AN ABSTRACT OF THE DISSERTATION OF

Brenna L. Brim for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on September 11, 2012.
Title: The Effects of Enhanced Expression of the GluN2B (NR2B) Subunit of the N-methyl-D-aspartate (NMDA) Receptor on Memory in Aged Animals.

Abstract approved ______________________________________________________________________

Kathy R. Magnusson

As the aging population continues to grow worldwide, age-related complications are becoming more apparent within the aging population. One of the first age-related complications to become apparent is age-associated memory impairment and it can make the elderly more dependent on caregivers early on. The N-methyl-D-aspartate (NMDA) receptor is important to learning and memory and appears to be especially vulnerable to the process of aging. The density of NMDA receptors declines with age more than any other ionotropic glutamate receptor. Both the density of NMDA receptors and the mRNA and protein expression of its subunits decline with age. In particular, the GluN2B subunit of the NMDA receptor shows the greatest age-related declines in expression across multiple brain regions, including the frontal lobe (including the prefrontal and frontal cortices), caudate nucleus and hippocampus. These declines are strongly correlated to age-related declines in spatial memory. Specifically, age-related decreases in the protein expression of the GluN2B subunit within crude synaptosomes of the frontal cortex of C57BL/6 mice show a relationship to the declines in performance in a long-term spatial memory task across age groups. However, within the population of aged mice, there was a subpopulation of aged mice in which higher expression of the GluN2B subunit within the synaptic membrane of the hippocampus was associated with poorer performance in the same task. Moreover, transgenic mice designed to express higher
levels of the GluN2B subunit from birth also possess superior memory, including spatial memory, across adulthood to middle-age. Taken together, these data led to the hypothesis that increasing the expression of the GluN2B subunit within the aged brain could potentially alleviate age-related declines in memory. However, increasing its expression regionally was first examined since higher expression of the GluN2B subunit within the hippocampus has been associated with poorer memory in aged animals.

Since age-related decreases in the protein expression of the GluN2B subunit within the frontal cortex show a relationship to impaired memory function, the first study was designed to determine if increasing GluN2B subunit expression in the frontal lobe would improve memory in aged mice. Mice received bilateral injections of either an adenoviral vector, containing cDNA specific for the GluN2B subunit and enhanced Green Fluorescent Protein (eGFP) (GluN2B vector); an adenoviral vector containing only the cDNA for eGFP (control vector); or vehicle into their frontal lobe. Spatial memory, cognitive flexibility and associative memory were assessed using the Morris water maze. Aged mice, with increased GluN2B subunit expression in the frontal lobe, exhibited improved long-term spatial memory, comparable to young mice, in the second day of training. Moreover, a higher concentration of the specific GluN2B antagonist, Ro 25-6981, was required to impair long-term spatial memory in aged mice with enhanced GluN2B subunit expression, as compared to aged controls. The requirement for greater antagonism in aged mice to block memory performance suggests that the number of GluN2B-containing receptors in their frontal lobe was enhanced and contributed to the improved memory. This study provides suggestive evidence that therapies that enhance GluN2B subunit expression within the aged brain could have the potential to ameliorate age-related memory loss.
Since higher expression of the GluN2B subunit within the hippocampus of aged mice is associated with poorer memory, the second study was designed to determine if increasing GluN2B subunit expression in the hippocampus would improve or further impair memory in aged mice. This would help to determine if a therapy aimed at enhancing the GluN2B subunit expression or function of GluN2B-containing receptors throughout the aged brain could help ameliorate age-associated memory loss. Mice were injected bilaterally with either the GluN2B vector, a control vector or vehicle into the hippocampus. Spatial memory, cognitive flexibility and associative memory were assessed using the Morris water maze. Aged mice, with increased GluN2B subunit expression in the hippocampus, exhibited improved long-term spatial memory, comparable to young mice, early in training. However, there was a trend for impaired memory later in the long-term spatial memory trials. Still, these data suggest that enhancing GluN2B subunit expression in the aged hippocampus could be more beneficial to memory than harmful. In addition, the results of this study suggest that enhancing GluN2B subunit expression in different brain regions may improve memory at different phases of learning. Therefore, therapies that enhance GluN2B subunit expression throughout the aged brain could help ameliorate age-related memory loss.

The first two studies demonstrated that enhancing the expression of the GluN2B subunit within either the frontal lobe or hippocampus of the aged brain has the potential to reduce age-related memory declines. However, the increase was not global nor specific to the synapse. Therefore, a third study was developed with the intent of garnering a more global increase in GluN2B subunit expression that was localized to the synapse. Cyclin dependent kinase 5 (Cdk5) enhances endocytosis of the GluN2B subunit-containing NMDA receptors from the synapse. Previous research has shown that inhibiting Cdk5 increases the number of GluN2B subunits at the synapse and within the whole cell and improves memory in young mice. This
study was designed to determine if using antisense phosphorodiamidate morpholino oligomers (Morpholinos) to decrease the expression of Cdk5 protein within the brain would improve memory in aged mice. Morpholinos were conjugated to a cell penetrating peptide, which enhances cellular uptake, and delivered bilaterally to the lateral ventricles of both young and aged mice via acute stereotaxic injection. Treatments consisted of equivalent volumes and concentrations of either vehicle, control Morpholino or a Morpholino targeting the mRNA of Cdk5 (Cdk5 Morpholino). Memory was evaluated in the Morris water maze and using a novel object recognition task. Aged mice treated with the Cdk5 Morpholino exhibited improved early acquisition and spatial bias in the long-term spatial memory trials, as well as improved performance overall, compared to control Morpholino-treated aged animals. However, aged mice treated with the Cdk5 Morpholino performed similarly to vehicle-treated aged animals. The presence of the peptide-conjugated Morpholinos within the brain may have worsened performance in the Morris water maze task since control Morpholino-treated animals performed significantly worse than vehicle-treated animals. In concurrence, there was significantly greater gliosis in peptide-conjugated Morpholino-treated animals over vehicle-treated brains, suggesting it was neurotoxic. In contrast, young mice treated with the Cdk5 Morpholino showed impaired early acquisition and spatial bias but a trend for improved later learning in the long-term spatial memory task compared to control Morpholino-treated animals. Treatment with the Cdk5 Morpholino had no significant effect on cognitive flexibility, associative memory or novel object recognition for young or aged animals. Immunohistochemistry revealed increased GluN2B subunit expression within cells with characteristics of neurons and astroglia in regions of the frontal lobe, caudate nucleus and hippocampus of aged mice who received the Cdk5 Morpholino compared to control treatments. However, the increased GluN2B subunit expression appeared to be greater within
the hippocampus. These results suggest that inhibiting the translation of Cdk5 using Morpholinos increased GluN2B subunit expression in both young and aged mice and may have contributed to the improved long-term spatial memory observed in aged mice, despite the Morpholino being administered at a presumably toxic concentration. An additional group of mice was used to determine a non-neurotoxic dosage of the peptide conjugated Morpholino. However, future studies are needed to determine the efficacy of the Cdk5 Morpholino at this dosage.

Taken together, the studies presented here suggest that increasing expression of the GluN2B subunit within the aged brain does improve age-associated memory declines. In addition, cell penetrating peptide-conjugated Morpholinos show promise as tools for genetic manipulation within the brain and Cdk5 could prove to be a novel target for enhancing GluN2B subunit expression within the aged brain. Though future studies are needed, the studies presented here do suggest that therapies that enhance GluN2B subunit expression within the aged brain have the potential to help ameliorate memory loss. However, since enhanced GluN2B subunit expression itself can increase the potential for excitotoxicity, an optimal dose of such a therapeutic would need to be determined.
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The Effects of Enhanced Expression of the GluN2B (NR2B) Subunit of the N-methyl-D-aspartate (NMDA) Receptor on Memory in Aged Animals.

by
Brenna L. Brim

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APPROVED:

Major Professor, representing the 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.

Brenna L. Brim, Author
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CONTRIBUTION OF AUTHORS

Chapter I: Conceptualized, prepared and edited by Brenna L. Brim and Dr. Kathy Magnusson.

Chapter II: Experiments were performed and the manuscript was prepared by Brenna L. Brim. The project was conceptualized by and the manuscript was edited by Dr. Kathy Magnusson. Authors: B. L. Brim, R. Haskell, R. Awedikian, N.M. Ellinwood, L. Jin, K. Magnusson.

Chapter III: Experiments were performed and the manuscript was prepared by Brenna L. Brim. The project was conceptualized by and the manuscript was edited by Dr. Kathy Magnusson. Authors: B. L. Brim, R. Haskell, R. Awedikian, N.M. Ellinwood, L. Jin, K. Magnusson.

Chapter IV: Experiments were performed and the manuscript was prepared by Brenna L. Brim. The project was conceptualized by Brenna L. Brim and the manuscript was edited by Dr. Kathy Magnusson. Authors: B. L. Brim, H. Moulton, V. Elias, K. Magnusson.

Chapter V: Conceptualized, prepared and edited by Brenna L. Brim and Dr. Kathy Magnusson.
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This work is dedicated to my beloved friend and husband,

Ryan David Brim.
THE EFFECTS OF ENHANCED EXPRESSION OF THE GLUN2B (NR2B) SUBUNIT OF THE N-METHYL-D-ASPARTATE (NMDA) RECEPTORS ON MEMORY IN AGED ANIMALS.
1.1 Aging and memory

As lifespan increases worldwide, especially in the developed world, so does the elderly population (UN 2005). It is predicted that by 2050, one in every three people in the developed world will be over sixty-five years of age (UN 2005). Aging is associated with multiple functional declines, including declines in strength, balance, motor coordination, cognitive flexibility and memory (Albert 1994; Timiras 2003). One of the earliest cognitive functions to show decline with age is memory. Memory deterioration is evident by the fifth decade in humans and is associated with significant impairment in memory recall (Albert and Funkenstein 1992; Singh-Manoux, Kivimaki et al. 2012). Memory decline can range in severity from normal age-associated memory loss to mild cognitive impairment (MCI) to various forms of dementia (Small, Gagnon et al. 2007; Albert 2011). Although memory decline is not uniform across individuals, it poses a serious risk to the quality of life of aging individuals and can predispose them to entering care facilities early, creating a huge economic burden (Glisky 2007; Okura and Langa 2011). Moreover, aging individuals who experience the more severe age-associated memory impairment, called “mild cognitive impairment”, are believed to be at greater risk for developing dementia later in life (Bowen, Teri et al. 1997; Levey, Lah et al. 2006). Given the importance of age-related memory decline, it is crucial to understand the molecular mechanisms that underlie age-associated memory impairment and determine possible interventions that could better prevent and even repair age-related memory deficits. The research presented here attempted to further understand the molecular mechanisms underlying memory decline during normal aging and the potential for possible interventions. This research is of importance because it could benefit our ever growing aging population by reducing the deficits associated with normal aging and may even help delay some of the debilitating effects of more severe forms age-related memory impairment, like mild cognitive impairment and Alzheimer’s disease.
1.2 Memory

Memory is the cognitive process by which information is encoded, stored and retrieved within the brain (Kandel, Kupfermann et al. 2000). The process of encoding information into a construct that can be stored within the brain and recalled later is termed acquisition. Consolidation (or storage) refers to the processes that stabilize a memory after the initial acquisition. Memory retrieval is the process by which events or information are recalled from the past (McGaugh 1966). Consolidation can include reconsolidation, in which previously consolidated memories can be made labile again through reactivation of the memory trace (Nader, Schafe et al. 2000). Memory can be categorized, by duration, into short-term and long-term memory. Short-term memory can be defined as remembering information over a period of several seconds to several minutes (Atkinson and Shiffrin 1971). Working memory is related to short-term memory in terms of duration but is distinct. Working memory can be defined as the processes that are used to temporarily store, organize and manipulate information. Short-term memory, on the other hand, refers only to the temporary storage of information in memory (Atkinson and Shiffrin 1968; Baddeley and Hitch 1974). Long-term memory can be defined as remembering information over a period of several days to months or years (Atkinson and Shiffrin 1971). Long-term memory can be further categorized by the level of consciousness into explicit (declarative; conscious) and implicit (non-declarative; unconscious) memory (Squire 1987). Explicit memory can be further subdivided into episodic memory (event-based memory) and semantic memory (fact-based memory (Eichenbaum 2000). Implicit memory can be further subdivided into priming (where exposure to one stimulus influences a response to a later stimulus), procedural, associative and non-associative memory (Graf and Schacter 1985) (Figure 1.1).

Of these above described forms of memory, working memory and explicit long-term spatial memory were the focus of the present studies, due to the ease with which they can be characterized in rodent models (refer to section 1.3). Moreover, spatial memory, which is responsible for the navigation of organisms
within their environment, is particularly affected by the aging process (Barnes 1988). Object recognition memory, another form of explicit memory, was also assessed in the third study presented here (refer to section 1.4).

