5'-UCUCUGCCUUGGACUCACGct-3'. The siRNA and control siRNAs were diluted to a concentration of 500μM with RNAse free water and stored in separate aliquots. On surgery days, an aliquot of both the siRNA and control siRNA were diluted with an equal volume of transfection reagent (siLentFect Lipid Reagent for RNAi, Bio-Rad, Hercules, CA) to give a final concentration of 250μM. Animals meant for vehicle alone treatments were injected with equal parts of transfection reagent and sterile water.

4.2.3 Stereotactic surgery

Injection of either vehicle, control siRNA or $GluN1_{0XX}$ siRNA was performed by stereotaxic surgery into the ventro-lateral orbital cortex of the brain as described by (Yoon et al., 1996). Briefly, twenty-four hours before surgery animals were provided with 120 mg of acetaminophen (Tylenol, McNeil-PPC Inc., Skillman, NJ) per 100 ml of water. On surgery days, each animal was anaesthetized by exposure to 4% isoflurane (Vet One, distributed by MWI, Meridian, ID) with 1 l/min O_2 via intranasal inhalation. The dose of isoflurane was adjusted to 1.5-2.25% as needed once the animals were non-responsive to painful stimuli. Each animal was then placed in the stereotaxic apparatus and the head was stabilized. Mouse body temperature was measured at the beginning of surgery and every 10 minutes after that. To prevent drying, the eyes were lubricated with sterile Puralube ophthalmic lubricant (Fougera & Co., Melville, NY). Care was taken to keep the surgery environment as sterile as possible during the surgery. Prior to incision, the scalp of each anaesthetized mouse was swabbed alternatively with alcohol and bedadine three times using sterile cotton swabs. A skin incision (7 to 9 mm) was made to access the dorsal surface of the skull over the prefrontal cortex. A cotton swab soaked in hydrogen peroxide was applied to

the skull for about a minute and then wiped off in order to visualize the frontal and longitudinal sutures on the skull. The point of junction of the frontal and longitudinal sutures, bregma, was used as a reference point for determining the skull coordinates for the injection into ventro-lateral orbital regions. Holes were drilled in the skull using an electric drill bit over both the hemispheres at points 2.3 mm rostral to bregma and 1.6 mm to the left and right of the longitudinal suture. Mice were injected with 5μl of either $GluN1_{0XX}$ specific siRNA, control siRNA or vehicle with the use of a fixed needle glass syringe (Hamilton Company, Reno, NV) at a depth of 2.7 mm from the surface of the skull at a rate of 500 nl/min with the use of an electric pump (UltraMicroPump 3 with SYS-Micro4 controller, World Precision Instruments, Sarasota, FL). To reduce back flow from the injection site, the syringe was left in place for 3 minutes after the pump delivered the appropriate amount into the injection site in order to allow diffusion. The skin incision was promptly sealed using glue (Super glue Corp., Rancho Cucamonga, CA) after removing the syringe. Mice were injected with 0.1cc of 0.03μM buprenorphine subcutaneously after they recovered from the anesthesia by showing signs of movement. Following surgery, mice were provided with acetaminophen plus codeine phosphate solution (Pharmaceutical Associates Inc., Greenville, SC) in their water (1ml per 20ml of water) for three days. After three days the water was changed to acetaminophen alone (120 mg in 100 ml water) and this was administered until they were euthanized. Animals in the group with no treatment did not undergo surgery, anesthesia or analgesic treatment.

4.2.4 Behavioral testing

Behavioral testing of the animals started on the 5^{th} day following surgery. Spatial reference memory and cued control task abilities were tested using the Morris Water Maze. A 1.2m diameter metal tank was covered with white contact paper and filled with water that was made opaque white with non-toxic tempera paint (Prang, Dixon Ticonderoga Company, Heathrow, FL). A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth. The cues were placed high on the walls of both the room

and the tank. There were seven different platform positions available at five different distances from the tank wall. Trials were video taped using a video camera (Sony Corp., Tokyo, Japan) placed above the center of the tank on the ceiling of the room. The animals' path was tracked with the "SMART" video tracking system (San Diego Instruments, San Diego, CA). There were different entry points for each trial and the mice were placed in the tank facing the wall.

4.2.4.1 Pretraining:

Three days prior to surgery, mice were pretrained for two consecutive days. Each pretraining session consisted of each mouse swimming for 60 seconds in the tank without the platform and then being trained to remain on the platform for 30 seconds each day. This platform position was different from the one used for subsequent reference memory testing. There was a one-day gap between pretraining and surgery.

4.2.4.2 Spatial reference memory:

On days 5 through 7 post-surgery, mice underwent spatial reference memory testing. The task consisted of 8 place trials per day and probe trials at the end of each day. There was also a probe trial at the beginning of the first day of reference memory testing. The platform was kept in the same quadrant for each place trial. Place trials consisted of 60 seconds maximum in the water searching for the platform, 30 seconds on the platform and 2 minutes of cage rest. If a mouse failed to find the platform within the designated 60 second swim time, it was led to the platform by the experimenter. Probe trials were performed to assess the animal's ability to show a bias for the platform location (Gallagher et al., 1993). During the probe trial, the platform was removed and the mouse was allowed to search in the water for 30 seconds. On the 8^{th} day post-surgery, a reversal reference memory task was performed wherein the platform was placed in the opposite quadrant in the tank. This task also consisted of 8 place and 1 probe trials and was similar to the reference memory task.

4.2.4.3 Cued control task:

Cued trials were designed to test motivation, visual acuity, and physical ability for the task. On the 9^{th} day following surgery, mice underwent 6 cued trials. The

platform was kept submerged but was marked by a 20.3 cm support with a flag. For each cued trial, the platform was changed to a different position and the mouse was placed into the tank facing the wall at one of the entry points and was allowed to search for the platform for 60 seconds. All mice were tested at one platform position before the platform was moved to a new position.

4.2.5 Brain sectioning

To visualize and obtain the mRNA densitometry for reductions in specific mRNA expression following siRNA treatment, the brains were prepared for *in situ* hybridization. Frozen brain from each animal was sectioned into 12 μm sections. Slides used for brain tissue sections were coated with 0.5% gelatin solution and air-dried before placement of sections on the slide. Each animal brain was sectioned coronally using a Leica CM1850 UV Cryostat (Leica Microsystems Inc., Bannockburn, IL). Brain sections representing at least one animal from each experimental group were placed on each slide belonging to one cutting group and kept frozen at -80 °C until further processing. Several slides were prepared to have a full coverage of the injection site. There were a total of seven cutting groups. Positions of animal brain sections representing each treatment group on slides belonging to each cutting group was determined randomly and was varied between cutting groups.

4.2.6 In situ hybridization

Oligonucleotide sequences used for probes for $GluN1_{0XX}$ was 5'-AACTGCAGCACCTTCTCTGCCTTGGACTCCCGTTCCTCCA-3' (Laurie and Seeburg, 1994) (Macromolecular Resources, Colorado State University, Fort Collins, CO); and for GAPDH was 5'-TGGGCCCTCAGATGCCTGCTTCACCACCTTCTT-GATGTCA-3' (Invitrogen Corp., Carlsbad, CA). They were labeled with ^{33}P-dATP (Perkin Elmer, Waltham, MA; specific activity: 3257 to 3839 Ci/mM) using terminal deoxyribonucleotidyl transferase (Invitrogen Corp., Carlsbad, CA) and purified with Microspin G-25 columns (Amersham Bioscience, Piscataway, NJ). The specific activities for the labeled oligonucleotides were calculated to be 17-69 dpm/fmol of

$GluN1_{0XX}$ probe and 30-58 dpm/fmol of GAPDH probe, depending on the labeling experiment.

In situ hybridization was performed as described by Watanabe and coworkers (Watanabe et al., 1993) and previous studies in our lab (Das and Magnusson, 2008; Magnusson et al., 2005). Briefly, each solution step was performed with gentle rotation on a rotating table except for the fixation and hybridization steps. Slides with sections were thawed, air-dried, fixed in 4% paraformaldehyde-PBS, pH 7.2 (25 °C) for 15 min, placed in 2 mg/ml glycine in PBS, pH 7.2 (25 °C) for 20 min, and placed in 0.25% acetic anhydride-0.1M triethanolamine, pH 8.0 (25 °C) for 10 min. Slides were immersed for 2 hr in a prehybridization solution (25 °C) consisting of 50% formamide, 0.1M Tris-HCl, pH 7.5, 4X SSC (1X SSC = 150mM NaCl and 15mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2% sarkosyl, and 250 µg/ml salmon testes DNA. Slides were then successively washed for 5 min each in 2X SSC, 70 and 100% ethanol, and air-dried for 15 min. Hybridization was performed by placing 150 µl of prehybridization solution containing 10% dextran sulfate and 0.33 pmoles of ^{33}P-labeled oligonucleotide probe onto the slides, covering the slides with parafilm, and incubating them for 18 hr in a 42 °C oven, humidified with 5X SSC. After incubation, coverslips were removed; slides were rinsed for 40 min in 2X SSC and 0.1% sarkosyl (25°C) and for 2×40 min in 0.1X SSC and 0.1% sarkosyl (55 °C) and air-dried. Nonspecific hybridization was determined by addition of 50-fold excess non-radiolabelled oligonucleotide to the hybridization solution on some slides. Slides were exposed to Kodak Biomax films for 8 days for $GluN1_{0XX}$ probes and for 1 day for GAPDH along with a slide containing ^{14}C standards. Brain and standard images were captured using a Macintosh G4 computer with a Powerlook 2100 XL scanner (UMAX, Taiwan) and NIH Image software. Quantitative densitometry was performed on the images from four sections for total hybridization and two sections for nonspecific hybridization from each animal with the use of NIH Image software (Version 1.63). The prefrontal cortex brain regions analyzed for mRNA expressions were deep (cortical layers IV-VI) and

superficial (cortical layers II-III) layers of ventral and lateral orbital cortex from both left and right sides of the brain (Fig. 4.2I). mRNA expressions from both sides of the brain were averaged for each animal to give one value per brain region per animal. Specific signal was determined by subtracting nonspecific hybridization from total hybridization. The ^{14}C standards were used to convert optical density to pmol of labeled ^{33}P-dATP/mm^2 tissue (Eakin et al., 1994).

4.2.7 Data analysis

Data for reference memory tests were analyzed as described earlier with a few modifications (Das and Magnusson, 2008). Briefly, the distance of the animal from the platform was measured every 0.2 second by the computer for the whole duration of the trial. Cumulative proximity was calculated by adding together the distance calculated at each 0.2 second interval. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle). A cumulative proximity measurement for the ideal path using the start position, average swim speed and platform position was calculated with the use of this macro. This cumulative proximity measure for the ideal path was subtracted from the cumulative proximity score for the whole track to obtain the corrected cumulative proximity scores for the place trials in reference memory tasks and the cued control tasks. For the probe trials of the reference memory tasks, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score. Learning index was then calculated from the average proximity measurements for probe trials (Gallagher et al., 1993).

Data from image analysis were normalized to the average of untreated mice to reduce variability between films and assays. Normalization factors were obtained by dividing the overall averages of the brain regions analyzed in all the uninjected mice by the averages for the uninjected animals within each assay group and were then multiplied by all animals' values within the assay. Differences in performance in reference memory and cued tasks and mRNA densities for $GluN_{0XX}$ and GAPDH were analyzed separately by two-way repeated measures ANOVA (treatment X trial or

treatment X brain region) followed by Fisher's protected post-hoc analysis using Statview software (SAS Institute Inc., Cary, NC).