1.3 Characterization of spatial memory in rodent models

Not all types of human memory can be assessed in animal models because of the differences in cognitive functions, particularly the absence of language skills. As a result, it has been difficult to investigate all the age-related declines in human memory in animal models. However, similar to humans, animals experience declines in spatial memory, a memory function that is important for successful navigation of an environment (Birren 1962; Gaylord and Marsh 1975; Barnes, Nadel et al. 1980; Wallace, Krauter et al. 1980; Perlmutter, Metzger et al. 1981; Gage, Dunnett et al. 1984; Rapp, Rosenberg et al. 1987; Gallagher and Pelleymounter 1988).

Spatial memory tasks have been used extensively for characterizing age-related declines in learning and memory (Goodrick 1968; Barnes 1979; Morris 1981; Ingram 1985; Lohninger, Strasser et al. 2001). Several spatial memory tasks such as the multiple T-maze (Goodrick 1968; Ingram 1985; Lohninger, Strasser et al. 2001), the Barnes maze (Barnes 1979), the eight-arm radial maze (Olton and Samuelson 1976), and the Morris water maze (Morris 1981), have been developed by researchers to assess spatial memory, primarily in rodents. Of these different maze-learning tasks, the Morris water maze has been most extensively used to assess age-related declines in memory (Penner and Barnes 2007). The Morris water maze typically consists of a large circular tank filled with opaque water and containing an escape platform hidden under the surface of the water (Morris 1981; Morris, Garrud et al. 1982) (Fig. 1.2A-C). Distal spatial cues, used as landmarks to locate the escape platform, are placed around the room and tank (Morris 1981; Morris, Garrud et al. 1982). On each trial, an animal is placed into the water (from different locations in the tank) and allowed to swim until either the escape platform is found or a specified amount
of time has elapsed (Morris 1981; Morris, Garrud et al. 1982) (Fig. 1.2A-B). After performing the task several times, young animals learn to find the escape platform and reach it more efficiently (Rapp, Rosenberg et al. 1987). In contrast, aged animals will spend a longer period of time searching and may require more trials before they reach it efficiently (Rapp, Rosenberg et al. 1987). Cumulative or average proximity measures can be used to quantify an animal’s spatial memory. These values are not based on latency to the platform but on the path taken and time spent in the water to reach the platform (Gallagher 1993). Values based on latency are biased toward young animals, which swim faster than their aged counterparts (Gallagher 1993). After multiple trials, the platform is removed and the animal’s spatial bias for the location of the escape platform is measured (Morris 1981; Morris, Garrud et al. 1982) (Fig. 1.2B). The animals that have learned the task and developed a spatial bias will spend considerably more time near the former location of the escape platform (Morris 1981; Morris, Garrud et al. 1982).

A variety of forms of spatial memory or related cognitive functions can be assessed using the Morris water maze by altering the task procedure. Specifically, long-term spatial memory can be characterized by keeping the escape platform in the same quadrant for several days (Morris 1984). Working spatial memory can be characterized by changing the escape 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 (Morris 1984; Means and Kennard 1991; van der Staay and de Jonge 1993). To assess cognitive flexibility, which also deteriorates with age, a reversal task can be employed (Stephens, Weidmann et al. 1985). In a reversal task, the escape platform is placed in the quadrant opposite to the previously learned position used to characterize spatial long-term memory (Morris 1984).

Associative memory, which is believed to not be age or NMDA receptor-dependent, can also be assessed in the Morris water maze and is typically used as a control task (Morris, Garrud et al. 1982; Morris 1984; Rapp, Rosenberg et al. 1987; Mei, Li et al. 2011). For this control task, all spatial cues are removed and
the escape platform is made visible with a flag (Morris 1984) (Fig. 1.2C). If a significant difference in performance is found within this control task, it may mean that animals have differences in motor ability, motivation, and/or visual acuity (Morris 1984). As a result, their performance in the other Morris water maze tasks cannot be interpreted as spatial memory (Morris 1984).

The Morris water maze was used to characterize long-term spatial memory, working spatial memory, cognitive flexibility and/or associative memory in one or more studies presented here. It should be noted that there are several limitations of the Morris water maze which may reduce the ability to detect differences in learning and memory independent of confounding factors. One of the greatest limitations of the Morris water maze is the stressful nature of the paradigm. Several studies confirm the stressful nature of the Morris water maze and the negative impact of glucocorticoids on performance (Barrett, Bennie et al. 2009; Harrison, Hosseini et al. 2009). Another potential limitation is that poor swimming ability could be mistaken for impairment in spatial memory (Whishaw and Tomie 1996).

1.4 Characterization of object recognition memory in rodent models

In addition to spatial memory, object recognition memory can also be characterized in rodent models (Ennaceur and Delacour 1988). Similar to spatial memory, both humans and animals can experience declines in object recognition memory, a memory function that allows individuals to recognize previously encountered objects (Flicker, Ferris et al. 1987; Wallace, Frankfurt et al. 2007; Wimmer, Hernandez et al. 2011). Novel object recognition tasks have been used for characterizing age-related declines in object recognition memory, which is a form of explicit memory (Bartolini, Casamenti et al. 1996; Liu, Gliddon et al. 2004; Wallace, Frankfurt et al. 2007; Wimmer, Hernandez et al. 2011). For rodents, novel object recognition tasks consist of different objects being placed in an arena, always in the same location (Ennaceur and Delacour 1988) (Figure 1.3A-C). Animals are habituated to the empty arena for a certain period of time and then familiarized to two identical objects (Ennaceur and Delacour 1988) (Figure 1.3 A-
B). After 1 and 24 hours, a familiar and a novel object are placed in the arena and the mice are allowed to explore the objects for a certain amount of time (Ennaceur and Delacour 1988; Carlini, Martini et al. 2008) (Figure 1.3C). The length of time spent exploring each object is manually recorded. Exploration is generally defined as time spent with head oriented toward the object and in close proximity to the object (Benice, Rizk et al. 2006). After familiarization, young animals remember the familiar object and so spend more time exploring the novel object (Wallace, Frankfurt et al. 2007; Wimmer, Hernandez et al. 2011). In contrast, aged animals will spend less time exploring the novel object than the young animals (Bartolini, Casamenti et al. 1996; Wallace, Frankfurt et al. 2007; Wimmer, Hernandez et al. 2011). The percentage of time exploring the novel objects can be used to quantify an animal’s object recognition memory (Carlini, Martini et al. 2008).

The novel object recognition task was used to characterize memory, in addition to the Morris water maze. However, it was not used in all of the studies presented here because it has only recently been developed and employed in our lab. A benefit of the novel object recognition task is that it is less stressful than the Morris water maze; however, it still has potential limitations. The greatest potential limitation of this task is that it is difficult to interpret whether the animal is exhibiting novelty preference or a preference for familiarity. Rodents generally exhibit a preference for novelty (Mumby 2002). However, preference for familiarity over novelty has been shown in humans in the visual paired comparisons task (Richmond, Colombo et al. 2007), and in rats in the open-field free exploration (Sheldon 1969). Therefore, novelty preference may not always be the most accurate index for object recognition memory.

### 1.5 Anatomical basis of memory

Specific brain regions are important for the acquisition, consolidation and retrieval of memory. In particular, regions of the frontal lobe and hippocampus are crucial for learning and memory. Early lesion studies in humans showed that the loss of areas within the frontal lobe (including prefrontal cortices) or
hippocampus resulted in impaired memory function (Milner 1959; Ghent, Mishkin et al. 1962; Corkin, Milner et al. 1964). Animal lesion studies have been used since to further elucidate the contributions of the sub-regions of the frontal lobe and hippocampus to learning and memory.

Lesions of the frontal lobe, including medial and orbital cortices, result in deficits in both spatial long- and working memory in the radial arm maze and the Morris water maze (Kolb, Sutherland et al. 1983; Kolb, Buhrmann et al. 1994). Working memory is maintained by reverberating circuits of neurons involved communication between the prefrontal cortex and other regions of the brain, including the sensory cortices (Funahashi, Inoue et al. 1993; Goldman-Rakic 1996; Jones and Wilson 2005). In particular, the frontal lobe, including medial prefrontal cortex, has been shown to be necessary for the encoding of short-term memories as well as contributing to long-term memory by maintaining the learned information (Granon, Vidal et al. 1994; Kessels, Postma et al. 2000). Specifically, the medial prefrontal cortex has been shown to be important for the retrieval of spatial information stored in the hippocampus (Jo, Park et al. 2007). The orbital, as well as the medial, prefrontal cortex has also been shown to be important for cognitive flexibility, (i.e. the ability to switch a behavioral response according to the context of a situation) (de Bruin JP 1994; Kim and Ragozzino 2005; Bissonnette, Martins et al. 2008) (Figure 1.4).

The hippocampus is responsible for the consolidation of less stable short-term memories into more stable long-term memories, which can be stored in the frontal lobe (Takehara, Kawahara et al. 2003; Frankland, Bontempi et al. 2004; Maviel, Durkin et al. 2004). In particular, spatial long-term memory has been shown to require the hippocampus in a variety of studies using hippocampal lesions (Olton, Walker et al. 1978; Morris, Garrud et al. 1982; Aggleton, Hunt et al. 1986; McNaughton, Barnes et al. 1989). In particular, selective electrolytic lesions of the entorhinal projection to the CA1 disrupts the consolidation of long-term memory (Remondes and Schuman 2004). In contrast, lesions of the dentate gyrus (DG) and
CA3, suggest that these regions of the hippocampus may be important for the acquisition of spatial memory (Steffenach, Sloviter et al. 2002; Jerman, Kesner et al. 2006) (Figure 1.4).

Other brain regions involved in memory include the caudate nucleus, amygdala, cerebellum, striatum and the entorhinal cortex. The caudate nucleus is believed to be involved in the acquisition and consolidation of spatial memory in the Morris water maze (Whishaw, Funk et al. 1987; Devan, Goad et al. 1996; Setlow and McGaugh 1999). The amygdala, in conjunction with the prefrontal cortex, is believed to be responsible for encoding of emotional and fear memory (Adolphs, Cahill et al. 1997; Ferry, Roozendaal 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 short- and long-term memory (Frank and Brown 2003) The striatum and medial temporal lobe are believed to be involved in the formation of associative memory (Mattfeld and Stark 2011) (Figure 1.4). Of these above described regions of the brain, the frontal lobe and hippocampus were the focus of the present studies, due to their important role in declarative learning and memory.

1.6 Cellular basis of memory

Long-term potentiation (LTP) is believed to be a major cellular mechanism underlying learning and memory (Lomo 1966; Bliss and Lomo 1973; Dolphin, Errington et al. 1982; Morris, Anderson et al. 1986) and occurs at synapses (Lomo 1966) (Fig. 1.5.1). LTP is the enhanced signal transmission which results from the stimulation of a post-synaptic neuron by several synchronous excitatory inputs. It has been established that the induction of LTP in many brain regions, including regions of the frontal lobe and hippocampus, is highly dependent on the NMDA receptor (Wigstrom and Gustafsson 1985; Collingridge and Bliss 1987; Laroche, Jay et al. 1990; Bliss and Collingridge 1993; Jay, Burette et al. 1995; Malenka
and Nicoll 1999). However, not all LTP is NMDA receptor dependent (Weisskopf, Bauer et al. 1999), (Harris and Cotman 1986; Aniksztejn and Ben-Ari 1991).