4.3. Results

4.3.1 Spatial reference memory

Mice treated with siRNA against the $GluN1_{0XX}$ splice variant of the GluN1 subunit of the NMDA receptors in young mice in the present study showed higher cumulative proximities (worse performance) during the early phase of reference memory testing compared to the mice treated with control siRNA and vehicle treated animals. There was no overall effect of treatment on the blocks of four place trials of the reference memory task ($F_{(3,44)} = .663$, P = .58), but there was a significant treatment by block interaction ($F_{(3,15)} = 1.79$, p = .038). When individual blocks were analyzed separately for effect of treatment, there was a significantly higher cumulative proximity in block one (p = .02, Fig. 4.1A) and block 3 (p = .049, Fig. 4.1A) of the reference memory task in mice injected with $GluN1_{0XX}$ specific siRNA as compared to mice injected with control siRNA. There was also a significantly higher cumulative proximity of mice injected with $GluN1_{0XX}$ specific siRNA than mice injected with the vehicle (transfection reagent) in reference memory task in the third block of four place trials (p = .03, Fig. 4.1A). In the probe trials for reference memory, there was no difference in performance between different treatment groups ($F_{(3,44)} = .90$, p = .45, Fig. 4.1B).

There was no overall significant difference in cumulative proximity of mice in different treatment groups ($F_{(3,44)} = .646$, Fig. 4.1C) in the reversal reference memory task (escape platform in an opposite quadrant) and no significant interaction between treatment and different reversal trials. Analyzing the eight reversal place trials individually, since analysis of the individual trials was part of our experimental plan, revealed a near-significant higher cumulative proximity for mice injected with $GluN1_{0XX}$ siRNA, as compared to mice injected with control siRNA in the second reversal place trial (Fig. 4.1C, p = .06), which disappeared by the third trial (p = .12,

Fig. 4.1C). There was no significant difference in the reversal probe trial between different treatment groups ($F_{(3,44)} = .255$, $p = .86$; data not shown)

4.3.2 Associative memory in cued control task

There was no overall difference ($F_{(3,44)} = 1.936$, $p = .1377$) in cumulative proximity scores of animals in the different treatment groups in the cued control task, where the platform position was made visible by putting a flag on top (Fig. 4.1D). On average, mice in no platform position except for the first (south) had higher cumulative proximity than any trials of the place trials of the reference memory task (Fig. 4.1A, D).

4.3.3 mRNA expression following treatment with siRNA

There was a 10-19% reduction in $GluN1_{0XX}$ splice variant mRNA hybridization density in superficial and deep layers of ventro-lateral orbital region of the brain after the treatment with siRNA against $GluN1_{0XX}$. There was an overall significant difference ($F_{(3,43)} = 4.840$, $p = .005$) in hybridization density (pmol $^{33}P/mm^2$ tissue) expression of $GluN1_{0XX}$ splice variant across all the brain regions analyzed among the different treatment groups. The deep ($p = .004$-$.01$) and superficial ($p = .002$-$.01$) layers of ventral orbital and the superficial layers of lateral orbital ($p = .001$-$.003$) regions had a significantly lower hybridization density of $GluN1_{0XX}$ splice variant in the animals treated with siRNA against $GluN1_{0XX}$ as compared to all other controls (Fig. 4.2B, D, F, H, 4.3A). In the deep layers of lateral orbital cortex, $GluN1_{0XX}$ mRNA in the animals treated with siRNA had a trend for lower hybridization density as compared to animals injected with control RNA ($p = .06$, Fig. 4.3A) or vehicle ($p = .07$, Fig. 4.3A) and a significantly lower expression than the animals left untreated. The animals treated with siRNA against $GluN1_{0XX}$ had an overall decline of 10-19% $GluN1_{0XX}$ mRNA across the various brain regions analyzed as compared to the animals treated with a control siRNA (Fig. 4.3A). There was no significant change in hybridization density ($F_{(3,43)} = 0.815$, $p = .49$) of GAPDH mRNA after treatment with either siRNA against $GluN1_{0XX}$ as compared to the various other control reagents (Fig. 4.2A, C, E, G, 4.3B). The injection site within the brain seemed

to be within the orbital regions and near the intended injection sites (Fig. 4.2J). The spread of grossly visible reduction in mRNA for $GluN1_{0XX}$ extended from 0.25mm to 2.0mm laterally from the midline, 1.5mm to 3.0mm ventrally from the surface of the skull and 1.94mm to 2.8mm rostral to the Bregma (Fig. 4.2J).

4.4 Discussion

The present study provided evidence for a role for the $GluN1_{0XX}$ subunit splice variant of the NMDA receptor subunit within the prefrontal cortex of the brain in spatial reference memory in young mice. Knockdown of the splice variant in the ventral and lateral orbital regions of the brain in young mice lead to a delay in early acquisition of spatial reference memory. There was no difference in behavior in the later phases of reference memory training. There was also no difference in performance in the reversal training of reference memory, when the platform was moved to an opposite quadrant.

Mice treated with siRNA against the $GluN1_{0XX}$ splice variant of the GluN1 subunit of the NMDA receptors in young mice in the present study showed delayed learning early on as compared to the mice treated with control siRNA and vehicle treated animals. There was a 10-19% decline in $GluN1_{0XX}$ splice variant mRNA hybridization density after the treatment in superficial and deep layers of ventro-lateral orbital region of the brain. Specificity of knockdown of $GluN1_{0XX}$ splice variant was verified by absence of reduction in hybridization density of a control mRNA, GAPDH after $GluN1_{0XX}$ specific siRNA treatment in the same brain regions. Hence it can be inferred that the delay in learning in the spatial reference memory task might be due to the reduction in expression of the $GluN1_{0XX}$ splice variants in the ventro-lateral orbital regions of the brain.

Mice treated with $GluN1_{0XX}$ specific siRNA splice variant had worse performance in reference memory as compared to control siRNA injected animals at the beginning of the training. However, the lack of difference from the vehicle-injected animals makes this difficult to interpret. At the beginning of the second day,

the group treated with siRNA against $GluN1_{0XX}$ splice variant performed worse than the groups treated with both control siRNA and vehicle. These $GluN1_{0XX}$ siRNA injected mice showed improved performance similar to all groups by block 4, suggesting that $GluN1_{0XX}$ splice variants play a role in early acquisition. Memory tasks involving several trials per day for several days in the Morris water maze involve both acquisition at the beginning and consolidation of memory at the end of each training session and it is difficult to differentiate between them. Memory consolidation in mice has been shown to occur within several hours to days after training for a task (Abel and Lattal, 2001). Leon et al., using inhibitors of protein kinases have shown that memory consolidation occurs 2 hours after acquisition of single day learning in the Morris water maze (Leon et al., 2010). Using inhibitors of protein synthesis, Artinian and co-workers (Artinian et al., 2008) observed initiation of memory consolidation as early as 4 hours after training in the Morris water maze. Based on the above findings it is possible that the deficit in memory in animals treated with $GluN1_{0XX}$ specific siRNA in the first half of training may be due to problems with early acquisition and/or early consolidation.

In the present study we observed about 10-19% decline in $GluN1_{0XX}$ mRNA expression after treatment with $GluN1_{0XX}$ specific siRNA across the ventro-lateral orbital region of the prefrontal cortex. The GluN1 subunit of the NMDA receptor has been identified as a necessary subunit for proper functioning of the NMDA receptor (Ishii et al., 1993; Meguro et al., 1992; Yamazaki et al., 1992). The $GluN1_{0XX}$ subunit splice variant which lacks the N1 cassette, have reduced affinity for agonist by almost five fold as compared to the ones with N1 cassette (Durand et al., 1993). These $GluN1_{0XX}$ subunit splice variants show increased mRNA expression in orbital, insular and medial prefrontal cortex of the brain in old mice after they have been subjected to behavioral testing experience (Das and Magnusson, 2008). Increase in $GluN1_{0XX}$ subunit splice variant mRNA expression in the orbital region also had a near-significant (corrected $p = .08$) association with performance in reference memory in old mice (Das and Magnusson, 2008). Using an inducible and region specific

knockout of GluN1 subunit has revealed involvement of the subunit in consolidation of hippocampal-independent nondeclarative taste memory (Cui et al., 2005). The prefrontal cortex has been shown to be important for reference memory function including formation of recent memory (Blum et al., 2006; Zhao et al., 2005) and recall of stored infomation (Nyberg et al., 1995; Paradiso et al., 1997). Tests of spatial memory using Morris water maze with multiple distant cues and varied number of trials have been shown to involve prefrontal cortex for acquisition of memory (Compton et al., 1997). The prefrontal cortex has been shown to be involved in consolidation and recall of recent spatial memory after training in the Morris water maze (Leon et al., 2010). Therefore reduction in $GluN1_{0XX}$ subunit splice variants by $GluN1_{0XX}$ specific siRNA treatment in the ventro-lateral orbital cortex might be responsible for the problem in early acquisition and consolidation of long term memory after training with Morris water maze.

We did not observe a difference between the animals left untreated and the animals receiving siRNA specific for $GluN1_{0XX}$ in the present study. The mice that were left untreated did not receive acetaminophen, codeine, buprenorphine or isoflurane. Prolonged exposure to isoflurane during development is shown to cause neurodegeneration in rodents (Loepke et al., 2009) but is shown to spare its effect on aged rodents (Stratmann et al., 2010). A study by Ishida and coworkers (Ishida et al., 2007) on the effects of acetaminophen on memory performance in Morris water maze shows that at a high dose (302.3 mg/kg body weight), causes memory impairment and at a low dose (15.1 mg/kg body weight) it facilitates memory performance. Our dose of acetaminophen was similar to this low dose of acetaminophen and so may have facilitated memory performance in mice receiving the drug.

Reduction of the $GluN1_{0XX}$ splice variant mRNA in the ventro-lateral orbital regions did not seem to cause problems in performance in the probe trials of the reference memory task where the escape platform was missing. Instead there appeared to be improvement in performance of the mice injected with $GluN1_{0XX}$ specific siRNA compared to the mice injected with control siRNA at the end of the second day

training, although there again was no difference from vehicle injected mice. The probe trials we used to measure the bias towards a previously learned escape platform location. If there was an absence of a difference among the different treatment groups, it would indicate a similar bias among the treatment groups for the previously learned platform position. However, the greatest difference between injected animals in the place trials occurred at the beginning of the second training day and the measurement of bias was at the end of that day, after the $GluN1_{0XX}$ specific siRNA injected mice showed equal or better performance to the other treatment groups. Animals possess redundant systems for different functions of the body, which deteriorate over time (Penner and Barnes, 2007). Better performance in probe trials observed in the mice injected with $GluN1_{0XX}$ siRNA compared to other treatment groups towards the end of the second training day could be due to use of other redundant systems, available to these young animals.

There was also no overall significant difference in performance of mice between different treatment groups when the escape platform was moved to the opposite quadrant. However, a tendency of mice injected with $GluN1_{0XX}$ specific siRNA to perform poorly early in the task was observed. This might suggest a reduced flexibility in animals with the specific siRNA treatment in the ventro-lateral orbital region. Prefrontal cortex has been shown to be involved in reversal training in both non-human primates (Mishkin, 1964) and rodents (De Bruin, 1994; Li and Shao, 1998). The role of the orbital prefrontal cortex in reversal learning has shown inconsistent results across different tasks. Object reversal learning was impaired in monkeys with orbital prefrontal lesions (Mishkin, 1964), whereas tasks involving serial reversal learning and response extinction was not affected (Kolb et al., 1974). Damage to medial prefrontal cortex, however, shows compelling evidence for problems in reversal learning during spatial learning tasks in Morris water maze (De Bruin, 1994; Ragozzino et al., 1999a; Ragozzino et al., 1999b). From the above findings it may be inferred that the reduced expression of $GluN1_{0XX}$ in the ventro-lateral orbital region may not cause the possible deficit in performance in reversal

reversal learning or that a 10-19% reduction of $GluN1_{0XX}$ is not sufficient to cause significant impairment in a young animal.

In conclusion, reducing the expression of the $GluN1_{0XX}$ subunit splice variant of the NMDA receptor in orbital cortex appeared to interfere with early acquisition and/or consolidation of spatial reference memory. In contrast the ability to switch to a different strategy did not appear to be altered by the reduction of the $GluN1_{0XX}$ subunit in the ventro-lateral orbital region. Overall this study indicated a role of $GluN1_{0XX}$ in early acquisition and/or consolidation of long-term spatial memory.

Acknowledgements

Funding sources: This research was supported by NIH grants AG016322 (KRM).

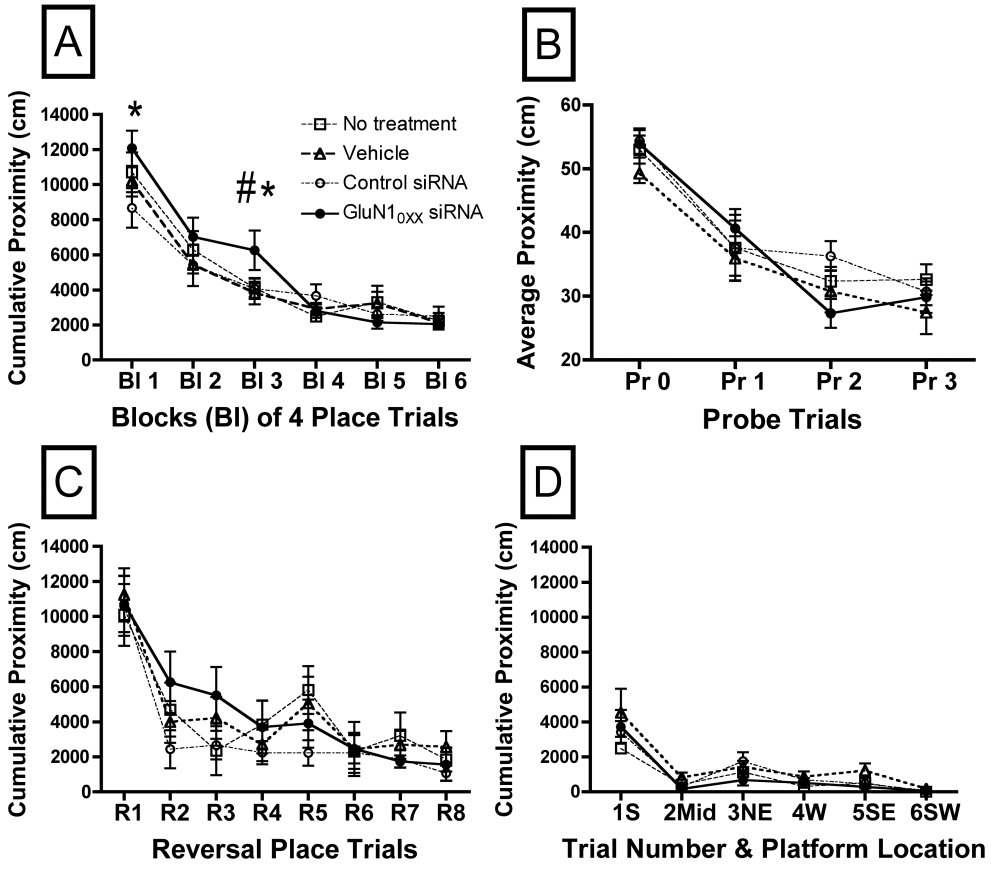

Fig. 4.1 – Performance of mice in various tasks of spatial reference or associative memory in the Morris water maze after various treatments. Performance of mice within blocks of four place learning trials for a 3-day spatial reference memory task (A), each probe trial of the same spatial reference memory task (B), each reversal trial (C) and each cued control (associative memory) trial (D). * $p < .05$ for difference in proximity measurement between animals injected with GluN1$_{0XX}$ siRNA and control siRNA. # $p < .05$ for difference in performance from animals injected with vehicle. Data indicate mean ± SEM. Bl = blocks, Pr = probe trial, R = reversal trial, S = south, Mid = middle, NE = northeast, W = west, SE = southeast, SW = southwest.

Fig. 4.2 – Representative images of brain sections showing mRNA expression of GAPDH (A, C, E, G) and $GluN1_{0XX}$ (B, D, F, H) following treatment with either $GluN1_{0XX}$ specific siRNA (A, B), vehicle (C, D), control siRNA (E, F) or left untreated (G, H). I) Image of the region of brain where the injections were applied (solid vertical lines) and regions where mRNA analysis was performed (regions 1= deep (layers IV-VI) ventral orbital, 2 = superficial (layers II-III) ventral orbital, 3 = deep lateral orbital, and 4 = superficial lateral orbital). J) Graphical representation of the injection site (vertical solid lines from top) and the spread of grossly visible reduction of mRNA for $GluN1_{0XX}$ (dotted area) from rostral to caudal. Distance from Bregma is indicated below each section in image (J). Image I and J adapted from Paxinos and Franklin (2001). A-H) Standard images and the equivalent pmol $^{33}P/mm^2$ tissue are shown to the right of each brain image.

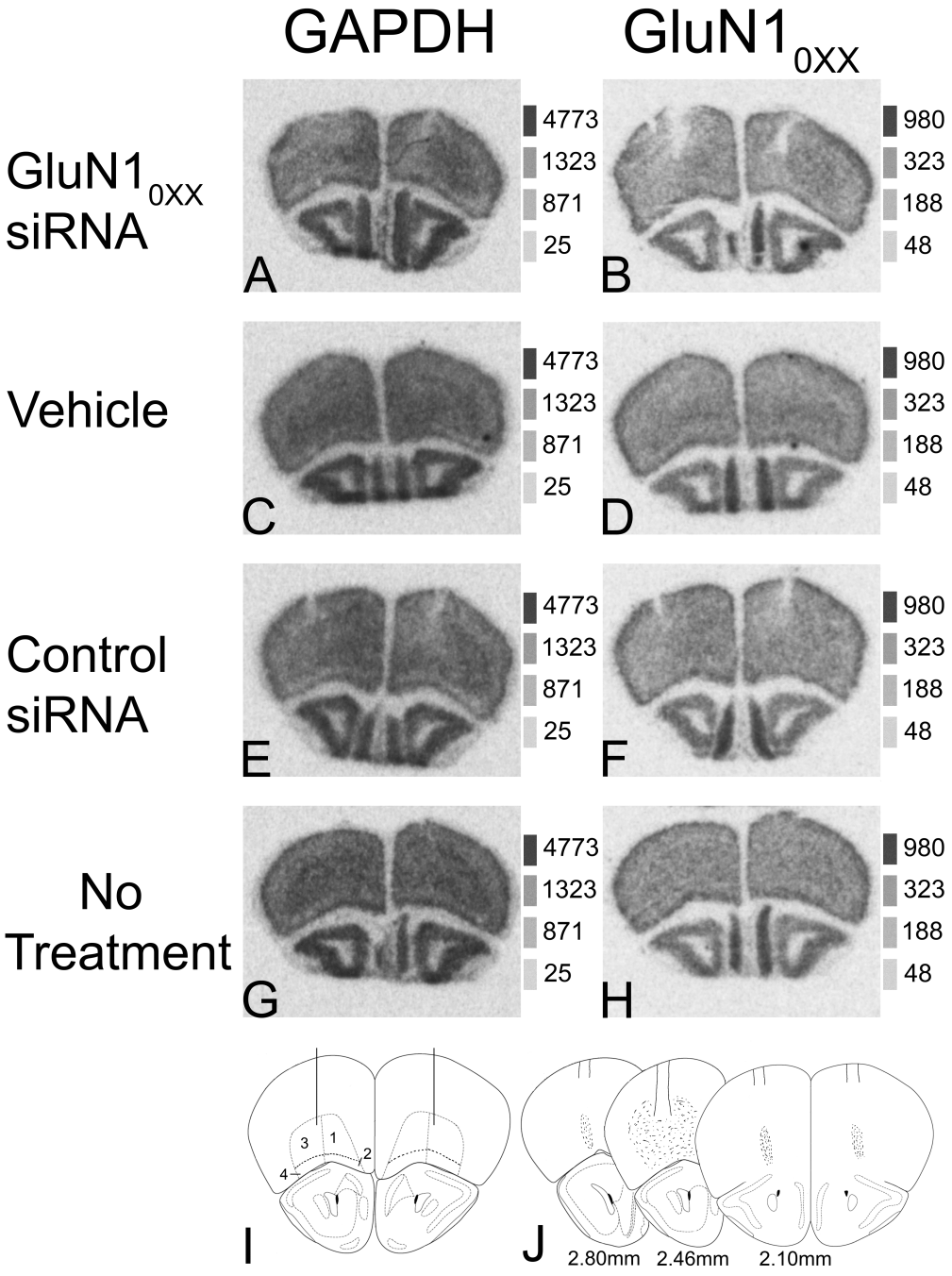
GAPDH
GluN1 0XX
GluN1 0XX siRNA
Vehicle
Control siRNA
No Treatment
4773
1323
871
25
980
323
188
48
A
B
C
D
E
F
G
H
I
J
3
1
2
4
2.80mm
2.46mm
2.10mm

Fig. 4.2

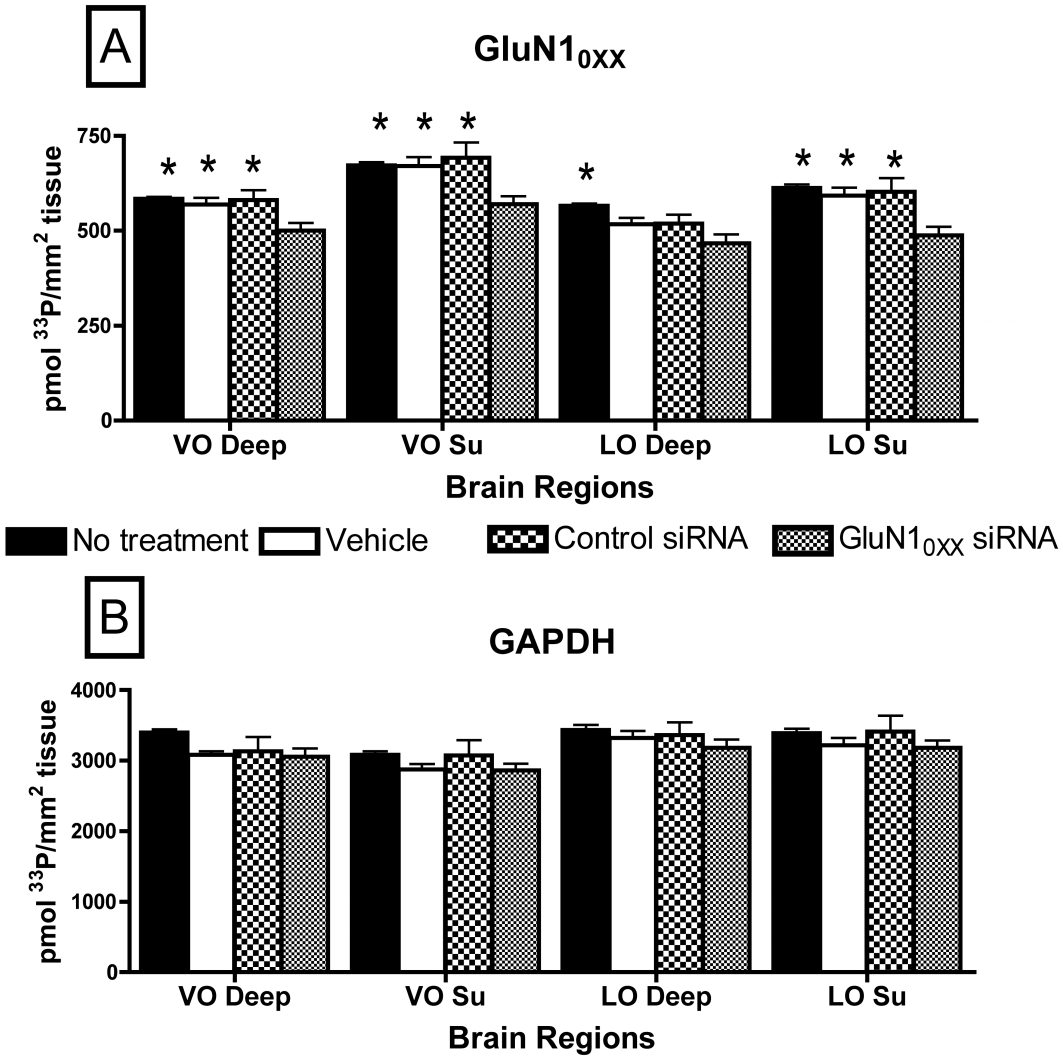

Fig. 4.3 – mRNA expression of GluN1$_{0XX}$ (A) and GAPDH in different regions of the prefrontal cortex of the brain. * $p < .05$ for difference in mRNA expression in animals injected with siRNA against GluN1$_{0XX}$. VO = ventral orbital, LO = lateral orbital, SU = superficial. Cortical layers II-III, deep = critical layers IV-VI