In NMDA-dependent LTP, glutamate is released from a depolarized presynaptic neuron and binds glutamate receptors on the post-synaptic membrane (Dolphin, Errington et al. 1982; Schroder, Schlichthaar et al. 1994). On the post-synaptic membrane, the ionotropic glutamate receptor, NMDA, works in concert with another ionotropic glutamate receptor, the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor, to generate LTP (Izumi, Miyakawa et al. 1987; Watt, Sjostrom et al. 2004). Excitation by glutamate opens the AMPA receptor channel allowing for the flux of both Na+ and K+ ions across the membrane, initiating depolarization of the post-synaptic membrane (Dingledine 1983; Hosli, Hosli et al. 1983) (Fig. 1.5.2A). If the depolarization event is sustained for several seconds and both glutamate and glycine are bound, NMDA receptors are relieved of their magnesium block and bind glycine (Fig. 1.5.2A). This opens the NMDA receptor channel and allows for an influx of Ca2+ into the post-synaptic neuron (Coan and Collingridge 1985; Collingridge and Watkins 1994). Within post-synaptic neurons, Ca2+ acts as a second messenger. In concert with calmodulin, Ca2+-activates several kinases, including protein kinase C (PKC) and calcium/calmodulin dependent kinase II (CAMKII) (Grant, Guttmann et al. 2001; Lee, Escobedo-Lozoya et al. 2009). These kinases aid in the phosphorylation of existing AMPA receptors and translocation of new AMPA receptors into the post-synaptic membrane (Barria, Muller et al. 1997; Shi, Hayashi et al. 1999; Lu, Man et al. 2001). This increase in AMPA receptors enhances and maintains the post-synaptic neuron’s response to glutamate and represents the expression of the early phase of LTP. Late phase LTP is achieved when various activated kinases, including PKA, CAMKII and mitogen-activated protein kinase (MAPK), lead to the activation of cAMP response element binding protein (CREB)-mediated gene expression and protein synthesis (Frey, Krug et al. 1988; Frey, Huang et al. 1993; Nguyen, Abel et al. 1994; Abel, Nguyen et al. 1997; Kandel 2001; Ahmed and Frey 2005; Racaniello, Cardinale et al. 2010) (Figure 1.5.2B).
At the cellular level, the difference between short-term memory and long-term memory is that long-term memory requires maintenance of LTP leading to protein synthesis, which occurs with late phase LTP (Barondes and Cohen 1966; Schafe, Nadel et al. 1999). In contrast, short-term memory does not require protein synthesis (Davis, Rosenweig et al. 1981; Schafe, Nadel et al. 1999). Instead, short-term memory requires only post-translational modification of proteins, such as phosphorylation, which can occur during the early phase LTP (Quevedo, Vianna et al. 2004). Clearly, NMDA receptors play an important role in initiating LTP, the cellular mechanism believed to underlie many types of learning and memory. Therefore, understanding the localization, structure and pharmacology of the NMDA receptor is important to fully understanding the cellular mechanisms underlying memory.

1.7 The NMDA receptor

1.7.1 Localization of the NMDA receptor

NMDA receptors are localized throughout the brain (Greenamyre, Olson et al. 1985; Monaghan, Yao et al. 1985). They are found abundantly throughout regions of the hippocampus and frontal lobe (Bockers, Zimmer et al. 1994; Scherzer, Landwehrmeyer et al. 1998). At the cellular level, these receptors are predominately present in the post-synaptic neurons and generally localized to the post-synaptic membrane (Fagg and Matus 1984; Bekkers and Stevens 1989). However, some NMDA receptors can be located extrasynaptically (Clark, Farrant et al. 1997; Rao and Craig 1997). Extrasynaptic NMDA receptors have been implicated in synaptic dysfunction, particularly excitotoxicity (Sattler, Xiong et al. 2000; Hardingham and Bading 2002; Okamoto, Pouladi et al. 2009; Xu, Kurup et al. 2009; Bordji, Becerril-Ortega et al. 2010; Li, Jin et al. 2011).
1.7.2 Structure of the NMDA receptor

Evidence from electron microscopy and X-ray crystallography studies suggest that the NMDA receptor is a tetramer (Tichelaar, Safferling et al. 2004; Furukawa, Singh et al. 2005). NMDA receptor tetramers are thought to be composed of combinations of subunits from different families of proteins, the GluN1 (NR1), GluN2 (NR2) and GluN3 (NR3) subunit families (Dingledine, Borges et al. 1999; Collingridge, Olsen et al. 2009). Eight different splice variants of the GluN1 subunit, 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 (Premkumar and Auerbach 1997; Laube, Kuhse et al. 1998; Furukawa, Singh et al. 2005; Ulbrich and Isacoff 2008; Collingridge, Olsen et al. 2009). A number of studies indicate that the NMDA receptors are heteromeric complexes composed primarily of two obligate GluN1 subunits and two GluN2 subunits (Moriyoshi, Masu et al. 1991; Monyer, Sprengel et al. 1992; Ishii, Moriyoshi et al. 1993; Tichelaar, Safferling et al. 2004; Furukawa, Singh et al. 2005; Stephenson, Cousins et al. 2008; Ulbrich and Isacoff 2008)(Figure 1.6.1). However, the GluN3 subunit can sometimes assemble with the GluN1-N2 complex to dampen glutamate currents or assemble with GluN1 subunit alone to form a glycine receptor (Cull-Candy, Brickley et al. 2001; Nishi, Hinds et al. 2001; Chatterton, Awobuluyi et al. 2002)

Each NMDA receptor subunit has three transmembrane segments (M1, M3 and M4), a re-entrant membrane loop (M2), an extracellular N-terminus and an intracellular C-terminus (Bennett and Dingledine 1995) (Figure 1.6.2). The re-entrant membrane loop (M2) forms a loop inside the membrane, which after assembly with the other subunits becomes part of the channel pore (Bennett and Dingledine 1995) (Figure 1.6.2). Like the other ionotropic glutamate receptors subtypes, the agonist-recognition region of an NMDA receptor subunit is defined by polypeptide segments S1 and S2 (Stern-Bach, Bettler et al. 1994) (Figure 1.6.2). The N-terminal domain is defined by the first ~400 amino acid residues and,
while it is not directly involved in agonist binding, it is implicated in subunit assembly (Perez-Otano, Schulteis et al. 2001) and in receptor modulation by protons, polyamines and Zn$^{2+}$ (Zheng, Erreger et al. 2001) (Figure 1.6.2). The intracellular C-terminal domain is a localization and regulatory module, interacting with numerous post-synaptic proteins (Raymond, Tingley et al. 1994; Sheng and Pak 1999) (Figure 1.6.2). The intracellular C-terminus of the NMDA subunits enables localization to the synaptic membrane through their interaction with either post-synaptic proteins, such as PSD-95 (Lin, Skeberdis et al. 2004; Cui, Hayashi et al. 2007). These scaffolding proteins have multiple protein-binding domains that help organize a specific cytoskeletal and signaling complex for the NMDA receptor (Lin, Skeberdis et al. 2004; Cui, Hayashi et al. 2007). The C-terminus of the NMDA receptor subunits also interact with a multitude of post-synaptic proteins that are involved in either mediating receptor function and/or downstream signaling, or receptor trafficking. In particular, GluN1 subunit is phosphorylated by protein kinase C (PKC) on several distinct sites, and most of these sites are located within a single alternatively spliced exon in the C-terminal domain, which potentiates NMDA-evoked current (Raymond, Tingley et al. 1994). In contrast, the GluN2B subunit is phosphorylated by Src family kinases, which also potentiates NMDA receptor mediated responses (Nakazawa, Komai et al. 2001; Zhang, Edelmann et al. 2008). The intracellular C-terminal domain of NMDA receptor subunits have been shown to be involved in mediating NMDA receptor endocytosis. Both the GluN1 and GluN2 subunits are believed to possess a tyrosine AP-2 binding motif in their C-terminus (Vissel, Krupp et al. 2001). Specifically, the GluN2B subunits have been shown to interact with the adaptor protein-2 (AP-2) and be endocytosed in a clathrin dependent manner (Roche, Standley et al. 2001; Vissel, Krupp et al. 2001; Lavezzari, McCallum et al. 2003).

1.7.3. Pharmacology of the NMDA receptor

The receptor contains several binding sites, including a glutamate/NMDA binding site, a glycine binding site (glycine acts as a co-agonist) and several other binding sites for noncompetitive antagonists (Davies
and Watkins 1983; Monaghan and Cotman 1985; Johnson and Ascher 1987) (Fig. 1.6.1). At the resting membrane potential, the channel of the NMDA receptor is kept closed by the presence of magnesium, which acts as a physical block of the channel (Nowak, Bregestovski et al. 1984). The block is relieved only when the cell is depolarized by the influx of positive ions through other nearby channels (Mayer, Westbrook et al. 1984). As a result, the NMDA receptor is both ligand and voltage-gated. To become fully active, the NMDA receptor requires the voltage-dependent removal of the magnesium block and the binding of neurotransmitters, glutamate and glycine (Nowak, Bregestovski et al. 1984; Johnson and Ascher 1987; Kleckner and Dingledine 1988; Lynch and Guttmann 2001). Once activated, NMDA receptors are permeable to both divalent (Ca$^{2+}$) and monovalent (Na$^+$, K$^+$) cations (MacDermott, Mayer et al. 1986; Mayer and Westbrook 1987; Ascher and Nowak 1988; Yu and Salter 1998).

In addition to L-glutamate or NMDA, other dicarboxylic amino acids, such as L-aspartate and L-homocysteate, can bind to the glutamate site of the GluN2 subunits and act as agonists (Perkins and Stone 1983; Patneau and Mayer 1990; Provini, Ito et al. 1991). However, there is no known agonist specific for the individual subunits of the GluN2 family, including the GluN2B subunit (Erreger 2007). Other compounds such as D-2-amino-5-phosphonopentanoic acid (AP5), and [(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP) can competitively inhibit the glutamate site (Olverman, Jones et al. 1984; Harris, Ganong et al. 1986). Several other agonists, such as D-serine and D-cycloserine, are specific for the glycine site of the GluN1 subunit (Kleckner and Dingledine 1988; Hood, Compton et al. 1989). The glycine site can be competitively inhibited by compounds such as 7-Chlorokynurenate and 5,7-dichlorokynurenate (Hartley, Monyer et al. 1990; McNamara, Smith et al. 1990; Khan, Seidman et al. 2000). Several other compounds, including (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine maleate (MK801), ketamine, phencyclidine (PCP), ifenprodil, Ro 25-6981, memantine and zinc can act as noncompetitive antagonists (Anis, Berry et al. 1983; Wong, Kemp et al. 1986; Westbrook and
Mayer 1987; Carter, Benavides et al. 1988; Bormann 1989; Fischer, Mutel et al. 1997) (refer to Table 1.1).

NMDA receptor function can also be modulated by additional compounds, including protons, polyamines and nitric oxide. NMDA receptors are sensitive to changes in proton concentration and slight decreases in extracellular pH (proton concentration) can inhibit the function of NMDA receptors (Traynelis and Cull-Candy 1991). Polyamines, such as spermine and spermidine, can either inhibit or potentiate the NMDA receptor function (Rock and Macdonald 1992; Rock and MacDonald 1992). Nitric oxide, which is believed to be produced by NMDA receptor activation, is thought to negatively regulate NMDA receptor function by blocking the channel (Lei, Pan et al. 1992; Fagni, Olivier et al. 1995).

1.8 Subunits of the NMDA receptor

As described in section 1.6, NMDA receptors are thought to be heteromeric tetramers composed of combinations of subunits from different families of proteins. the GluN1, GluN2 and GluN3 subunit families (Dingledine, Borges et al. 1999). However, NMDA receptors are typically composed of two obligate GluN1 subunits and two GluN2 subunits (Moriyoshi, Masu et al. 1991; Monyer, Sprengel et al. 1992; Ishii, Moriyoshi et al. 1993; Tichelaar, Safferling et al. 2004; Furukawa, Singh et al. 2005; Stephenson, Cousins et al. 2008; Ulbrich and Isacoff 2008). For the purpose of the studies presented here, the focus will be on the GluN2B subunit of the GluN2 family of proteins.

1.8.1 GluN2B subunit of the NMDA receptor

Similar to the other members of the GluN2 family of subunits, the GluN2B subunit contains the glutamate binding site in the loop region between the third and fourth transmembrane domain, making it an important subunit for proper functioning of the receptor (Laube, Hirai et al. 1997)(refer to Figure 1.6). However, unlike the GluN1 subunits, it is not necessary or sufficient for NMDA receptor function
(Yamazaki, Mori et al. 1992). Also, unlike the GluN1 family of subunits, there is a developmental switch from expression of the GluN2B subunit to the GluN2A subunit (Watanabe, Inoue et al. 1992; Williams, Russell et al. 1993; Monyer, Burnashev et al. 1994; Watanabe 1996). The GluN2B subunit increases in expression until around post-natal day 20, and then starts to decline in expression (Monyer, Burnashev et al. 1994). It is hypothesized that the decline of GluN2B subunit expression seen in aging could be a continuation of the developmental decline (Ontl, Xing et al. 2004). Across species, the GluN2B subunit is expressed in high levels across the frontal lobe and hippocampus and, at a lower extent, in the basal ganglia (amygdala and striatum). However, it is absent from adult cerebellar granule cells (Monyer, Burnashev et al. 1994; Laurie, Bartke et al. 1997; Schito, Pizzuti et al. 1997).