CHAPTER V

CONCLUSION

The elderly population is rapidly growing in the United States and elsewhere in the world. In the next twenty years, by the year 2030, one in every five people in the US will be over 65 years of age (Federal Inter-agency forum on aging related statistics, 2010). Because of this demographic shift in population, there is a severe need to combat diseases related to the elderly. Memory is one of the functions that is affected by the process of aging and many elderly complain about memory decline. Although memory decline is not the same across individuals, it poses a serious risk to the life of elderly individuals and makes them predisposed to entering care facilities early in life, creating a huge economic burden for them. Given the importance of age related memory decline, it is imperative to understand its details and find a possible cure or at least delay its onset. The current research is an attempt to further understand the molecular mechanisms of memory decline during aging. The present work describes effects of experience in a learning task and aging on expression of the individual splice variants of the NMDA receptor subunit GluN1. It also describes the effects of reduction of one of the splice variants on memory performance in a spatial long-term memory task.

Age-related changes in the protein and mRNA expression of some or all of the splice forms of the GluN1 subunit of the NMDA receptor have been seen in mice and rats (Magnusson, 2000; Magnusson et al., 2002; Magnusson et al., 2005; Magnusson et al., 2007). mRNA for C-terminal splice variants, $GluN1_{X11}$ and $GluN1_{X10}$ showed significant declines during aging in several brain regions even though overall GluN1 mRNA expression was not affected by aging in all of these regions (Magnusson et al., 2005). This suggests that these splice forms are more influenced by aging than the subunit as a whole.

In the present study, there was an increase in the expression of the $GluN1_{0XX}$ splice form in the behaviorally-experienced old mice relative to the younger groups. Old mice with high levels of mRNA expression for the $GluN1_{0XX}$ splice form in orbital cortex showed the best performances in the working and reference memory tasks, but the poorest performances in the cued, associative learning task. The orbital

cortex is involved with sensory integration, decision making and value expectation (Kringlebach, 2005). Good performance with high expression of $GluN1_{0XX}$ in orbital cortex thus may have indicated that the mice with high expression of $GluN1_{0XX}$ were good with decision making during a working or reference memory task. The $GluN1_{0XX}$ splice variant lacks the N1 cassette, protein expression of which could not be confirmed due to lack of an available antibody against the splice form. NMDA receptors expressed in Xenopus oocytes containing GluN1 subunits without the N1 cassette have reduced affinity for agonist, NMDA by almost five fold and nearly abolished potentiation by polyamine, spermine at saturating glycine concentration (Durand et al., 1993). High mRNA expression in the old animals therefore might indicate a reduced activity of the receptor due to reduced affinity for agonists.

Declines in expression of GluN1 splice variants $GluN1_{X10}$ and $GluN1_{X11}$ are observed with increasing age in the present study. Protein expression of GluN1 subunits containing C-terminal cassettes C2 or C2' were observed to decline with increasing age. The $GluN1_{X11}$ splice variant contains the C2 cassette and $GluN1_{X10}$ splice variant contains the C2' cassette. So the protein and mRNA expression of the individual cassette and splice variants are in accordance with each other. The two splice variants with decreased mRNA expression with increasing age also contain a C1 cassette along with a C2 or C2' cassette. Protein expression of the C1 cassette was however, not changed with increasing age. Differences between these results could be due to the fact that mRNA analysis was done in individual brain regions where as the protein expression analysis was done in the homogenized prefrontal cortex isolated from one half of the brain. Also protein expression of the C1 cassette involves analysis of four splice variants that contain the C1 cassette and not just the two splice variants ($GluN1_{X10}$ and $GluN1_{X11}$) we observed aging differences in mRNA expression.

Among the aged animals, higher protein expression of GluN1 subunits containing C1 cassettes and the whole population of GluN1 subunits were found to be associated with better performance in the final phase of probe trials, but this appeared to be due to maintenance of perseveration or delays in applying an accurate search

strategy. In middle-age animals, higher expressions of the GluN1 subunit and C2' cassette proteins were associated with good reference memory on initial search for the platform. The C1 cassette contains phosphorylation sites, for PKC and PKA activity (Tingley et al., 1997). It also appears to interact with other scaffolding proteins important for clustering of receptors (Ehlers et al., 1998; Lin et al., 1998). High expression in old animals therefore would give rise to increased activity of the NMDA receptor, which may have been important for the maintenance of perseveration during the search for platform. Potentiation due to phosphorylation of GluN1 subunits of NMDA receptors depends on the presence or absence of C1 (Tingley et al., 1997) or C2 cassettes (Durand et al., 1992). Maintenance of normal spine density and stabilization of synapses in rat pyramidal neurons depends on the expression of C2 cassettes (Alvarez et al., 2007). Thus, reduction in expression of the GluN1 subunits containing the C2 cassette may result in altered phosphorylation state of the receptor due to the loss of phosphorylation sites. It may also result in instability of the synapses dominated by the NMDA receptor in the prefrontal/frontal cortex. The C2' cassette contains a signal responsible for export of the GluN1 subunit from the endoplasmic reticulum (Mu et al., 2003; Standley et al., 2000). Splicing out of the C2 cassette results in seven-fold increase of potentiation of NMDA receptors by PKC (Durand et al., 1992). High expression of the C2' cassette in the middle-age animals therefore means a higher activity of the NMDA receptor which may be important for initial search strategy.

In order to determine if the $GluN1_{0XX}$ subunit splice variant was important for spatial reference memory performance, young mice were injected with either siRNA specific for $GluN1_{0XX}$, control siRNA, or vehicle alone bilaterally into ventro-lateral orbital regions or left untreated. When tested five days post-injection, in a Morris water maze task, there was a decline in performance in the early stages of a reference memory task in the mice receiving siRNA against the $GluN1_{0XX}$ splice form, as compared to the mice injected with control siRNA and/or vehicle. This corresponded to a 10-19% reduction in $GluN1_{0XX}$ splice variant hybridization density after

behavioral assessment. In addition, flexibility of the animals at the beginning of the reversal task was nearly affected by the 10-19% reduction of $GluN1_{0XX}$ in the ventro-lateral orbital region. These results suggest an important role of the $GluN1_{0XX}$ splice variant in orbital regions for early acquisition and/or consolidation of spatial reference memory.

Collectively, the current work describes an important and complex role of the GluN1 splice variants during aging and learning experience. Of importance in particular are the splice variants containing the C2 or C2' and the ones without the N1 cassette. Given the results of the current study, a follow up study could be to enhance memory performance in the old animals by trying to increase the splice variants not containing N1 cassettes or containing C2 and C2' cassettes. Another area that the current work didn't describe in detail was the difference between memory acquisition, consolidation and recall in the Morris water maze. Although results in Chapter – IV are suggestive of problems with memory consolidation and acquisition, it has not been explicitly investigated. Understanding impairments during the exact stage of memory formation would be beneficial to develop therapeutic strategies in future.

BIBLIOGRAPHY

Abel, T., Lattal, K.M., 2001. Molecular mechanisms of memory acquisition, consolidation and retrieval. Curr Opin Neurobiol. 11**,** 180-187.

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., Bourtchouladze, R., 1997. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell. 88**,** 615-626.

Adolphs, R., Cahill, L., Schul, R., Babinsky, R., 1997. Impaired declarative memory for emotional material following bilateral amygdala damage in humans. Learn Mem. 4**,** 291-300.

Aggleton, J.P., Hunt, P.R., Rawlins, J.N., 1986. The effects of hippocampal lesions upon spatial and non-spatial tests of working memory. Behavioural Brain Research. 19**,** 133-146.

Akaneya, Y., Jiang, B., Tsumoto, T., 2005. RNAi-induced gene silencing by local electroporation in targeting brain region. J Neurophysiol. 93**,** 594-602.

Albert, M.S., Funkenstein, H.H., 1992. The effects of age: Normal variation and its relation to disease. In: Diseases of the Nervous System: Clinical Neurobiology. Vol., A.K. Asbury, G.M. McKhann, W.I. McDonald, eds. Saunders, Philadelphia, pp. 598-611.

Alessandri, B., Battig, K., Welzl, H., 1989. Effects of ketamine on tunnel maze and water maze performance in the rat. Behav Neural Biol. 52**,** 194-212.

Alexander, G.E., Crutcher, M.D., 1990. Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci. 13**,** 266-271.

Alvarez, V.A., Ridenour, D.A., Sabatini, B.L., 2007. Distinct structural and ionotropic roles of NMDA receptors in controlling spine and synapse stability. J. Neurosci. 27, 7365-7376.

Anantharam, V., Panchal, R.G., Wilson, A., Kolchine, V.V., Treistman, S.N., Bayley, H., 1992. Combinatorial RNA splicing alters the surface charge on the NMDA receptor. FEBS Lett. 305**,** 27-30.

Artinian, J., McGauran, A.M., De Jaeger, X., Mouledous, L., Frances, B., Roullet, P., 2008. Protein degradation, as with protein synthesis, is required during not only long-term spatial memory consolidation but also reconsolidation. Eur J Neurosci. 27**,** 3009-3019.

Artola, A., Singer, W., 1994. NMDA receptors and developmental plasticity in visual neocortex. In: The NMDA Receptor. Vol., G.L. Collingridge, J.C. Watkins, eds. Oxford University Press, Oxford, pp. 313-339.

Atkinson, R.C., Shiffrin, R.M., 1971. The control of short-term memory. Sci Am. 225**,** 82-90.

Atkinson, R.C., Siffrin, R.M. (Eds.), 1968. Human memory: A proposed system. Academic Press, New York.

Baddeley, A.D., 1999. Essentials of human memory, Psychology Press, East Sussex.

Baddeley, A.D., Bressi, S., Della Sala, S., Logie, R., Spinnler, H., 1991. The decline of working memory in Alzheimer's disease. A longitudinal study. Brain. 114 (Pt 6)**,** 2521-2542.

Baddeley, A.D., Hitch, G.J., 1974. Working memory. In: The psychology of learning and motivation. Vol., G.A. Bower, eds. Academic Press, New York, pp. 47-89.

Bagasra, O., Prilliman, K.R., 2004. RNA interference: the molecular immune system. J Mol Histol. 35**,** 545-553.

Barense, M.D., Fox, M.T., Baxter, M.G., 2002. Aged rats are impaired on an attentional set-shifting task sensitive to medial frontal cortex damage in young rats. Learn Mem. 9**,** 191-201.

Barnes, C.A., 1979. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol. 93**,** 74-104.