The GluN2B subunit of the NMDA receptor has been shown to be necessary for survival because mice lacking the GluN2B subunit gene do not survive past the first week of birth (Kutsuwada, Sakimura et al. 1996). This finding suggests a role for the GluN2B subunit in development. In concurrence, several studies have shown that the GluN2B subunit is preferentially localized to developing synapses. The GluN2B subunit has been localized to axonal growth cones and the processes of immature neurons, suggesting a role for this subunit in the regulation of neurite outgrowth and migration (Herkert, Rottger et al. 1998). Moreover, other studies have shown that immature synapses contain a higher concentration of GluN1/GluN2B heteromers as compared to mature synapses which contain more triheteromeric NMDA receptors (Tovar and Westbrook 1999).

Functionally, LTP experiments have shown that the GluN2B subunit contributes to slow deactivation kinetics for NMDA receptor-mediated excitatory post-synaptic currents (EPSC), thus keeping the NMDA receptor channel open longer compared to NMDA receptors lacking the GluN2B subunit (Tovar, Sprouffske et al. 2000). Other studies have supported this by showing that the presence of the GluN2B subunit enhances NMDA-mediated calcium increases (Grant, Bacskaï et al. 1998). As mentioned
previously, calcium influx through the NMDA-type of glutamate receptor and activation of calcium/calmodulin-dependent kinase II (CaMKII) are critical events in certain forms of LTP (refer to section 1.6). The GluN2B subunit appears to directly interact with phosphorylated CaMKII, which binds directly to the C-terminus of the GluN2B subunit (Strack and Colbran 1998; Strack, McNeill et al. 2000).

The GluN2B subunit appears to play an important role in both LTP, a mechanism believed to underlie learning and memory, as well as various forms of memory including spatial, fear and object recognition memory. Pharmacological and genetic blockade of the GluN2B subunit within the prefrontal cortex results in diminished long term potentiation and impaired fear conditioning (Zhao, Toyoda et al. 2005). Region specific knockouts of the GluN2B subunit show that expression of the GluN2B subunit within the frontal lobe and CA1 region of the hippocampus is necessary for long term potentiation and depression, and learning and memory in spatial and fear conditioning tasks (Brigman, Wright et al. 2010). Moreover, transgenic mice designed to express higher levels of the GluN2B subunit from birth also possess superior memory, including spatial and object recognition memory, across adulthood to middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). Therefore, it is evident that the GluN2B subunit plays an important role in the pharmacology of the NMDA receptor as well as in LTP and learning and memory. Moreover, its decline from development onward could be responsible for the age-related declines observed in memory.

1.8.1.1 GluN2B subunit and Cyclin dependent kinase 5 (Cdk5)

When GluN2B is localized to the post-synaptic density it directly binds post-synaptic density protein 95 (PSD-95), a scaffolding protein (Bekenstein, Bennett et al. 1990; Kornau, Schenker et al. 1995) (Figure 1.8). It is well documented that tyrosine kinases phosphorylate NMDA receptors and potentiate NMDA receptor function (Moon, Apperson et al. 1994; Lau and Huganir 1995; Yu, Askalan et al. 1997; Lu, Roder et al. 1998; Yu and Salter 1998). Specifically, NMDA receptor surface expression can be increased
by LTP-inducing stimulation via tyrosine kinase-dependent mechanisms in the adult hippocampus (Grosshans, Clayton et al. 2002). After LTP induction, there is an increase in tyrosine phosphorylation of the GluN2B subunit. This suggests that phosphorylation of this residue may promote maintenance of LTP (Wang and Salter 1994; Rosenblum, Dudai et al. 1996; Rostas, Brent et al. 1996). Specifically, phosphorylation of tyrosine 1472 residue in the c-terminus of the GluN2B subunit has been shown to be associated with LTP (Nakazawa, Komai et al. 2001). Src family kinases, such as Fyn and Src, can both phosphorylate this residue (Yu, Askalan et al. 1997; Cheung, Teves et al. 2001; Nakazawa, Komai et al. 2001). Src family kinase phosphorylation of the tyrosine 1472 of the GluN2B subunit inhibits clathrin-mediated endocytosis of GluN2B subunit-containing NMDA receptors (Zhang, Edelmann et al. 2008)(Figure 1.8). Clathrin-mediated endocytosis is promoted by phosphorylation of PSD-95 (Zhang, Edelmann et al. 2008) (Figure 1.8). This phosphorylation blocks Src family kinases (Figure 1.8). Cdk5 is responsible for the phosphorylation of PSD-95 (Zhang, Edelmann et al. 2008) (Figure 1.8). If Cdk5 is inhibited, then GluN2B subunit-containing receptors are maintained at the post-synaptic membrane (Zhang, Edelmann et al. 2008) (Figure 1.7). In addition, research suggests that excitatory glutamatergic neurotransmission, such as NMDA receptor function, may regulate Cdk5 activity through degradation of its regulator, p35. Therefore, NMDA receptor activity may ultimately down-regulate the activity of Cdk5 (Wei, Tomizawa et al. 2005). Moreover, transgenic studies in adult mice have shown that removing Cdk5 results in increased levels of GluN2B mRNA and protein expression and increased levels of GluN2B phosphorylated at tyrosine 1472, as well as enhanced memory (Hawasli, Benavides et al. 2007).

Cyclin-dependent kinase 5 (Cdk5), also termed neuronal Cdc2-like kinase (NCLK) is predominately expressed within the brain and its expression levels do not significantly decline with age (Hellmich, Pant et al. 1992; Wu, Yu et al. 2000). NCLK can refer to Cdk5 bound to its neuronal activator (Hellmich, Pant et al. 1992; Lew, Huang et al. 1994). Its expression has been shown to be elevated in multiple neurodegenerative diseases, including Alzheimer’s disease (Baumann, Mandelkow et al. 1993; Pei,
Therefore, in addition to enhancing GluN2B subunit expression, Cdk5 could be a more specific target for increasing GluN2B-containing receptors at the synapse.

1.9 Aging, memory and the NMDA receptor

NMDA receptors, as a whole, are implicated in cognitive functions because of their role in memory formation as described in section 1.6. Antagonists of the receptor block initiation of long-term potentiation, a cellular mechanism believed to underlie learning and memory, in both the hippocampus and the regions of the frontal lobe (Harris, Ganong et al. 1984; Morris, Anderson et al. 1986; Bashir, Alford et al. 1991; Castro-Alamancos, Donoghue et al. 1995; Tsien, Huerta et al. 1996; Trepel and Racine 1998; Escobar, Alcocer et al. 2002). Memory and learning, including spatial memory, is also impaired by use of NMDA receptor specific antagonists such as AP5, MK801 and ketamine (Morris, Anderson et al. 1986; Alessandri, Battig et al. 1989; Morris 1989; Heale and Harley 1990; Lee and Kim 1998; Winters, Tucci et al. 2009). In addition, correlations have been seen between NMDA-displaceable $[^3]$H glutamate binding and NMDA subunit expression and spatial memory performance in the Morris water maze (Wenk, Walker et al. 1991; Magnusson 1998; Magnusson 2000; Magnusson 2001; Ontl, Xing et al. 2004; Magnusson, Kresge et al. 2006; Magnusson, Scruggs et al. 2007; Das and Magnusson 2008).

Aging animals exhibit declines in NMDA receptor binding densities. The NMDA binding site has been shown to be more affected by aging than any other ionotropic glutamate receptor (Magnusson and Cotman 1993; Magnusson and Cotman 1993; 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 receptor in the frontal lobe, including the prefrontal cortices, and in the hippocampus of the mouse brain (Magnusson and Cotman 1993; Magnusson and Cotman 1993; Magnusson 1995; Magnusson 1997). Similar results were also observed in
other species, including rats (Kito, Miyoshi et al. 1990; Nicolle, Bizon et al. 1996; Nicolle and Baxter 2003), canids (Magnusson 2000), non-human primates (Wenk, Walker et al. 1991) and humans (Piggott, Perry et al. 1992).

Several studies have used spatial memory tasks to characterize the relationship between age related declines in memory and NMDA receptor expression (Gage, Dunnett et al. 1984; Rapp, Rosenberg et al. 1987; Pellemounter, Beatty et al. 1990; Davis, Markowska et al. 1993; Gallagher and Nicolle 1993; Nicolle, Bizon et al. 1996; Magnusson 1998; Magnusson 2001; Magnusson, Scruggs et al. 2003; Magnusson, Scruggs et al. 2007; Das and Magnusson 2008; Zhao, Rosenke et al. 2009; Das and Magnusson 2011). Specifically, the age-related decline in densities of NMDA receptor binding and expression of its subunits in regions of the frontal lobe and hippocampus of the rodent brain have been shown to be associated with declines in spatial memory during aging (Pellemounter, Beatty et al. 1990; Nicolle, Bizon et al. 1996; Magnusson 1998; Magnusson 2001; Magnusson, Scruggs et al. 2003; Magnusson, Scruggs et al. 2007; Das and Magnusson 2008; Zhao, Rosenke et al. 2009; Das and Magnusson 2011). 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 we and animals experience during the aging process. Since the focus of these studies is on the GluN2B subunit of the NMDA receptor, in the next sections I will discuss the effect of aging on the GluN2B subunit alone and its relationship to memory.

1.9.1 Effects of aging on the GluN2B subunit within the brain

Of the NMDA receptor subunits, the GluN2B subunit is most affected by the aging process. The GluN2B subunit exhibits the greatest declines in both protein and mRNA expression with age within the frontal lobe, caudate nucleus and sub-regions of the hippocampus across species (Magnusson 2000; Magnusson, Nelson et al. 2002; Bai, Hof et al. 2004). The GluN2B subunit has been shown to be especially vulnerable
to the effects of aging in the cerebral cortex, including the prefrontal cortices, and the dentate gyrus of 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; Magnusson, Kresge et al. 2006). Significant declines in GluN2B mRNA expression have also been observed in aged macaques in the prefrontal cortex and caudate nucleus and in the hippocampus of aged rats (Clayton and Browning 2001; Bai, Hof et al. 2004). In the frontal lobes of C57BL/6 mice, it appears that the decline in mRNA during adult aging may be a continuation of the developmental decline and, therefore, may be programmed (Ontl, Xing et al. 2004). Protein expression of the GluN2B subunit also declines with age across regions of the frontal lobe and hippocampus (Clayton and Browning 2001; Magnusson, Nelson et al. 2002; Magnusson, Scruggs et al. 2007). However, the protein levels of the GluN2B subunit show a greater decline with age within the synaptic membrane of the frontal lobe, than in the tissue as a whole (Zhao, Rosenke et al. 2009). This suggests that there might be an additional effect of aging on the localization of the GluN2B subunit within the synaptic environment, in addition to an overall programmed decline. Within the hippocampus, there is a significant decline in GluN2B subunit expression in the synapse with age, similar to the tissue changes (Zhao, Rosenke et al. 2009). However, although there is an overall decline with age, there is a subset of the aged animals who have the highest expression of the GluN2B subunit within the synapse for an aged animal, but the worst memory (Zhao, Rosenke et al. 2009). This may suggest that there is a change with aging in the NMDA receptor themselves or downstream signaling pathways within the hippocampus that causes GluN2B-containing receptors to no longer be beneficial to memory in aged animals. Functional studies of NMDA receptors also suggest that aging results in a decrease in or loss of functional GluN2B-containing receptors. Specifically, NMDA receptors from aged animals appear to be less sensitive to ifenprodil, a GluN2B specific antagonist, and have an increased rate of deactivation (faster channel closing) (Kuehl-Kovarik, Magnusson et al. 2000; Kuehl-Kovarik, Partin et al. 2003). Through this current work, I will attempt to determine if enhancing GluN2B subunit expression in a region specific manner is beneficial to memory in normal aging.
1.9.2 Consequence of an aging GluN2B subunit on memory

The GluN2B subunit is important for spatial memory and its decrease, via experimental manipulation, has been shown to be sufficient to account for the degree of spatial memory impairment seen in aged rodents (Clayton, Mesches et al. 2002). The effect of aging on GluN2B subunit expression is most evident in regions of the frontal lobe and hippocampus (Clayton and Browning 2001; Magnusson, Nelson et al. 2002; Bai, Hof et al. 2004; Magnusson, Scruggs et al. 2007). Aging studies have shown that there is a significant correlation between decreased GluN2B subunit expression and impaired spatial memory in aged animals (Magnusson, Scruggs et al. 2007). Specifically, age-related decreases in the protein expression of the GluN2B subunit within crude synaptosomes of the frontal cortex of C57BL/6 mice show a relationship to the declines in performance in the long-term spatial memory task across age groups. However, higher expression of the GluN2B subunit within the synaptic membrane of the hippocampus of a subset of aged mice is associated with poorer performance in the same task (Zhao, Rosenke et al. 2009). Previous research has shown that increasing GluN2B subunit expression throughout multiple brain regions from birth is beneficial to memory, including long-term and working spatial memory, and remains beneficial even into middle-age (18 months) (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). These data suggest that maintaining higher levels of the GluN2B subunit during aging than are seen in normal aged mice is beneficial for memory. Through this current work, I will attempt to determine if enhancing GluN2B subunit expression in a region specific manner is beneficial to memory in normal aging.