Barnes, C.A., 1988. Aging and the physiology of spatial memory. Neurobiol Aging. 9, 563-568.

Bashir, Z.I., Alford, S., Davies, S.N., Randall, A.D., Collingridge, G.L., 1991. Long-term potentiation of NMDA receptor-mediated synaptic transmission in the hippocampus. Nature. 349, 156-158.

Bashir, Z.I., Berretta, N., Bortolotto, Z.A., Clark, K., Davies, C.H., Frenguelli, B.G., Harvey, J., Potier, B., Collingridge, G.L., 1994. NMDA receptors and long-term potentiation in the hippocampus. In: The NMDA Receptor. Vol., G.L. Collingridge, J.C. Watkins, eds. Oxford University Press, Oxford, pp. 294-312.

Benke, D., Wenzel, A., Scheuer, L., Fritschy, J.M., Mohler, H., 1995. Immunobiochemical characterization of the NMDA-receptor subunit NR1 in the developing and adult rat brain. J Recept Signal Transduct Res. 15**,** 393-411.

Bermudez-Rattoni, F., Introini-Collison, I.B., McGaugh, J.L., 1991. Reversible inactivation of the insular cortex by tetrodotoxin produces retrograde and anterograde amnesia for inhibitory avoidance and spatial learning. Proc Natl Acad Sci U S A. 88, 5379-5382.

Blackstone, C.D., Moss, S.J., Martin, L.J., Levey, A.I., Price, D.L., Huganir, R.L., 1992. Biochemical characterization and localization of a non-N-methyl- D-aspartate glutamate receptor in rat brain. J Neurochem. 58**,** 1118-1126.

Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 361**,** 31-39.

Blum, S., Hebert, A.E., Dash, P.K., 2006. A role for the prefrontal cortex in recall of recent and remote memories. NeuroReport. 17**,** 341-344.

Bockers, T.M., Zimmer, M., Muller, A., Bergmann, M., Brose, N., Kreutz, M.R., 1994. Expression of the NMDA R1 receptor in selected human brain regions. Neuroreport. 5, 965-969.

Bradley, J., Carter, S.R., Rao, V.R., Wang, J., Finkbeiner, S., 2006. Splice variants of the NR1 subunit differentially induce NMDA receptor-dependent gene expression. J Neurosci. 26**,** 1065-1076.

Brose, N., Gasic, G.P., Vetter, D.E., Sullivan, J.M., Heinemann, S.F., 1993. (1993) Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. The Journal of biological chemistry 268(30):22663-22671. Journal of Biological Chemistry. 268**,** 22663-22671.

Chung, H.J., Huang, Y.H., Lau, L.F., Huganir, R.L., 2004. Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. J Neurosci. 24**,** 10248-10259.

Clarke, H.F., Dalley, J.W., Crofts, H.S., Robbins, T.W., Roberts, A.C., 2004. Cognitive inflexibility after prefrontal serotonin depletion. Science. 304, 878-880.

Clayton, D.A., Browning, M.D., 2001. Deficits in the expression of the NR2B subunit in the hippocampus of aged Fisher 344 rats. Neurobiol. Aging. 22, 165-168.

Clayton, D.A., Grosshans, D.R., Browning, M.D., 2002. Aging and surface expression of hippocampal NMDA receptors. J Biol Chem. 277**,** 14367-14369.

Collingridge, G.L., Bliss, T.V.P., 1987. NMDA receptors - their role in long-term potentiation. Trends Neurosci. 10**,** 288-293.

Collingridge, G.L., Olsen, R.W., Peters, J., Spedding, M., 2009. A nomenclature for ligand-gated ion channels. Neuropharmacology. 56, 2-5.

Collingridge, G.L., Watkins, J.C., 1994. The NMDA receptor, Oxford University Press, Oxford.

Compton, D.M., Griffith, H.R., McDaniel, W.F., Foster, R.A., Davis, B.K., 1997. The flexible use of multiple cue relationships in spatial navigation: a comparison of water maze performance following hippocampal, medial septal, prefrontal cortex, or posterior parietal cortex lesions. Neurobiology of Learning and Memory. 68**,** 117-132.

Conneely, K.N., Boehnke, M., 2007. So many correlated tests, so little time! Rapid adjustment of p values for multiple correlated tests. Am J Hum Genet. 81, 1158-1168.

Cook, I.A., Bookheimer, S.Y., Mickes, L., Leuchter, A.F., Kumar, A., 2007. Aging and brain activation with working memory tasks: an fMRI study of connectivity. Int J Geriatr Psychiatry. 22, 332-342.

Cooke, S.F., Bliss, T.V., 2006. Plasticity in the human central nervous system. Brain. 129**,** 1659-1673.

Corkin, S., 2002. What's new with the amnesic patient H.M.? Nat Rev Neurosci. 3**,** 153-160.

Cotman, C.W., Bridges, R.J., Taube, J.S., Clark, A.S., Geddes, J.W., Monaghan, D.T., 1989. The role of the NMDA receptor in central nervous system plasticity and pathology. J NIH Res. 1, 65-74.

Coultrap, S.J., Nixon, K.M., Alvestad, R.M., Valenzuela, C.F., Browning, M.D., 2005. Differential expression of NMDA receptor subunits and splice variants among the CA1, CA3 and dentate gyrus of the adult rat. Brain Res Mol Brain Res. 135**,** 104-111.

Craik, F.I.M., Morris, R.G., Gick, M.L. (Eds.), 1990. Adult age differences in working memory. Cambridge University Press, Cambridge.

Crook, T.H., Ferris, S.H., 1992. Age associated memory impairment. BMJ. 304**,** 714.

Crook, T.H., Larrabee, G.J., Youngjohn, J.R., 1990. Diagnosis and assessment of age-associated memory impairment. Clin Neuropharmacol. 13 Suppl 3, S81-91.

Cui, Z., Lindl, K.A., Mei, B., Zhang, S., Tsien, J.Z., 2005. Requirement of NMDA receptor reactivation for consolidation and storage of nondeclarative taste memory revealed by inducible NR1 knockout. European Journal of Neuroscience. 22**,** 755-763.

Cull-Candy, S., Brickley, S., Farrant, M., 2001. NMDA receptor subunits: diversity, development and disease. Curr. Opin. Neurobiol. 11**,** 327-335.

Das, S.R., Magnusson, K.R., 2008. Relationship between mRNA expression of splice forms of the zeta1 subunit of the N-methyl-D-aspartate receptor and spatial memory in aged mice. Brain Res. 1207**,** 142-154.

Davis, S., Markowska, A.L., Wenk, G.L., Barnes, C.A., 1993. Acetyl-L-carnitine: behavioral, electrophysiological, and neurochemical effects. Neurobiol Aging. 14**,** 107-115.

De Bruin, J.P.C., 1994. Evolution of prefrontal cortex: comparative aspects of its functioning. In: Flexibility and constraints in Behavioral Systems. Vol., R.J. Greenspan, C.P. Kyriacou, eds. John Wiley & Sons, Chichester, pp. 185-192.

Dean, R.L., 3rd, Scozzafava, J., Goas, J.A., Regan, B., Beer, B., Bartus, R.T., 1981. Age-related differences in behavior across the life span of the C57BL/6J mouse. Exp. Aging Res. 7, 427-451.

Deutsch, J.A., 1971. The cholinergic synapse and the site of memory. Science. 174**,** 788-794.

Dingledine, R., Borges, K., Bowie, D., Traynelis, S.F., 1999. The glutamate receptor ion channels. Pharmacol Rev. 51**,** 7-61.

Divac, I., 1971. Frontal lobe system and spatial reversal in the rat. Neuropsychologia. 9**,** 175-183.

Dunah, A.W., Standaert, D.G., 2001. Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. J. Neurosci. 21, 5546-5558.

Durand, G.M., Bennett, M.V., Zukin, R.S., 1993. Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. Proc Natl Acad Sci U S A. 90, 6731-6735.

Durand, G.M., Gregor, P., Zheng, X., Bennett, M.V., Uhl, G.R., Zukin, R.S., 1992. Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. Proc Natl Acad Sci U S A. 89, 9359-9363.

Eakin, T.J., Baskin, D.G., Breininger, J.F., Stahl, W.L., 1994. Calibration of 14C-plastic standards for quantitative autoradiography with 33P. J Histochem Cytochem. 42, 1295-1298.

Ehlers, M.D., Fung, E.T., O'Brien, R.J., Huganir, R.L., 1998. Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments. J Neurosci. 18, 720-730.

Ehlers, M.D., Tingley, W.G., Huganir, R.L., 1995. Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. Science. 269, 1734-1737.

Eriksson, M., Nilsson, A., Froelich-Fabre, S., Akesson, E., Dunker, J., Seiger, A., Folkesson, R., Benedikz, E., Sundstrom, E., 2002. Cloning and expression of

the human N-methyl-D-aspartate receptor subunit NR3A. Neurosci Lett. 321, 177-181.

Fagni, L., Olivier, M., Lafon-Cazal, M., Bockaert, J., 1995. Involvement of divalent ions in the nitric oxide-induced blockade of N-methyl-D-aspartate receptors in cerebellar granule cells. Mol Pharmacol. 47**,** 1239-1247.

Federal interagency forum on aging-related statistics, 2010. Older Americans 2010: Key indicators of well being. Washington DC, pp. 156. (available online at http://www.agingstats.gov).

Ferry, B., Roozendaal, B., McGaugh, J.L., 1999. Role of norepinephrine in mediating stress hormone regulation of long-term memory storage: a critical involvement of the amygdala. Biol Psychiatry. 46**,** 1140-1152.

Fletcher, P.C., Henson, R.N., 2001. Frontal lobes and human memory: insights from functional neuroimaging. Brain. 124, 849-881.

Frank, L.M., Brown, E.N., 2003. Persistent activity and memory in the entorhinal cortex. Trends Neurosci. 26**,** 400-401.

Frankiewicz, T., Pilc, A., Parsons, C.G., 2000. Differential effects of NMDA-receptor antagonists on long-term potentiation and hypoxic/hypoglycaemic excitotoxicity in hippocampal slices. Neuropharmacology. 39**,** 631-642.

Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L., Silva, A.J., 2004. The involvement of the anterior cingulate cortex in remote contextual fear memory. Science. 304**,** 881-883.

Frick, K.M., Baxter, M.G., Markowska, A.L., Olton, D.S., Price, D.L., 1995. Age-related spatial reference and working memory deficits assessed in the water maze. Neurobiol Aging. 16, 149-160.

Frick, K.M., Fernandez, S.M., 2003. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. Neurobiol. Aging. 24, 615-626.

Furukawa, H., Singh, S.K., Mancusso, R., Gouaux, E., 2005. Subunit arrangement and function in NMDA receptors. Nature. 438, 185-192.

Gage, F., Dunnett, S., Bjorklund, A., 1984. Spatial learning and motor deficits in aged rats. Neurobiol. Aging. 5**,** 43-48.

Gage, F.H., Dunnett, S.B., Bjorklund, A., 1984. Spatial learning and motor deficits in aged rats. Neurobiol Aging. 5, 43-48.

Galea, L.A., Ormerod, B.K., Sampath, S., Kostaras, X., Wilkie, D.M., Phelps, M.T., 2000. Spatial working memory and hippocampal size across pregnancy in rats. Horm Behav. 37, 86-95.

Gallagher, M., Bizon, J.L., Hoyt, E.C., Helm, K.A., Lund, P.K., 2003. Effects of aging on the hippocampal formation in a naturally occurring animal model of mild cognitive impairment. Exp Gerontol. 38, 71-77.

Gallagher, M., Burwell, R., Burchinal, M., 1993a. Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behav Neurosci. 107, 618-626.

Gallagher, M., McMahan, R.W., Schoenbaum, G., 1999. Orbitofrontal cortex and representation of incentive value in associative learning. J Neurosci. 19, 6610-6614.