1.9.3 Aging interventions that enhance the GluN2B subunit

Many different diet or drug interventions have been used to successfully attenuate the effects of aging and have indirectly affected the expression levels or phosphorylation state of the GluN2B subunit. A popular aging intervention, caloric restriction, is an aging intervention that has been shown to increase longevity.
(Davis, Bales et al. 1983) as well as memory performance (Witte, Fobker et al. 2009), especially in spatial tasks (Algeri, Biagini et al. 1991; Pitsikas and Algeri 1992; Magnusson 1997; Adams, Shi et al. 2008). A recent study employed the long-term (6-8 months), intermittent fasting diet (L-IFD), a variant of caloric restriction, which involves alternating periods of fasting and non-fasting. The L-IFD enhanced learning and memory in multiple tasks in aged mice, including object recognition and LTP (Fontan-Lozano, Saez-Casanelli et al. 2007). This was associated with an increase in protein expression of the GluN2B subunit in both the hippocampus and perirhinal cortex (Fontan-Lozano, Saez-Casanelli et al. 2007). In addition, the enhancements in learning and LTP were blocked by region specific GluN2B subunit antagonism suggesting that they were the result of enhanced expression of the GluN2B subunit (Fontan-Lozano, Saez-Casanelli et al. 2007).

Dietary supplementation, not just restriction, has also shown promise for alleviating memory declines associated with aging. Aging is associated with declines in omega-3 polyunsaturated fatty acids (n-3 PUFA) in the brain (Ullmann, Mimouni et al. 2001; Bader Lange, Cenini et al. 2008) and deficits in n-3 PUFA is associated with memory impairment (Kalmijn 2000; Grant, Campbell et al. 2002; Morris 2003). Dietary supplementation with n-3 PUFA reversed deficits in the expression of the GluN2B subunit in aged rats (Dyall, Michael et al. 2007). Dietary supplementation with blueberry extract in aged rats rescued NMDA receptor-dependent LTP in the hippocampus and increased phosphorylation of the GluN2B subunit at Tyrosine 1472 in aged rats. However, it did not prevent aged related declines in the protein expression of the GluN2B subunit (Coultrap, Bickford et al. 2008).

Drug-related interventions have also ameliorated the effects of aging on the GluN2B subunit. Insulin-like growth factor 1 (IGF-1) repaired the decline in expression of the GluN2B subunits seen in older rats (Sonntag, Bennett et al. 2000). Similarly, nicotine reversed the effects of aging on the GluN2B subunit in the hippocampus in aged rats (Delibas, Doguc et al. 2005). Sulindac, a non-steroidal anti-inflammatory
drug (NSAID), attenuated age-related deficits in memory, decreased inflammation and increased expression levels of the GluN2B subunit in aged rats (Mesches, Gemma et al. 2004).

Many of these aging interventions only had selective effects on GluN2B subunit; however, these various aging interventions do suggest that the levels and/or phosphorylation state of the GluN2B subunit is an important factor in aging and age-related memory declines. It also provides further evidence that manipulation of this subunit in aging could potentially be used to repair this deficit. However, it is not clear whether these interventions had a direct influence over the GluN2B subunit or whether other proteins were also affected. In addition, many of these interventions do not demonstrate how memory is affected by these changes to the GluN2B subunit, or how these changes compare to young mice. In contrast to these aging interventions, the studies presented here do target the expression of the GluN2B subunit directly. Moreover, our studies do address whether up-regulating the expression of the GluN2B subunit within aged mice is beneficial to memory. They also demonstrate how memory is affected compared to young animals and attempt to address whether the enhanced GluN2B subunit expression is functionally responsible for the changes observed in memory in aged animals.

Still, there has been some evidence that decreasing the expression of the GluN2B subunit could be beneficial to improving age-associated memory impairment. Restricting the dietary intake of calories to 60% of ad-libitum fed animals, beginning from three months of age, resulted in the maintenance of slighter higher levels of [3H]glutamate binding to the NMDA site in older C57Bl/6 mice (Magnusson 1997; Magnusson 2001) and this effect is correlated with improved spatial memory performance in the water maze (Magnusson 1998; Magnusson 2001). However, there was no reversal of the age-related declines in GluN2B subunit (Magnusson 2001; Newton, Forbes et al. 2007). In fact, aged animals on the restricted diets actually show the greatest decreases in GluN2B subunit mRNA densities in the dentate gyrus (Magnusson 2001; Newton, Forbes et al. 2007). This could suggest that enhancing GluN2B subunit
expression within in the aged brain, specifically the hippocampus, may further impair memory function in aging.

1.10 Tools for manipulating gene expression

1.10.1 Adenoviral vectors

An adenoviral vector was used to enhance GluN2B subunit expression because adenoviral vectors have previously been used to effectively deliver genes to the central nervous system (CNS) (Benitez and Segovia 2003). An adenoviral vector was chosen because of its packaging capacity (Berkner 1992). Specifically, the GluN2B vector expresses two transgenes, GluN2B and eGFP, from two independent promoters (Figure 1.8.1). This cannot be accomplished in an adenovirus-associated viral vector because the packaging capacity is too small (Dong, Fan et al. 1996). A lentivector, such as feline immunodeficiency virus (FIV), can be engineered to express two transgenes using an internal ribosomal entry site (IRES) sequence; however, the expression levels of the gene under the control of the IRES is much lower than is seen with an adenoviral vector (Mizuguchi, Xu et al. 2000). Finally we can obtain higher infectious titers with adenoviral vector compared to a lentivector (Thomas, Ehrhardt et al. 2003). However, there are limitations to using adenoviral vectors over other viral vectors. Specifically, enhanced expression with adenoviral vectors is transient because the message is not integrated into the host genome (Harui, Suzuki et al. 1999). Also, adenoviral vectors can also result in a substantial immune response (Gregory, Nazir et al. 2011). There are also limitations to using any viral vector. Primarily, a viral vector does not provide as much control over the localization and timing of the expression of your protein of interest, like a transgenic model (Jaenisch 1988).
1.10.2 Morpholinos

Phosphorodiamidate morpholino oligomers (Morpholinos) are synthetic uncharged P-chiral analogs of nucleic acids that act as antisense oligomers and inhibit the interactions of macromolecules with mRNA by base pairing with the targeted mRNA in a complimentary manner (Moulton 2008) (Figure 1.8.2). Morpholinos have been used systemically to reduce expression of inhibitory factors or enhance splicing of genes long-term (Alter, Lou et al. 2006; Summerton 2007; Moulton and Moulton 2010). Morpholinos do not trigger an immune response like siRNA or viral vectors, do not interact strongly with proteins like the negatively-charged antisense oligonucleotides, and are not rapidly degraded in cell cultures or in animals (Amantana, Moulton et al. 2007; Youngblood, Hatlevig et al. 2007). Peptide-conjugated Morpholinos are far more effective at entering cells at lower doses than unmodified Morpholinos (Moulton, Hase et al. 2003; Moulton and Moulton 2010) (Figure 1.9.2). These peptide-conjugated Morpholinos have an increased half-life and a greater volume of distribution in the body when given systemically than unmodified Morpholinos (Amantana, Moulton et al. 2007). The intracellular activity of peptide-conjugated Morpholinos is also distributed more evenly across tissues than is the activity of unmodified Morpholinos (Moulton, Hase et al. 2003; Moulton and Moulton 2010). However, although a lot is known about the effects of unmodified and peptide-conjugated Morpholinos outside the central nervous system (CNS), there very little information on their effects within the brain (Oh, Shimizu et al. 2005; Kurokawa 2008; Sekiguchi, Zushida et al. 2009). Only recently, a study using Vivo-Morpholinos, which are similar to peptide-conjugated Morpholinos except that they are conjugated to a non-peptide transporter for enhanced cell-permeability, showed that intracranially delivered cell-penetrating Morpholinos, though effective, can result in neurotoxicity at higher doses (Reissner, Sartor et al. 2012). This finding may explain some of the result found in the third study presented here.
1.11 Gliosis

The brain and spinal cord are considered "immune privileged" organs in that they are separated from the rest of the body by a series of endothelial cells known as the blood–brain barrier, which prevents most foreign matter, including infectious agents and many drugs, from reaching the brain (Broman, Radner et al. 1949; Broman 1955). Therefore, when administering substances directly to the brain, it is important to ensure those substances did not cause excessive damage and/or inflammation. Damage to the central nervous system (CNS) elicits the activation of glial cells, specifically astrocytes and microglia, and can be termed gliosis (Morshead and van der Kooy 1990; Zhang, Hu et al. 2010). Astrocytes are star-shaped glial cells that are involved in the regulation of blood flow, participation in synaptic function and plasticity, and maintenance of the extracellular balance of ions and neurotransmitters as well as in the repair and scarring process of the brain and spinal cord following traumatic injuries (Pasti, Zonta et al. 2001; Lian and Stringer 2004; Piet, Vargova et al. 2004; Filosa, Bonev et al. 2006; Voskuhl, Peterson et al. 2009). Proliferation of astrocytes in damaged areas of the CNS can be a histopathological sign of neurotoxicity (O Callahagn 1991). Microglia are the resident macrophages of the brain and spinal cord, and provide immune defense to the CNS (Gehrmann, Matsumoto et al. 1995). Activated microglia release pro-inflammatory cytokines when activated resulting in an inflammatory response (Hanisch 2002). Proliferation of microglia in areas of the CNS can be a histopathological sign of inflammation (Herber, Maloney et al. 2006).

1.11.1 Glial fibrillary acidic protein (GFAP) and neurofilament

For the studies involving the adenoviral vector, it was important to differentiate between glial cells and neurons with enhanced GluN2B subunit expression because the promoter of the vectors was not neuron specific. However, it should be noted that neuron specific promoters do exist, and one highly neuron-specific promoter is Human synapsin 1 gene promoter (Kugler, Kilic et al. 2003). Glial cells comprise the
majority of cells within the CNS and adenoviral vectors more readily transduce astrocytic glial cells; therefore, astrocytes were most likely to show enhanced GluN2B subunit expression besides neurons (Miyaguchi, Maeda et al. 1999; Azevedo, Carvalho et al. 2009). A common marker used to identify astroglia is glial fibrillary acidic protein (GFAP) (Raff, Fields et al. 1979). GFAP is an intermediate filament protein that is expressed by astrocytes (Chiu, Norton et al. 1981). GFAP is thought to help to maintain astrocyte mechanical strength, as well as the shape (Eng 1985). GFAP expression has also been shown to be enhanced upon neurotoxic insult (Lumpkins, Bochicchio et al. 2008). In order to identify neurons, a neuronal marker, neurofilament was used (Schilling, Scherbaum et al. 1988). Neurofilaments, similar to GFAP, are intermediate filaments but are found exclusively in neurons and comprise the major component of a neuron’s cytoskeleton, and provide support for normal axonal radial growth (Huneeus and Davison 1970; Rao, Campbell et al. 2003).

1.12 Summary

Memory is one of the first cognitive functions to show decline with age (Albert and Funkenstein 1992) and NMDA receptors, which are critical to memory function, have been shown to be especially vulnerable to the effects of aging (Magnusson, Brim et al. 2010). Of its subunits, the GluN2B subunit of the NMDA receptor shows the greatest and most consistent declines with age in 26-30 month old animals (Magnusson 2000; Magnusson, Bai et al. 2002; Magnusson, Bai et al. 2005). However, there may be a differential effect on aging on this subunit in different brain regions. In the frontal cortex, it is hypothesized that the decline in GluN2B subunit expression associated with aging might be a continuation of the developmental switch from GluN2B to GluN2A-containing receptors and may contribute to memory declines as we age (Watanabe, Inoue et al. 1992; Williams, Russell et al. 1993; Monyer, Burnashev et al. 1994; Watanabe 1996; Ontl, Xing et al. 2004). In the hippocampus, since a correlation has been observed between higher expression of the GluN2B subunit and poorer performance
in the Morris water maze in a subpopulation of aged mice, increasing expression of the subunit could actually further impair memory in aged animals (Zhao, Rosenke et al. 2009). However, transgenic mice designed to express higher levels of the GluN2B subunit from birth also possess superior memory, including spatial and object recognition memory, across adulthood into middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). These data led to the hypothesis that the decline in the expression of the GluN2B subunit is responsible, at least in part, for the declines observed in spatial memory with age in these age mice. However, increasing its expression regionally was first examined since higher expression of the GluN2B subunit within the hippocampus has been associated with poorer memory in aged animals. In chapter II and III, I have focused on enhancing the expression of the GluN2B subunit directly using a replication deficient adenoviral vector to deliver the cDNA of the GluN2B subunit to the frontal lobe (Chapter II) and the hippocampus (Chapter III) of aged (and young mice) to determine if it might simply be a loss of GluN2B subunits that is responsible for the memory decline observed in aging. In chapter IV, I have also included a study that attempts to use a peptide-conjugated Morpholino to inhibit the translation of the Cdk5 protein in order to enhance post-synaptic GluN2B-containing receptors in aged animals.

An adenoviral vector was used to enhance GluN2B subunit expression because adenoviral vectors have previously been used to effectively deliver genes to the central nervous system (CNS) (Benitez and Segovia 2003). The results of the study presented in the Chapter II provide compelling evidence that increasing the expression of the GluN2B subunit of the NMDA receptor within the frontal lobe and the caudate nucleus of aged mice is beneficial to memory. Aged mice with enhanced GluN2B subunit expression across the frontal lobe and rostro-dorsal caudate nucleus had improved learning on the second day of long-term memory training, as compared to aged controls, and their improved performance was similar to that seen in young vehicle-treated mice. By the end of the second day of the long-term spatial memory task, these aged mice showed a stronger bias for the platform location compared to either aged
control and, in fact, showed a similar bias for the platform location as compared to young vehicle-treated mice. Moreover, there was a need for a higher dose of Ro 25-6981 to impair memory in aged mice with enhanced GluN2B subunit expression. In concurrence, immunohistochemistry revealed increased GluN2B subunit expression within cells with either neuronal or glial characteristics across the frontal lobe and caudate of aged mice treated with the GluN2B vector compared to control treatments. From these data, we concluded that increasing the expression of the GluN2B subunit of the NMDA receptor in the frontal lobe appeared to restore later learning and memory (later acquisition and/or consolidation) and that a therapeutic aimed at enhancing the number or function of the GluN2B containing NMDA receptors in old age could potentially be used to help ameliorate age-related memory loss.

The results of the study presented in the Chapter III provide evidence that increasing the expression of the GluN2B subunit of the NMDA receptor within the aged hippocampus is beneficial to memory, despite the previous correlation that increased GluN2B subunit expression in the aged hippocampus may impair memory. Aged mice with increased GluN2B subunit expression in the hippocampus actually exhibited improved early learning in the first day of the long-term spatial memory task compared to aged controls, and performed similarly to young vehicle-treated mice. However, there was a trend for impaired learning in later trials. Similar to the first study, immunohistochemistry revealed increased GluN2B subunit expression within cells with either neuronal or astroglial characteristics across the hippocampus of aged mice treated with the GluN2B vector compared to control treatments. From these data, we concluded that enhancing GluN2B subunit expression within the hippocampus of aged mice might improve early learning (acquisition) and that a therapeutic aimed at enhancing the number or function of the GluN2B subunit-containing NMDA receptors throughout the aged brain could potentially be used to help ameliorate age-related memory impairment.
In order to achieve a more global, yet specific, enhanced localization of the GluN2B subunit to the synaptic membrane, Cdk5 was targeted to enhance GluN2B-containing receptors at the synapse within the aged brain. Chapter IV explored the benefit of enhancing the expression of the GluN2B subunit within the brains of aged mice using antisense phosphorodiamidate morpholino oligomers (Morpholinos), conjugated to a peptide that enhances cellular uptake, in order to decrease the expression of Cdk5 protein. The purpose of the present study was to determine whether the decline in spatial memory observed in aging could be improved by enhancing the expression of the GluN2B subunit at the synapse within the aged brain. This would help determine whether a therapy aimed at enhancing the number of GluN2B subunit-containing NMDA receptors in old age would be beneficial to memory. In Chapter IV, I have discussed the effects of the peptide-conjugated Morpholino targeted against Cdk5 on memory and its potential as a genetic tool. The results of the studies presented here will delineate the effects of increased GluN2B subunit expression on memory in aging. It will also indicate the potential of peptide-conjugated Morpholinos as a tool for gene manipulation and the potential of the Cdk5 Morpholino to enhance expression of the GluN2B subunit within the brain.
Fig. 1.1- Schematic diagram of memory classified by duration and consciousness. Image adapted from Miyashita et al. (Miyashita 2004).
Figure 1.2
Fig. 1.2—Schematic representation of the different alterations of the Morris water maze task for assessment of spatial and associative memory and cognitive flexibility. (A) Long-term and working spatial memory is assessed in conditions where the escape platform is hidden under the water and distal cues are present around the tank and room. Cognitive flexibility is assessed in the same conditions except that the escape platform is located in the quadrant opposite of the position used for long-term spatial memory assessment. Animals should navigate to the platform with the help of the distal spatial cues. (B) Spatial bias for the escape platform location is assessed under similar conditions except that the escape platform has been removed. Animals with a spatial bias (recall of its previous location) for the escape platform’s location should navigate near this location. (C) Associative memory is characterized under conditions where the escape platform is made visible by a proximal cue (flag) and all distal cues are removed from around the tank and room. This is a control condition to assess motivation, motor performance and visual acuity. Unimpaired and motivated animals should navigate to the cued platform. Image adapted from Branchi et al. (Branchi, D'Andrea et al. 2006).
Novel Object Recognition Arena

A  Habituation

B  Familiarization

C  Novel Object Recognition

Figure 1.3
Fig. 1.3– Schematic representation of the different alterations of the novel object recognition task for the assessment of object recognition memory. (A) Habituation sessions, in which animals are acclimated to the arena, occur in an empty arena. (B) Familiarization sessions, in which animals are familiarized to two identical objects, occur in the same arena containing two identical objects. (C) Object recognition memory is characterized in the same arena containing one of the objects from the familiarization trial and a new object. To assess object recognition memory, animals are placed in the arena with a familiar and a novel object one hour post-familiarization and again with the same familiar object and a new novel object twenty-four hours post-familiarization. Image adapted from Bevins et al. (Bevins and Besheer 2006).
Fig. 1.4- Schematic diagram of a rodent brain depicting various sub-regions of the brain. Image adapted from Gene Expression Nervous System Atlas (GENSAT) (Heintz 2004).
Fig. 1.5.1 – Schematic representation of a synapse between two neurons, the presynaptic and post-synaptic neuron. Image adapted from McKinley et al. (McKinley and O'Loughlin 2008).
Fig. 1.5.2 – Schematic diagram of a cellular mechanism underlying learning and memory, long-term potentiation (LTP). (A) Representation of a synapse between two neurons, depicting both a presynaptic terminal of the axon of the presynaptic neuron and a post-synaptic dendritic spine on the post-synaptic neuron. 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 post-synaptic neuron. In response to glutamate, AMPA receptor channels open and allow sodium ions into the cell, leading to depolarization. NMDA receptors are closed initially because of the magnesium block in the channel. (B) However, if the depolarization event triggered by AMPA receptor activation is sufficient to remove the magnesium block and it binds glycine, the NMDA receptor channel will open and conduct calcium into the post-synaptic neuron. 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. Towards the late phase, the kinases lead to subsequent protein synthesis, further strengthening the synapse and leading to late phase long-term potentiation. Image adapted from Sadava et al. (Sadava 2008).
Fig. 1.6.1 – Schematic representation of the assembly of NMDA receptors with its various binding sites. Image adapted from Kalia et al. (Kalia, Kalia et al. 2008).
Fig. 1.6.2– Schematic representation of the structure of the NMDA receptor and the GluN1 and GluN2 subunits. Both the GluN1 and GluN2 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) of each subunit. The agonist recognition region is defined by polypeptide segments S1 and S2. Image adapted from Yamakura et al. (Yamakura and Shimoji 1999).
Table 1.1- Compounds that modulate NMDA receptor function

<table>
<thead>
<tr>
<th>Agents</th>
<th>Type</th>
<th>Binding site</th>
<th>NR1/NR2A</th>
<th>NR1/NR2B</th>
<th>NR1/NR2C</th>
<th>NR1/NR2</th>
<th>Selectivity</th>
<th>Limitations</th>
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<tbody>
<tr>
<td>L-glutamate</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NMDA</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-homocysteine</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycine</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>D-serine</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-cycloserine</td>
<td>agonist</td>
<td>Gln2 ABD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>(R)/AP5</td>
<td>competitive antagonist</td>
<td>Gln2 ABD</td>
<td>0.3</td>
<td>0.5</td>
<td>1.6</td>
<td>3.7</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td>Poor selectivity</td>
</tr>
<tr>
<td>(R)/AP7</td>
<td>competitive antagonist</td>
<td>Gln2 ABD</td>
<td>0.5</td>
<td>4</td>
<td>6</td>
<td>17</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td>(≤10 fold)</td>
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<tr>
<td>(R)/CPP</td>
<td>competitive antagonist</td>
<td>Gln2 ABD</td>
<td>0.04</td>
<td>0.3</td>
<td>0.06</td>
<td>2</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
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<tr>
<td>7-CKA</td>
<td>competitive antagonist</td>
<td>Gln1 ABD</td>
<td>0.6</td>
<td>0.2</td>
<td>0.1</td>
<td>0.6</td>
<td>2C&lt;2B&lt;2A&lt;2D</td>
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<tr>
<td>5,7-DCKA</td>
<td>competitive antagonist</td>
<td>Gln1 ABD</td>
<td>0.03</td>
<td>0.05</td>
<td>0.2</td>
<td>0.09</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td></td>
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<tr>
<td>Mg2+</td>
<td>noncompetitive antagonist</td>
<td>Channel pore</td>
<td>20</td>
<td>20</td>
<td>80</td>
<td>20</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td>Poor selectivity</td>
</tr>
<tr>
<td>PCP</td>
<td>noncompetitive antagonist</td>
<td>Channel pore</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td></td>
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<tr>
<td>Ketamine</td>
<td>noncompetitive antagonist</td>
<td>Channel pore</td>
<td>0.7</td>
<td>0.5</td>
<td>0.5</td>
<td>0.7</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td></td>
</tr>
<tr>
<td>Memantine</td>
<td>noncompetitive antagonist</td>
<td>Channel pore</td>
<td>0.9</td>
<td>0.8</td>
<td>-</td>
<td>0.5</td>
<td>2A&lt;2B&lt;2D</td>
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<tr>
<td>(+)-MK-801</td>
<td>noncompetitive antagonist</td>
<td>Channel pore</td>
<td>0.01</td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>noncompetitive antagonist</td>
<td>Gln2A NTD</td>
<td>0.2</td>
<td>2</td>
<td>20</td>
<td>10</td>
<td>2A&lt;2B&lt;2C&lt;2D</td>
<td></td>
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<tr>
<td>Ifenprodil</td>
<td>noncompetitive antagonist</td>
<td>Gln2B NTD</td>
<td>&gt;30</td>
<td>0.15</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>2B&gt;2A&gt;2C&lt;2D</td>
<td></td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>noncompetitive antagonist</td>
<td>Gln2B NTD</td>
<td>&gt;30</td>
<td>0.009</td>
<td>-</td>
<td>-</td>
<td>2B&gt;2&gt;2A&gt;2C&lt;2D</td>
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Table 1.1- Table listing compounds that can either potenti ate or inhibit NMDA receptor function. Characteristics such as binding site, selectivity and limitations are listed when applicable. Table adapted from Paoletti et al. (Paoletti and Neyton 2007). ABD=agonist binding domain. NTD=N-terminal domain.
Fig. 1.7– Schematic representation of the endocytosis of the GluN2B containing NMDA receptors. Image adapted from Chen et al. (Chen and Roche 2007).
Fig. 1.8.1 – Schematic representation of the GluN2B vector.
Fig. 1.8.2- Schematic representation of an unconjugated Morpholino (PMO) and peptide-conjugated Morpholino (PPMO) structure. The structure of the peptide conjugate is (RXR)$_4$XB, where R=arginine, X= 6-aminohexanoic acid and B= beta-alanine. Image adapted from Moulton et al. (Moulton and Yan 2008).
CHAPTER II

MEMORY IN AGED MICE IS RESCUED BY INCREASED EXPRESSION OF THE GLUN2B (NR2B) SUBUNIT OF THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR IN THE FRONTAL LOBE.
MEMORY IN AGED MICE IS RESCUED BY INCREASED EXPRESSION OF THE GLUN2B (NR2B) SUBUNIT OF THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR IN THE FRONTAL LOBE.