Gallagher, M., Nicolle, M.M., 1993. Animal models of normal aging: Relationship between cognitive decline and markers in hippocampal circuitry. Behav Brain Res. 57**,** 155-162.

Genz, A., Bretz, F., Hothorn, T., 2007. mvtnorm: Multivariate Normal and T Distribution. R package version 0.8.1.

Goldman-Rakic, P.S., 1987. Circuitry of the primate prefrontal cortex and the regulation of behavior by representational memory. In: Handbook of Physiology, the Nervous System, Higher Function of the Brain. Vol., F. Plum, eds. American Psychological Society, Bethsda, MD, pp. 373-417.

Goldman-Rakic, P.S., 1990. Cortical localization of working memory. In: Brain Organization and Memory. Vol., J.L. McGaugh, N.M. Weinberger, G. Lynch, eds. Oxford University Press, New York, pp. 285-298.

Goodrick, C., 1968. Learning, retention and extinction of a complex maze habit for mature young and senescent Wistar albino rats. J Gerontol. 8**,** 294-304.

Grady, C.L., McIntosh, A.R., Craik, F.I., 2005. Task-related activity in prefrontal cortex and its relation to recognition memory performance in young and old adults. Neuropsychologia. 43**,** 1466-1481.

Grant, E.R., Guttmann, R.P., Seifert, K.M., Lynch, D.R., 2001. A region of the rat N-methyl-D-aspartate receptor 2A subunit that is sufficient for potentiation by phorbol esters. Neurosci Lett. 310**,** 9-12.

Greenwood, P.M., 2000. The frontal aging hypothesis evaluated. J Int Neuropsychol Soc. 6, 705-726.

Gustafsson, B., Wigstrom, H., AbrahamW.C., Huang, Y.Y., 1987. Long-term potentiation in the hippocampus using depolarizing current pulses as the conditioning stimulus to single volley synaptic potential. J Neurosci. 7: 774-780.

Gutchess, A.H., Welsh, R.C., Hedden, T., Bangert, A., Minear, M., Liu, L.L., Park, D.C., 2005. Aging and the neural correlates of successful picture encoding: frontal activations compensate for decreased medial-temporal activity. Journal of Cognitive Neuroscience. 17**,** 84-96.

Hampshire, A., Gruszka, A., Fallon, S.J., Owen, A.M., 2008. Inefficiency in self-organized attentional switching in the normal aging population is associated with decreased activity in the ventrolateral prefrontal cortex. J. Cogn. Neurosci. 20, 1670-1686.

Harburger, L.L., Lambert, T.J., Frick, K.M., 2007. Age-dependent effects of environmental enrichment on spatial reference memory in male mice. Behav. Brain Res. 185, 43-48.

Harris, E.W., Ganong, A.H., Cotman, C.W., 1984. Long-term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors. Brain Res. 323, 132-137.

Hartley, D.M., Monyer, H., Colamarino, S.A., Choi, D.W., 1990. 7-Chlorokynurenate Blocks NMDA Receptor-Mediated Neurotoxicity in Murine Cortical Culture. Eur J Neurosci. 2**,** 291-295.

Head, D., Kennedy, K.M., Rodrigue, K.M., Raz, N., 2009. Age differences in perseveration: cognitive and neuroanatomical mediators of performance on the Wisconsin Card Sorting Test. Neuropsychologia. 47, 1200-1203.

Heale, V., Harley, C., 1990. MK-801 and AP5 impair acquisition, but not retention, of the Morris milk maze. Pharmacol Biochem Behav. 36**,** 145-149.

Hirai, H., Kirsch, J., Laube, B., Betz, H., Kuhse, J., 1996. The glycine binding site of the N-methyl-D-aspartate receptor subunit NR1: identification of novel determinants of co-agonist potentiation in the extracellular M3-M4 loop region. Proc Natl Acad Sci U S A. 93**,** 6031-6036.

Hof, P.R., Duan, H., Page, T.L., Einstein, M., Wicinski, B., He, Y., Erwin, J.M., Morrison, J.H., 2002. Age-related changes in GluR2 and NMDAR1 glutamate receptor subunit protein immunoreactivity in corticocortically projecting neurons in macaque and patas monkeys. Brain Res. 928**,** 175-186.

Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G., Heinemann, S., 1993. Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. Neuron. 10, 943-954.

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand J Stat. 6, 65-70.

Hood, W.F., Sun, E.T., Compton, R.P., Monaghan, J.B., 1989. 1-aminocyclobutane-1-carboxylate (ACBC): A specific antagonist of the N-methyl-D-aspartate receptor coupled glycine receptor. Eur. J. Pharmac. 161**,** 281-282.

Ikeda, K., Nagasawa, M., Mori, H., Araki, K., Sakimura, K., Watanabe, M., Inoue, Y., Mishina, M., 1992. Cloning and expression of the epsilon 4 subunit of the NMDA receptor channel. FEBS Lett. 313, 34-38.

Ingram, D.K., 1985. Analysis of age-related impairments inlearning and memory in rodent models. Ann NY Acad Sci. 444**,** 312-331.

Inman-Wood, S.L., Williams, M.T., Morford, L.L., Vorhees, C.V., 2000. Effects of prenatal cocaine on Morris and Barnes maze tests of spatial learning and memory in the offspring of C57BL/6J mice. Neurotoxicol Teratol. 22, 547-557.

Ishida, T., Sato, T., Irifune, M., Tanaka, K., Nakamura, N., Nishikawa, T., 2007. Effect of acetaminophen, a cyclooxygenase inhibitor, on Morris water maze task performance in mice. J Psychopharmacol. 21**,** 757-767.

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., et al., 1993. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem. 268, 2836-2843.

Jarvis, M.F., Murphy, D.E., Williams, M., 1987. Quantitative autoradiographic localization of NMDA receptors in rat brain using [3H]CPP: comparison with [3H]TCP binding sites. Eur. J. Pharmacol. 141, 149-152.

Johnson, J.W., Ascher, P., 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature. 325**,** 529-531.

Kametani, H., Bresnahan, E.L., Chachich, M.E., Spangler, E.L., Ingram, D.K., 1989. Comparison of retention performance between young rats with fimbria-fornix lesions and aged rats in a 14-unit T-maze. Behav Brain Res. 35**,** 253-263.

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

Kandel, E.R., Kupfermann, I., Iversen, S., 2000. Learning and Memory. In: Principles of Neural Sciences. Vol., E.R. Kandel, J.H. Schwartz, T.M. Jessel, eds. McGraw-Hill, New York, pp. 1227-1245.

Karp, S.J., Masu, M., Eki, T., Ozawa, K., Nakanishi, S., 1993. Molecular cloning and chromosomal localization of the key subunit of the human N-methyl-D-aspartate receptor. J Biol Chem. 268**,** 3728-3733.

Kessels, R.P., Postma, A., Wijnalda, E.M., de Haan, E.H., 2000. Frontal-lobe involvement in spatial memory: evidence from PET, fMRI, and lesion studies. Neuropsychol Rev. 10**,** 101-113.

Khan, M.J., Seidman, M.D., Quirk, W.S., Shivapuja, B.G., 2000. Effects of kynurenic acid as a glutamate receptor antagonist in the guinea pig. Eur Arch Otorhinolaryngol. 257**,** 177-181.

Kikusui, T., Tonohiro, T., Kaneko, T., 1999. Simultaneous evaluation of spatial working memory and motivation by the allocentric place discrimination task in the water maze in rats. J Vet Med Sci. 61, 673-681.

Kirasic, K.C., Bernicki, M.R., 1990. Acquisition of spatial knowledge under conditions of temporospatial discontinuity in young and elderly adults. Psychol. Res. 52, 76-79.

Kito, S., Miyoshi, R., Nomoto, T., 1990. Influence of age on NMDA receptor complex in rat brain studied by in vitro autoradiography. J Histochem Cytochem. 38, 1725-1731.

Kohama, S.G., Urbanski, H.F., 1997. Distribution of glutamate receptor subunits in the primate temporal cortex and hippocampus. Brain Res. 769, 44-56.

Kolb, B., Buhrmann, K., McDonald, R., Sutherland, R.J., 1994. Dissociation of the medial prefrontal, posterior parietal, and posterior temporal cortex for spatial navigation and recognition memory in the rat. Cereb Cortex. 4**,** 664-680.

Kolb, B., Nonneman, A.J., Singh, R.K., 1974. Double dissociation of spatial impairments and perseveration following selective prefrontal lesions in rats. J Comp Physiol. 87**,** 772-780.

Kolb, B., Sutherland, R.J., Whishaw, I.Q., 1983. A comparison of the contributions of the frontal and parietal association cortex to spatial localization in rats. Behav Neurosci. 97**,** 13-27.

Kral, V.A., 1962. Senescent forgetfulness: benign and malignant. Can Med Assoc J. 86**,** 257-260.

Kringlebach, M.L., 2005. The orbitofrontal cortex: linking reward to hedonic experience. Nature Reviews Neuroscience. 6**,** 691-702.

Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., et al., 1992. Molecular diversity of the NMDA receptor channel. Nature. 358, 36-41.

Larrabee, G.J., Crook, T.H., 3rd, 1994. Estimated prevalence of age-associated memory impairment derived from standardized tests of memory function. Int Psychogeriatr. 6, 95-104.

Laube, B., Kuhse, J., Betz, H., 1998. Evidence for a tetrameric structure of recombinant NMDA receptors. J. Neurosci. 18, 2954-2961.

Laurie, D.J., Putzke, J., Zieglgänsberger, W., Seeburg, P.H., Tölle, T.R., 1995. The distribution of splice variants of the NMDAR1 subunit mRNA in adult rat brain. Brain Res Mol Brain Res. 32**,** 94-108.

Laurie, D.J., Seeburg, P.H., 1994. Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. J Neurosci. 14, 3180-3194.

Le Bourdelles, B., Wafford, K.A., Kemp, J.A., Marshall, G., Bain, C., Wilcox, A.S., Sikela, J.M., Whiting, P.J., 1994. Cloning, functional coexpression, and pharmacological characterisation of human cDNAs encoding NMDA receptor NR1 and NR2A subunits. J Neurochem. 62**,** 2091-2098.

Lehmann, O., Jeltsch, H., Lehnardt, O., Pain, L., Lazarus, C., Cassel, J.C., 2000. Combined lesions of cholinergic and serotonergic neurons in the rat brain using 192 IgG-saporin and 5,7-dihydroxytryptamine: neurochemical and behavioural characterization. Eur J Neurosci. 12, 67-79.

Lei, S.Z., Pan, Z.H., Aggarwal, S.K., Chen, H.S., Hartman, J., Sucher, N.J., Lipton, S.A., 1992. Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. Neuron. 8**,** 1087-99.

Leite-Almeida, H., Almeida-Torres, L., Mesquita, A.R., Pertovaara, A., Sousa, N., Cerqueira, J.J., Almeida, A., 2009. The impact of age on emotional and cognitive behaviours triggered by experimental neuropathy in rats. Pain. 144**,** 57-65.

Leon, W.C., Bruno, M.A., Allard, S., Nader, K., Cuello, A.C., 2010. Engagement of the PFC in consolidation and recall of recent spatial memory. Learn Mem. 17**,** 297-305.

Li, H.B., Matsumoto, K., Tohda, M., Yamamoto, M., Watanabe, H., 1997. NMDA antagonists potentiate scopolamine-induced amnesic effect. Behav. Brain Res. 83**,** 225-228.

Li, L., Shao, J., 1998. Restricted lesions to ventral prefrontal subareas block reversal learning but not visual discrimination learning in rats. Physiol Behav. 65**,** 371-379.

Liang, J., 2004. The influence of morphine GR89696 on NMDA receptor phosphorylation. J Undergrad Res. 5, 5.