Authors and affiliations
B. L. Brim\textsuperscript{a,b,c}, R. Haskell\textsuperscript{d}, R. Awedikian\textsuperscript{e}, N.M. Ellinwood\textsuperscript{e}, L. Jin\textsuperscript{a,b}, K. Magnusson\textsuperscript{a,b,c}

\textsuperscript{a}Molecular and Cellular Biosciences Program, Oregon State University, Corvallis, OR, 97331, U.S.A.
Department of Biomedical Sciences, Oregon State University, Corvallis, OR, 97331, U.S.A.\textsuperscript{b} Healthy Aging Program, Linus Pauling Institute, Oregon State University, Corvallis, OR; 97331, U.S.A.\textsuperscript{c}
ViraQuest, Inc., North Liberty, IA; 52317, U.S.A.\textsuperscript{d} Department of Animal Sciences, Iowa State University, Ames, IA, 50011, U.S.A.\textsuperscript{e}

In revision for \textit{Behavioural Brain Research}
Abstract

The GluN2B subunit of the $N$-methyl-D-aspartate (NMDA) receptor shows age-related declines in expression across the frontal cortex. This decline is strongly correlated to age-related memory declines. This study was designed to determine if increasing GluN2B subunit expression in the frontal lobe would improve memory in aged mice. Mice were injected bilaterally with either the GluN2B vector, containing cDNA specific for the GluN2B subunit and enhanced Green Fluorescent Protein (eGFP); a control vector or vehicle. Spatial memory, cognitive flexibility and associative memory were assessed using the Morris water maze. Aged mice, with increased GluN2B subunit expression, exhibited improved long-term spatial memory, comparable to young mice, on the second day of training. A higher concentration of the GluN2B antagonist, Ro 25-6981, was required to impair long-term spatial memory in aged mice with enhanced GluN2B subunit expression, as compared to aged controls. Therapies that enhance GluN2B subunit expression within the aged brain could help ameliorate memory loss.

Keywords: Aging, spatial memory, long-term memory, reference memory, working memory, cognitive flexibility, associative memory, frontal lobe, caudate nucleus, NMDA receptor, NR2B subunit, $\varepsilon$2 subunit, Ro 25-6981, Morris water maze, stereotaxic injection, C57BL/6 mice, adenoviral vector

2.1 Introduction

Aging causes declines in several cognitive functions and one of the earliest cognitive functions to show decline with age is memory (Albert 1994). Memory deterioration is evident in humans by the fifth decade (Albert and Funkenstein 1992). Spatial memory, which is responsible for the navigation of organisms within their environment, is particularly affected by the aging process (Barnes 1988).

Specific brain regions are important for the acquisition, consolidation and retrieval of spatial memory, including the prefrontal cortices within the frontal lobe and hippocampus (Spiers and Maguire 2007).
Regions of the frontal lobe have been shown to also be important for cognitive flexibility, (i.e. the ability to switch a behavioral response according to the context of a situation) (de Bruin JP 1994; Kim and Ragozzino 2005; Bissonnette, Martins et al. 2008). Cognitive flexibility is also vulnerable to the aging process and shows declines with age (Barense, Fox et al. 2002).

One subtype of glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor, has been shown to be important for learning and memory (Collingridge 1987). NMDA receptors are especially important for spatial memory (Morris 1989) and are also thought to be involved in cognitive flexibility (Nicolle and Baxter 2003). They are highly expressed throughout the frontal lobe, caudate nucleus and hippocampus (Watanabe, Inoue et al. 1993; Rigby, Le Bourdelles et al. 1996), regions shown to play a role in learning and memory (Spiers and Maguire 2007). NMDA receptors are heteromeric tetramers composed of combinations of subunits from different families of proteins, the GluN1 (NR1), GluN2 (NR2) and GluN3 (NR3) subunit families (Dingledine, Borges et al. 1999).

Neurotransmitter binding to NMDA receptors, as well as the expression levels of its subunits, decline with increasing age throughout the frontal lobe, caudate nucleus and hippocampus (Magnusson, Brim et al. 2010). These declines in expression correlate with declines in spatial memory (Magnusson, Scruggs et al. 2007; Magnusson, Brim et al. 2010). Of the NMDA receptor subunits, the expression of the GluN2B subunit exhibits the greatest declines in both protein and mRNA expression with age within the frontal lobe, caudate nucleus and sub-regions of the hippocampus (Magnusson 2000; Magnusson, Nelson et al. 2002; Bai, Hof et al. 2004). In the frontal lobe, the protein levels of the GluN2B subunit show a greater decline with age within the synaptic membrane than the cell as a whole and this decrease correlates with impaired memory (Zhao, Rosenke et al. 2009). The GluN2B subunit is important for spatial memory and its decrease, via experimental manipulation, has been shown to be sufficient to account for the degree of spatial memory impairment seen in aged rodents (Clayton, Mesches et al. 2002). Moreover, previous
research has shown that increasing GluN2B subunit expression throughout multiple brain regions from birth is beneficial to memory and remains beneficial even into middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). However, present research does not address whether increasing the expression of the GluN2B subunit within the aged brain would be beneficial to memory or not.

The present paper explored the effects of increasing the expression of the GluN2B subunit within the frontal lobe of aged mice using a replication deficient adenoviral vector to deliver cDNA specific to the GluN2B subunit. Adenoviral vectors have previously been used to successfully deliver genes to the central nervous system (CNS) (Benitez and Segovia 2003). The purpose of the present study was to determine whether the decline in spatial memory observed in aging could be improved by increasing the expression of the GluN2B subunit within the frontal lobe of the aged brain. This would help determine whether a therapy aimed at enhancing the number of GluN2B containing NMDA receptors in old age would be beneficial to memory.

2.2 Methods

2.2.1 Injection solutions

2.2.1.1 Adenoviral vectors

Custom adenoviral vectors were designed by Viraquest, Inc. (North Liberty, IA). A plasmid containing the cDNA for the GluN2B subunit derived from the mouse genome, a gift from M. Mishina, was subcloned into a human replication deficient type 5 adenoviral vector with a cytomegalovirus (CMV) promoter and reporter gene for enhanced green fluorescence protein (eGFP). The GluN2B vector contains the cDNA of the GluN2B subunit gene and eGFP. The control vector contains only the cDNA of eGFP. The GluN2B vector and control vector were diluted with the A195\textsuperscript{Viraquest} buffer to give a final
concentration of approximately $2.0 \times 10^8$ PFU/mL. Animals meant for vehicle alone were injected with equivalent volumes of the A195Viraquest buffer.

2.2.1.2 GluN2B Antagonist

The GluN2B antagonist, Ro 25-6981 (Sigma Aldrich, St. Louis, MO, USA), was diluted in 100% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) to a final concentration of 5 or 10 mg/mL, as previously published (Fontan-Lozano, Saez-Cassanelli et al. 2007). Each mouse received a subcutaneous injection of either 5 or 10 mg/kg Ro 25-6981 or an equivalent volume of DMSO 30 minutes prior to beginning each block of 4 place trials or cued trials. Each mouse received 1-2 injections per day, depending on the task.

2.2.1.3 Lipopolysaccharide

Lipopolysaccharide from Escherichia coli (Sigma Aldrich, St. Louis, Mo) was diluted in sterile water to give a final concentration of 1mg/mL and then stored at -80°C until used.

2.2.2 Transfection of cells

A laboratory-generated variety of rabbit skin cells were transfected with a 1:10 dilution of either the control vector or the GluN2B vector, followed by an overnight incubation. GFP expression was monitored using a Leica DM LB microscope (Leica Microsystems, Wetzlar, Germany). To visualize GluN2B subunit expression, cells were fixed with methanol and then stained for the GluN2B subunit (see below).
2.2.3 Animals

A total of one hundred and forty-six male C57BL/6 mice (National Institute on Aging, NIH, Bethesda, MD and The Jackson Laboratories, Bar Harbor, ME) representing 2 different age groups (3 and 22-26 months of age) were used for this study. The animals were fed *ad libitum* and housed individually in micro-isolator cages on a 12/12 hour light/dark cycle. The animals within each age group were randomly divided into 3 vector treatment groups (each containing 12-15 animals) and bilaterally injected with either: vehicle, control vector, or GluN2B vector. A separate group of aged mice, which had received bilateral injections of either the GluN2B vector or vehicle solution, were randomly divided into 3 antagonist treatment groups. The antagonist treatment groups received subcutaneous injections of 5mg/kg Ro 25-6981, 10mg/kg Ro 25-6981 or DMSO alone. Following behavioral testing (see below), approximately 21 days post-treatment, the mice were euthanized by exposure to CO₂, followed by decapitation. The brains were removed, frozen on dry ice, and stored at -80°C.

2.2.4 Stereotaxic surgery

All mice underwent stereotaxic surgery and received bilateral injections centered on the orbital cortex, as described by Das et al. with some modifications (Das, Jensen et al. 2012). Twenty-four hours prior to surgery all animals were provided with acetaminophen (1.2 mg/mL) in the drinking water (Tylenol, McNeil-PPC Inc., Skillman, NJ). Each animal was induced with isoflurane and placed in the stereotaxic apparatus. 1-2 mm holes were drilled in the skull using the stereotaxic coordinates 2.58 mm rostral to bregma and ±1.5 mm lateral to the longitudinal suture. Injections were delivered 2.3 mm ventral to the skull surface for the left and right ventral orbital cortices at a rate of 500 nL/minute with the use of an UltraMicroPump III with SYS-Micro4 controller (World Precision Instruments, Sarasota, FL). Injections consisted of equivalent volumes (orbital cortices: 5µl per injection) and equivalent concentrations of GluN2B vector, control vector or vehicle. For the mouse receiving lipopolysaccharide, the volume per
injection was 1µL (1µg lipopolysaccharide). Mice were injected subcutaneously with buprenorphine (0.1mg/kg) (Hospira, Lake Forest, IL) following recovery from anesthesia. Post-surgery, mice were provided with acetaminophen plus codeine phosphate solution (0.12 mg/mL) (Pharmaceutical Associates Inc., Greenville, SC) in their water for 72 hours followed by acetaminophen (1.2 mg/ ml) until they were euthanized. The mouse receiving lipopolysaccharide was treated with buprenorphine (0.1mg/kg) every 12 hours until euthanized 4 days post-injection.

2.2.5 Memory testing

2.2.5.1 Acclimation

Six to seven days post-surgery, mice were acclimated to the water maze for two consecutive days. Each session consisted of each mouse swimming for 60 seconds in the tank without the platform and then being placed on the platform in the tank and trained to remain there for 30 seconds. This platform position was different from those used for memory testing.

Spatial long-term and working memory, cognitive flexibility and associative memory were assessed using the Morris water maze. The water maze consisted of a 1.2 m diameter white tank filled with water made opaque by non-toxic white paint (Prang, Dixon Ticonderoga Company, Heathrow, FL) with spatial cues placed around the tank. An escape platform was hidden 1 cm below water level when present, except in cued trials, in which it was 0.5 cm above the surface. Trials were recorded and analyzed 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 tank wall.
2.2.5.2 Long-term spatial memory task

Following acclimation, mice underwent long-term spatial memory testing for two days. The task consisted of 8 place trials (platform present but hidden) per day and 1 probe trial (platform absent) at the end of each day. There was an hour rest period after every four trials. There was also a naïve probe trial at the beginning of the first day of spatial long-term memory testing. Probe trials were performed to assess any bias for the area of the tank in which the platform would be (naïve probe) or was localized during place trials (Gallagher, Burwell et al. 1993). During each probe trial the platform was inaccessible and the mouse was allowed to search in the water for 30 seconds. 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 120 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 handler.

2.2.5.3 Cognitive flexibility task

Following the long-term spatial memory task, a reversal task was performed to assess cognitive flexibility for 1 day. This task consisted of 8 place trials followed by 1 probe trial. There was an hour rest period following every four trials. The escape platform was placed in the quadrant diagonally opposite to the previous position for all reversal trials. Reversal trials consisted of 60 seconds maximum in the water searching for the platform and 30 seconds on the platform. A 30 second probe trial was performed at one hour after the last reversal trial.

2.2.5.4 Working spatial memory task

Following the cognitive flexibility task, a working spatial memory task was performed. The task consisted of two daily sessions of 4 place trials each for 8 days. The platform position changed for each session. Trials consisted of a naïve trial ($T_{\text{naive}}$) followed by a trial after a 10 minute delay, called $T_{\text{delay}}$.
and 2 more trials at 2 minute delays (not used for evaluation). Trials consisted of 60 seconds maximum in the water searching for the platform and 30 seconds on the platform.

2.2.5.5 Associative memory (control) task

Following the working spatial memory task, an associative (control) memory task was employed to test motivation, visual acuity, and physical ability for memory testing. The task consisted of 6 cued trials with the platform made visible with a 20.3 cm flag. For each trial, the platform was changed to a different position and each mouse 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.