Lin, J.W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J.U., Sheng, M., 1998. Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. J Neurosci. 18, 2017-2027.

Loepke, A.W., Istaphanous, G.K., McAuliffe, J.J., 3rd, Miles, L., Hughes, E.A., McCann, J.C., Harlow, K.E., Kurth, C.D., Williams, M.T., Vorhees, C.V., Danzer, S.C., 2009. The effects of neonatal isoflurane exposure in mice on brain cell viability, adult behavior, learning, and memory. Anesth Analg. 108**,** 90-104.

Lohninger, S., Strasser, A., Bubna-Littiz, H., 2001. The effect of l-carnatine on T-maze learning ability in aged rats. Arch Gerontol Geriatr. 32**,** 245-253.

Lynch, D.R., Guttmann, R.P., 2001. NMDA receptor pharmacology: perspectives from molecular biology. Current drug targets. 2**,** 215-231.

Lynch, G., 1998. Memory and the brain: unexpected chemistries and a new pharmacology. Neurobiol Learn Mem. 70, 82-100.

Maei, H.R., Zaslavsky, K., Teixeira, C.M., Frankland, P.W., 2009. What is the Most Sensitive Measure of Water Maze Probe Test Performance? Front Integr Neurosci. 3, 4.

Magnusson, K.R., 1995. Differential effects of aging on binding sites of the activated NMDA receptor complex in mice. Mech. Ageing Dev. 84**,** 227-243.

Magnusson, K.R., 1997. Influence of dietary restriction on ionotropic glutamate receptors during aging in C57B1 mice. Mech Ageing Dev. 95, 187-202.

Magnusson, K.R., 1998a. Aging of glutamate receptors: correlations between binding and spatial memory performance in mice. Mech Ageing Dev. 104, 227-248.

Magnusson, K.R., 1998b. The aging of the NMDA receptor complex. Front Biosci. 3, e70-80.

Magnusson, K.R., 2000. Declines in mRNA expression of different subunits may account for differential effects of aging on agonist and antagonist binding to the NMDA receptor. J Neurosci. 20, 1666-1674.

Magnusson, K.R., 2001. Influence of diet restriction on NMDA receptor subunits and learning during aging. Neurobiol Aging. 22, 613-627.

Magnusson, K.R., Bai, L., Zhao, X., 2005. The effects of aging on different C-terminal splice forms of the zeta1(NR1) subunit of the N-methyl-d-aspartate receptor in mice. Brain Res Mol Brain Res. 135, 141-149.

Magnusson, K.R., Brim, B.L., Das, S.R., 2010. Selective vulnerabilities of N-methyl-D-aspartate (NMDA) receptors during brain aging. Frontiers in Aging Neuroscience. 2**,** 11.

Magnusson, K.R., Cotman, C.W., 1993a. Age-related changes in excitatory amino acid receptors in two mouse strains. Neurobiol. Aging. 14**,** 197-206.

Magnusson, K.R., Cotman, C.W., 1993b. Effects of aging on NMDA and MK801 binding sites in mice. Brain Res. 604**,** 334-337.

Magnusson, K.R., Nelson, S.E., Young, A.B., 2002. Age related changes in the protein expresssion of subunits of the NMDA receptor. Mol Brain Res. 99**,** 40-45.

Magnusson, K.R., Nelson, S.E., Young, A.B., 2002. Age-related changes in the protein expression of subunits of the NMDA receptor. Brain Res Mol Brain Res. 99, 40-45.

Magnusson, K.R., Scruggs, B., Aniya, J., Wright, K.C., Ontl, T., Xing, Y., Bai, L., 2003. Age-related deficits in mice performing working memory tasks in a water maze. Behav Neurosci. 117, 485-495.

Magnusson, K.R., Scruggs, B., Zhao, X., Hammersmark, R., 2007. Age-related declines in a two-day reference memory task are associated with changes in NMDA receptor subunits in mice. BMC Neurosci. 8, 43.

Maguire, E.A., Burgess, N., Donnett, J.G., Frackowiak, R.S., Frith, C.D., O'Keefe, J., 1998. Knowing where and getting there: a human navigation network. Science. 280**,** 921-924.

Malenka, R.C., Nicoll, R.A., 1999. Long-term potentiation--a decade of progress? Science. 285**,** 1870-1874.

Martin, S.J., Clark, R.E., 2007. The rodent hippocampus and spatial memory: from synapses to systems. Cell. Mol. Life Sci. 64, 401-431.

Matsuda, K., Kamiya, Y., Matsuda, S., Yuzaki, M., 2002. Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces calcium permeability. Brain Res Mol Brain Res. 100, 43-52.

Maviel, T., Durkin, T.P., Menzaghi, F., Bontempi, B., 2004. Sites of neocortical reorganization critical for remote spatial memory. Science. 305**,** 96-99.

Mayer, M.L., Armstrong, N., 2004. Structure and function of glutamate receptor ion channels. Annual review of physiology. Annual Review of Physiology. 66**,** 161-181.

Mayer, M.L., Benveniste, M., Patneau, D.K., Vyklicky, L., Jr., 1992. Pharmacologic properties of NMDA receptors. Ann N Y Acad Sci. 648**,** 194-204.

Mayer, M.L., Westbrook, G.L., 1987. Permeation and block of N-methyl-D- aspartic acid receptor channels by divalent cations in mouse cultured central neurones. The Journal of Physiology. 394.

Mayer, M.L., Westbrook, G.L., Guthrie, P.B., 1984. Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature. 309**,** 261-263.

McNaughton, B.L., Barnes, C.A., Meltzer, J., Sutherland, R.J., 1989. Hippocampal granule cells are necessary for normal spatial learning but not for spatially-selective pyramidal cell discharge. Experimental Brain Research. 76**,** 485-496.

Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., Mishina, M., 1992. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature. 357, 70-74.

Mishkin, M., 1964. Perseveration of central sets after frontal lesions in monkeys. In: The Frontal Granular Cortex and Behavior. Vol., J. Warren, K. Ekert, eds. McGraw-Hill, New York, pp. 219-241.

Moffat, S.D., Elkins, W., Resnick, S.M., 2006. Age differences in the neural systems supporting human allocentric spatial navigation. Neurobiology of Aging. 27**,** 965-972.

Mondadori, C., Weiskrantz, L., Buerki, H., Petschke, F., Fagg, G.E., 1989. NMDA receptor antagonists can enhance or impair learning performance in animals. Exp Brain Res. 75, 449-456.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., Seeburg, P.H., 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science. 256**,** 1217-1221.

Moore, T.E., Richards, B., Hood, J., 1984. Aging and the coding of spatial information. J. Gerontol. 39, 210-212.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., Nakanishi, S., 1991. Molecular cloning and characterization of the rat NMDA receptor. Nature. 354**,** 31-37.

Morris, R.G., 1981. Spatial localization doesn't require the presence of local cues. Learn Motiv. 12**,** 239-261.

Morris, R.G., 1989. Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. J. Neurosci. 9, 3040-3057.

Morris, R.G., Anderson, E., Lynch, G.S., Baudry, M., 1986. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature. 319, 774-776.

Morris, R.G.M., Davis, M., 1994. The role of NMDA receptors in learning and memory. In: The NMDA Receptor. Vol., G.L. Collingridge, J.C. Watkins, eds. Oxford University Press, Oxford, pp. 340-375.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., O'Keefe, J., 1982. Place-navigation impaired in rats with hippocampal lesions. Nature. 297**,** 681-683.

Mu, Y., Otsuka, T., Horton, A.C., Scott, D.B., Ehlers, M.D., 2003. Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. Neuron. 40, 581-594.

Nakanishi, N., Axel, R., Shneider, N.A., 1992. Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A. 89, 8552-8556.

Nakanishi, S., 1992. Molecular diversity of glutamate receptors and implications for brain function. Science. 258**,** 597-603.

Nicoll, R.A., Malenka, R.C., 1999. Expression mechanisms underlying NMDA receptor-dependent long-term potentiation. Ann N Y Acad Sci. 868**,** 515-525.

Nicolle, M.M., Baxter, M.G., 2003. Glutamate receptor binding in the frontal cortex and dorsal striatum of aged rats with impaired attentional set-shifting. Eur J Neurosci. 18**,** 3335-3342.

Nicolle, M.M., Bizon, J.L., Gallagher, M., 1996. *In vitro* autoradiography of ionotropic glutamate receptors in hippocampus and striatum of aged Long-Evans rats: Relationship to spatial learning. Neuroscience. 74**,** 741-756.

Nyberg, L., Tulving, E., Habib, R., Nilsson, L.G., Kapur, S., Houle, S., Cabeza, R., McIntosh, A.R., 1995. Functional brain maps of retrieval mode and recovery of episodic information. Neuroreport. 7, 249-252.

O'Donnell, K.A., Rapp, P.R., Hof, P.R., 1999. Preservation of prefrontal cortical volume in behaviorally characterized aged macaque monkeys. Exp Neurol. 160, 300-310.

O'Keefe, J., Dostrovsky, J., 1971. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Research. 34**,** 171-175.

Olton, D.S., 1983. Memory functions and the hippocampus. In: Neurobiology of the Hippocampus. Vol., W. Seifert, eds. Academic Press, London, pp. 335-373.

Olton, D.S., Samuelson, R.J., 1976. Remembrane of places passed: Spatial memory in rats. Journal of Experimental Psychology: Animal behavior processes. 2**,** 97-116.

Olton, D.S., Walker, J.A., Gage, F.H., 1978. Hippocampal connections and spatial discrimination. Brain Research. 139**,** 295-308.

Ontl, T., Xing, Y., Bai, L., Kennedy, E., Nelson, S., Wakeman, M., Magnusson, K., 2004. Development and aging of N-methyl-D-aspartate receptor expression in the prefrontal/frontal cortex of mice. Neuroscience. 123, 467-479.

Paoletti, P., Neyton, J., 2007. NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol. 7**,** 39-47.

Paradiso, S., Crespo Facorro, B., Andreasen, N.C., O'Leary, D.S., Watkins, L.G., Boles Ponto, L., Hichwa, R.D., 1997. Brain activity assessed with PET during recall of word lists and narratives. Neuroreport. 8, 3091-3096.

Paxinos, G., Franklin, K.B.J., 2001. The mouse brain in stereotaxic coordinates, Vol., Academic Press, London.

Pelleymounter, M.A., Beatty, G., Gallagher, M., 1990. Hippocampal 3H-CPP binding and spatial learning deficits in aged rats. Psychobiology. 18**,** 298-304.

Penner, M.R., Barnes, C.A., 2007. Memory changes with age: Neurobiological correlates. In: Neurobiology of learning and memory. Vol., R.P. Kesner, J.L. Martinez, eds. Academic Press, New York, pp. 483-518.

Persson, J., Nyberg, L., Lind, J., Larsson, A., Nilsson, L.G., Ingvar, M., Buckner, R.L., 2006. Structure-function correlates of cognitive decline in aging. Cereb Cortex. 16, 907-915.

Premkumar, L.S., Auerbach, A., 1997. Stoichiometry of recombinant N-methyl-D-aspartate receptor channels inferred from single-channel current patterns. J Gen Physiol. 110**,** 485-502.

Prybylowski, K.L., Wolfe, B.B., 2000. Developmental differences in alternative splicing of the NR1 protein in rat cortex and cerebellum. Brain Res Dev Brain Res. 123, 143-150.

Quinlan, E.M., Lebel, D., Brosh, I., Barkai, E., 2004. A molecular mechanism for stabilization of learning-induced synaptic modifications. Neuron. 41, 185-192.

R Development Core Team, 2007. R: A language and environment for statistical computing. Vienna, Austria. (available online at http://www.r-project.org).

Ragozzino, M.E., Detrick, S., Kesner, R.P., 1999a. Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. J Neurosci. 19**,** 4585-4594.

Ragozzino, M.E., Wilcox, C., Raso, M., Kesner, R.P., 1999b. Involvement of rodent prefrontal cortex subregions in strategy switching. Behav Neurosci. 113**,** 32-41.

Rapp, P.R., Rosenberg, R.A., Gallagher, M., 1987. An evaluation of spatial information processing in aged rats. Behav Neurosci. 101, 3-12.

Roberson, E.D., English, J.D., Sweatt, J.D., 1996. A biochemist's view of long-term potentiation. Learn Mem. 3, 1-24.

Rock, D.M., MacDonald, R.L., 1992a. Spermine and related polyamines produce a voltage-dependent reduction of N-methyl-D-aspartate receptor single-channel conductance. Mol Pharmacol. 42**,** 157-164.

Rock, D.M., Macdonald, R.L., 1992b. The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. Mol Pharmacol. 41**,** 83-88.

Salthouse, T.A., 2003. Memory aging from 18 to 80. Alzheimer Dis Assoc Disord. 17, 162-167.

Saywell, N., Taylor, D., 2008. The role of the cerebellum in procedural learning – are there implications for physiotherapists' clinical practice? Physiother Theory Practice. 24**,** 321-328.

Scherzer, C.R., Landwehrmeyer, G.B., Kerner, J.A., Counihan, T.J., Kosinski, C.M., Standaert, D.G., Daggett, L.P., Velicelebi, G., Penney, J.B., Young, A.B., 1998. Expression of N-methyl-D-aspartate receptor subunit mRNAs in the human brain: hippocampus and cortex. J Comp Neurol. 390**,** 75-90.

Schorge, S., Colquhoun, D., 2003. Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. J Neurosci. 23**,** 1151-1158.

Schwabe, K., Enkel, T., Klein, S., Schutte, M., Koch, M., 2004. Effects of neonatal lesions of the medial prefrontal cortex on adult rat behaviour. Behav. Brain Res. 153, 21-34.

Scott, D.B., Blanpied, T.A., Swanson, G.T., Zhang, C., Ehlers, M.D., 2001. An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. J. Neurosci. 21, 3063-3072.

Sperling, G., 1960. The information available in brief visual presentations. Psychological Monographs: General and Applied. 74**,** 1-29.

Standley, S., Roche, K.W., McCallum, J., Sans, N., Wenthold, R.J., 2000. PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. Neuron. 28, 887-898.

Steele, R.J., Morris, R.G., 1999. Delay-dependent impairment of a matching-to-place task with chronic and intrahippocampal infusion of the NMDA-antagonist D-AP5. Hippocampus. 9, 118-136.

Stephenson, F.A., Cousins, S.L., Kenny, A.V., 2008. Assembly and forward trafficking of NMDA receptors. Molecular Membrane Biology. 25**,** 311-320.

Stratmann, G., Sall, J.W., Bell, J.S., Alvi, R.S., May, L.V., Ku, B., Dowlatshahi, M., Dai, R., Bickler, P.E., Russell, I., Lee, M.T., Hrubos, M.W., Chiu, C., 2010. Isoflurane does not affect brain cell death, hippocampal neurogenesis, or long-term neurocognitive outcome in aged rats. Anesthesiology. 112**,** 305-315.

Sugihara, H., Moriyoshi, K., Ishii, T., Masu, M., Nakanishi, S., 1992. Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. Biochem Biophys Res Commun. 185, 826-832.

Sun, W., Mercado, E., 3rd, Wang, P., Shan, X., Lee, T.C., Salvi, R.J., 2005. Changes in NMDA receptor expression in auditory cortex after learning. Neurosci Lett. 374, 63-68.

Takehara, K., Kawahara, S., Kirino, Y., 2003. Time-dependent reorganization of the brain components underlying memory retention in trace eyeblink conditioning. J Neurosci. 23**,** 9897-9905.

Tamaru, M., Yoneda, Y., Ogita, K., Shimizu, J., Nagata, Y., 1991. Age-related decreases of the N-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus. Brain Res. 542, 83-90.

Tashiro, A., Sandler, V.M., Toni, N., Zhao, C., Gage, F.H., 2006. NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. Nature. 442**,** 929-933.

Thakker, D.R., Natt, F., Husken, D., Maier, R., Muller, M., van der Putten, H., Hoyer, D., Cryan, J.F., 2004. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. Proc Natl Acad Sci U S A. 101**,** 17270-17275.

Thakker, D.R., Natt, F., Husken, D., van der Putten, H., Maier, R., Hoyer, D., Cryan, J.F., 2005. siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. Mol Psychiatry. 10**,** 782-789

Tichelaar, W., Safferling, M., Keinanen, K., Stark, H., Madden, D.R., 2004. The Three-dimensional Structure of an Ionotropic Glutamate Receptor Reveals a Dimer-of-dimers Assembly. Journal of molecular biology 344(2):435-442. Journal of Molecular Biology. 344**,** 435-442.

Timiras, P.S., 2003. The nervous system: functional changes. In: Physiological basis of aging and geriatrics. Vol., P.S. Timiras, eds. CRC Press, Boca Raton, pp. 119-140.

Tingley, W.G., Ehlers, M.D., Kameyama, K., Doherty, C., Ptak, J.B., Riley, C.T., Huganir, R.L., 1997. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J Biol Chem. 272, 5157-5166.

Tingley, W.G., Roche, K.W., Thompson, A.K., Huganir, R.L., 1993. Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. Nature. 364**,** 70-73.

Tisserand, D.J., Jolles, J., 2003. On the involvement of prefrontal networks in cognitive ageing. Cortex. 39, 1107-1128.

Traynelis, S.F., Cull-Candy, S.G., 1991. Pharmacological properties and H+ sensitivity of excitatory amino acid receptor channels in rat cerebellar granule neurones. J Physiol. 433**,** 727-763.

Traynelis, S.F., Hartley, M., Heinemann, S.F., 1995. Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. Science. 268, 873-876.

Tsein, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R., Tonegawa, S., 1996a. Subregion- and cell type-restricted gene knockout in mouse brain. Cell. 87**,** 1317-1326.

Tsien, J.Z., Huerta, P.T., Tonegawa, S., 1996b. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell. 87**,** 1327-1338.

Ulbrich, M.H., Isacoff, E.Y., 2008. Rules of engagement for NMDA receptor subunits. Proc Natl Acad Sci U S A. 105**,** 14163-14168.

United Nations Department of Economic and Social Affairs, Population Division, 2007. World population ageing, United Nations, New York.

Wang, H., Ferguson, G.D., Pineda, V.V., Cundiff, P.E., Storm, D.R., 2004. Overexpression of type-1 adenylyl cyclase in mouse forebrain enhances recognition memory and LTP. Nat Neurosci. 7**,** 635-642.

Wang, H., Hu, Y., Tsien, J. G., 2006. Molecular and systems mechanisms of memory consolidation and storage. Prog Neurobiol. 79, 123-135.

Watanabe, M., Inoue, Y., Sakimura, K., Mishina, M., 1993. Distinct distributions of five N-methyl-D-aspartate receptor channel subunit mRNAs in the forebrain. J Comp Neurol. 338, 377-390.

Wigstrom, H., Gustafsson, B., 1985. On long-lasting potentiation in the hippocampus: a proposed mechanism for its dependence on coincident pre- and postsynaptic activity. Acta Physiol Scand. 123**,** 519-522.

Wigstrom, H., Gustafsson, B., Huang, Y.Y., Abraham, W.C., 1986. Hippocampal long-term potentiation is induced by pairing single afferent volleys with intracellularly injected depolarizing current pulses. Acta Physiol Scand. 126: 317-319.

Williams, K., 1997. Interactions of polyamines with ion channels. Biochem J. 325 (Pt 2)**,** 289-297.

Wilson, M.A., McNaughton, B.L., 1993. Dynamics of the hippocampal ensemble code for space. Science. 261**,** 1055-1058.

Winocur, G., 1992. A comparison of normal old rats and young adult rats with lesions of the hippocampus or prefrontal cortex on a test of matching-to-sample. Neuropsychologia. 30**,** 769-781.

Yamakura, T., Mori, H., Masaki, H., Shimoji, K., Mishina, M., 1993. Different sensitivities of NMDA receptor channel subtypes to non-competitive antagonists. Neuroreport. 4**,** 687-690.

Yamazaki, M., Mori, H., Araki, K., Mori, K.J., Mishina, M., 1992. Cloning, expression and modulation of a mouse NMDA receptor subunit. FEBS Lett. 300**,** 39-45.

Yoon, S.O., Lois, C., Alvirez, M., Alvarez-Buylla, A., Falck-Pedersen, E., Chao, M.V., 1996. Adenovirus-mediated gene delivery into neuronal precursors of the adult mouse brain. Proc Natl Acad Sci U S A. 93**,** 11974-11979.

Zhao, M.-G., Toyoda, H., Lee, Y.-S., Wu, L.-J., Ko, S.W., Zhang, X.-H., Jia, Y., Shum, F., Xu, H., Li, B.-M., Kaang, B.-K., Zhuo, M., 2005. Roles of NMDA NR2B subtype receptor in prefrontal long-term potentiation and contextual fear memory. Neuron. 47**,** 859-872.

Zhao, X., Rosenke, R., Kronemann, D., Brim, B., Das, S.R., Dunah, A.W., Magnusson, K.R., 2009. The effects of aging on N-methyl-D-aspartate

receptor subunits in the synaptic membrane and relationships to long-term spatial memory. Neuroscience. 162, 933-945.

Zheng, X., Zhang, L., Durand, G.M., Bennett, M.V., Zukin, R.S., 1994. Mutagenesis rescues spermine and Zn2+ potentiation of recombinant NMDA receptors. Neuron. 12, 811-818.

Zhong, J., Carrozza, D.P., Williams, K., Pritchett, D.B., Molinoff, P.B., 1995. Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem. 64, 531-539.

Zukin, R.S., Bennett, M.V., 1995. Alternatively spliced isoforms of the NMDARI receptor subunit. Trends Neurosci. 18, 306-313.

APPENDIX

Table 1: Pearson correlation coefficients for mRNA expression of the different splice forms in different brain regions of the prefrontal and frontal cortex in 26-month old behaviorally-characterized mice and reference memory performance.

Cortical regions		GluN1-pan	$GluN1_{X11}$	$GluN1_{X10}$	$GluN1_{0XX}$	$GluN1_{1XX}$
Ins. cor.	-deep	-0.14	-0.24	-0.06	0.07	0.63
	-superficial	0.41	-0.21	0.25	0.06	0.34
VO cor.	-deep	0.04	0.12	-0.64	-0.46	-0.56
	-superficial	0.17	-0.27	-0.70	-0.60	-0.14
LO cor.	-deep	0.10	-0.04	-0.61	-0.54	-0.42
	-superficial	-0.21	-0.42	-0.74*	-0.76*	-0.32
MO cor.	-deep	0.41	0.02	-0.17	0.09	0.13
	-superficial	0.04	-0.15	-0.28	-0.14	-0.02
MII cor.	-deep	0.34	0.06	-0.28	0.25	0.25
	-superficial	0.63	0.12	0.54	0.49	0.49
MI cor.	-deep	0.37	0.09	0.12	0.31	0.36
	-superficial	0.67	0.34	0.19	0.45	0.50
SI cor.	-deep	-0.01	0.11	-0.27	0.34	0.55
	-superficial	0.25	0.21	0.21	0.36	0.69*

* Uncorrected $p < .05$

deep= cortical layers IV-VI; superficial= cortical layers II-III; cor = cortex; Ins = insular; VO = ventral orbital; LO = lateral orbital; MO = medial orbital; MII = secondary motor; M1 = primary motor; SI = somatosensory cortex