2.2.6 Tissue sectioning

The brain of each animal was cut in half longitudinally and one half (alternating sides across animals) was sectioned coronally using a Leica CM1850 UV Cryostat (Leica Microsystems Inc., Bannockburn, IL). The 20 µm sections were cold-mounted onto plus slides (Thermo Fisher Scientific, Waltham, MA). Brain sections representing at least one animal from each experimental group (age by treatment) were placed on each slide and the order was determined by block design. Slides were kept at -80 degrees Celsius until further processing.

2.2.7 Immunohistochemistry

2.2.7.1 Peptide and antibodies

Representative coronal tissue sections from each animal were labeled with either 20 µg/mL dilution of biotin-conjugated isolectin B4 (Sigma Aldrich, St. Louis, MO) to visualize microglia (inflammation marker), a 1:100 dilution of an anti-NMDA ε2 (GluN2B) goat polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), and/or a 1:500 dilution of an anti-glial fibrillary acidic protein (GFAP)
antibody (Abcam, Cambridge, MA) to visualize astrocytes or a 1:500 dilution of an anti-neurofilament antibody (Abcam, Cambridge, MA) to visualize neurons. Secondary antibodies used for visualization included rhodamine-conjugated donkey anti-goat antibody (Millipore, Billerica, MA) or Alexa Fluor 350 goat anti-rabbit antibody (Molecular Probes, Eugene, OR).

2.2.7.2 Staining protocol

Slides were fixed in 4% paraformaldehyde for 15 min. Slides were treated with 0.3% H₂O₂ solution for 15 min, followed by a 1 hour block in 0.1% BSA (isolectin B₄) or 5% serum (GluN2B or GFAP). Slides were then either incubated in primary antibody or isolectin B₄ for 48 hours. Slides were then treated with either 1:1000 dilution of secondary antibody and/or 1:800 avidin-biotin complex solution (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 1 hour each. Slides were visualized with 3,3′-Diaminobenzidine (DAB) (Sigma Fast DAB, Sigma Aldrich, St. Louis, MO) by incubation for either 5 min (isolectin B₄) or 30 min (GluN2B subunit). Nonspecific slides were incubated in either the absence of biotin-conjugated isolectin B₄ or primary antibody. Images were captured using a Leica DM LB microscope and SPOT camera (Diagnostic Instruments Incorporated, Sterling Heights, MI).

2.2.8 Injection site mapping

Representative coronal (rostral to caudal) sections from each animal were used to map the location of the injection site and the distribution of increased GluN2B subunit expression (or to verify its absence in control animals). All representative sections used for mapping were labeled for GluN2B and visualized with DAB staining.
2.2.9 Data Analysis

The data for each memory task was 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 seconds by the computer for the whole duration of the trial. Cumulative proximity was calculated by summing those distances. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle, WA). 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 the long-term spatial memory and cognitive flexibility tasks and all trials in working and associative memory (control) task. The difference between naïve and delayed trials was calculated from their respective corrected cumulative proximity measurements in the working memory tasks. For any probe trial, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score. Animals were removed from the study if they consistently floated throughout trials (1 mouse), performed significantly worse in cued trials from the rest of the animals (greater than the mean plus two standard deviations; 5 mice) or if there was considerable difference in injection location and protein expression from their counterparts (5 mice). These excluded animals were from both ages and most treatment groups.

2.2.10 Statistical Analysis

The data for memory tasks were statistically analyzed by Analysis of Variance (ANOVA) using Statview software. Performance in long-term spatial memory, cognitive flexibility, working spatial memory and associative memory tasks were analyzed separately by repeated measures ANOVA and two-way ANOVA followed by Fisher’s protected least significant difference post-hoc analysis as indicated. Analysis of
place trials within days was planned because of previous work showing greater age differences on day 2 (Magnusson, Scruggs et al. 2007). The response variable was performance in behavioral trials while the independent variables were age and/or treatment. For all comparisons, only $p \leq 0.05$ were considered statistically significant.

2.3 Results

2.3.1 Effect of the GluN2B vector on spatial and associative memory

There was an overall effect of age on cumulative proximity scores in the long-term spatial memory task, with young animals spending more time closer to the platform than aged animals ($F_{(1,76)}=26.3$, $p=0.0001$; young=6546±1092, aged=9436±1172). There was also an age by treatment interaction ($F_{(2,76)}=4.7$, $p=0.01$). For the cognitive flexibility task, where the location of the platform was moved to the opposite quadrant from that used in the long-term spatial memory task, there was an overall significant effect of age on cumulative proximity scores ($F_{(1,76)}=3.8$, $p=0.05$; young=4444±957, aged=5581±988), with young animals spending more time closer to the platform. The average difference in cumulative proximity between $T_{\textit{naïve}}$ and $T_{\textit{delay}}$ trials over 16 sessions was used to evaluate spatial short-term memory with a 10 minute delay. There was an overall significant effect of age on cumulative proximity ($F_{(1,76)}=11.3$, $p=0.0012$; young=2231±299, aged=1103±506), with young animals having greater differences between the naïve and delayed trials than old. Within the associative memory (control) task, all spatial cues were removed and the platform position was made visible with a flag as a cue to its position for the cued trials. For this control task, there was no overall significant effect of age ($F_{(1,76)}=0.6$, $p=0.44$; young=870±244, aged= 874±275).

There was a significant interaction between age and treatment in place trials and probes ($F_{(2,75)}=3.5$, $p=0.03$). Therefore, the young and aged mice were analyzed separately for treatment effects.
Comparisons between aged treatments and young vehicle treatment were included to assess the degree of improvement. There were no significant effects of treatment on the young mice in any of the behavioral tasks (Appendix Fig. 1, $p=0.15$-0.87), with the exception of the probe trial at the end of the first day of spatial long-term memory. Young mice treated with the GluN2B vector showed a higher average proximity to the platform location compared to either young control group by the end of the first day (Appendix Fig. 1, $p=0.02$-0.025; vehicle= 37±3, control vector= 38±2, GluN2B vector= 55±9). However, their average proximity scores for the platform location was similar to the young control animals by the end of the second day (Appendix Fig. 1, $p=0.60$-0.79, vehicle= 33±3, control vector= 31±2, GluN2B vector= 36±10).

For aged mice, there was an overall significant effect of treatment across blocks of four place trials (Fig. 2.1A, $F_{(2,37)}=3.3$, $p=0.05$) and a significant interaction between treatments and blocks of place trials (Fig. 2.1A, $F_{(6,111)}=3.8$, $p=0.0016$). Aged mice treated with the GluN2B vector had significantly lower cumulative proximity scores than both of the aged control groups by the second day of long-term spatial memory trials (Fig. 2.1B, $p=0.001$-0.015). The cumulative proximity scores of the aged GluN2B vector-treated mice were similar to the cumulative proximity scores of the young vehicle-treated mice by the second day (Fig. 2.1B, $F_{(1,27)}=0.6$, $p=0.44$). Young vehicle-treated mice had significantly lower proximity scores than aged control vector-treated mice on day 1 of long-term spatial memory trials and significantly lower proximity scores than both aged control groups by the second day (Fig. 2.1B, $p<0.0001$-0.0016).

Within probe trials, aged mice treated with the GluN2B vector exhibited lower average proximity to the platform location compared to either aged control group by the end of the second day (Fig. 2.1C, $p=0.0006$-0.05). The average proximity to the platform location of aged mice treated with the GluN2B vector was similar to young vehicle-treated animals by the end of the second day (Fig. 2.1C, $F_{(1,27)}=0.45$, $p=0.52$).
There was no significant effect of treatment in aged mice on average swim speed ($F_{(2,37)}=2.3$, $p=0.11$; aged vehicle: $14 \pm 0.3$, aged control vector: $15 \pm 0.5$, aged GluN2B vector: $16 \pm 0.5$).

There were no significant effects of treatment on cumulative proximity in reversal trials in the aged animals (Fig. 2.1D, $F_{(2,37)}=0.01$, $p=0.99$). There was no overall significant effect of treatment on the average difference in cumulative proximity between trials with a 10 minute delay in aged animals (Fig. 2.1E, $F_{(2,37)}=0.3$, $p=0.73$). Within the associative memory (control) task, there was no significant effect of treatment within aged animals (Fig. 2.1F, $F_{(2,37)}=2.3$, $p=0.12$). There was also no overall significant difference in the cumulative proximity scores in the control task between young vehicle-treated animals and aged GluN2B vector-treated animals (Fig. 2.1F, $F_{(1,27)}=1.0$, $p=0.33$) or aged controls ($p=0.13-0.54$).

### 2.3.2 Effect of a GluN2B antagonist on long-term spatial and associative memory

Aged mice, treated with either GluN2B vector or vehicle within their frontal lobe, were treated systemically with the GluN2B specific antagonist, Ro 25-6981. This was designed to determine whether the increased expression of the GluN2B subunit was responsible for the improved long-term spatial memory observed in aged mice. Systemic delivery of this GluN2B specific antagonist has previously been shown to impair object recognition memory (Fontan-Lozano, Saez-Casanelli et al. 2007). Due to the previous results with enhanced GluN2B subunit expression in the frontal lobe, the focus for the antagonist study was on the second day of spatial long-term memory testing. There were overall significant effects of antagonist treatment (Fig. 2.2A-B, $F_{(2,47)}=4.5$, $p=0.02$), and a significant vector by antagonist interaction (Fig. 2.2A-B, $F_{(2,47)}=3.1$, $p=0.05$). Aged vehicle-treated animals that received $5\text{mg/kg} \text{ Ro 25-6981}$ had significantly higher cumulative proximity scores than vehicle-treated dimethyl sulfoxide (DMSO) animals (Fig. 2.2B, $p=0.002$) in the second day. However, aged GluN2B vector-treated animals that received $5\text{mg/kg} \text{ Ro 25-6981}$ had similar cumulative proximity scores to GluN2B vector-treated DMSO animals (Fig. 2.2B, $p=0.22$). There was a near significant difference in cumulative
proximity between aged vehicle-treated animals that received 5mg/kg Ro 25-6981 compared to GluN2B vector-treated animals that received the same dose of Ro 25-6981, with GluN2B vector treated- animals spending more time closer to the platform than vehicle-treated animals (Fig. 2.2B, F_{(1,15)}=3.5 p=0.08). Aged GluN2B vector-treated mice that received a higher dose of Ro 25-6981 (10 mg/kg) spent less time near the platform than either aged GluN2B vector-treated DMSO (Fig. 2.2B, p=0.002) or aged GluN2B vector-treated animals that received a lower dose of Ro 25-6981 (5 mg/kg) (Fig. 2.2B, p=0.03). Aged GluN2B vector-treated animals that received the higher dose of Ro 25-6981 (10 mg/kg) had similar cumulative proximity scores to aged vehicle-treated mice that received the lower dose of Ro 25-6981 (5 mg/kg) (Fig. 2.2B, F_{(1,12)}=0.63, p=0.44). Within the associative memory (control) task, there were no significant effects of either vector treatment (Fig. 2.2C, F_{(1,45)}=0.05, p=0.83) or antagonist treatment (Fig. 2.2C, F_{(2,45)}=0.08, p=0.93) and no significant vector by antagonist interaction (Fig. 2.2C, F_{(2,45)}=0.01, p=0.99).

### 2.3.3 Effect of vectors on GluN2B protein expression

The efficacy of the adenoviral vectors used was first confirmed in vitro. Rabbit skin cells were transfected with the GluN2B vector and expressed both the GluN2B subunit and GFP, which co-localized (Fig. 2.3A-C). Rabbit skin cells transfected with the control vector only expressed GFP only (Fig 2.3D-E). In vivo, there was increased GluN2B immunoreactivity within the frontal lobe, corpus callosum, and caudate nucleus in mice treated with the GluN2B vector as compared to endogenous expression (Fig. 2.4C, F). Neither control treatments, vehicle (Fig. 2.4A, D) or control vector (Fig. 2.4B, E) elicited a change in GluN2B immunoreactivity from endogenous expression. Increased GluN2B subunit expression was visible in, but was not restricted to, neurons and neuronal processes (Fig.2.4C, F; Fig. 2.5). GFAP-positive cells also showed enhanced GluN2B immunoreactivity (Fig. 2.4F). There was lipofuscin (yellow profiles) present in the images of aged brains (Fig. 2.4A-F). Injection site mapping of strongly labeled
cells following the frontal lobe injections indicated that increased GluN2B subunit expression was predominantly localized to the frontal lobe, including the orbital and motor cortices, but was also seen in the corpus callosum, the caudate nucleus and in the ependymal cells lining the lateral ventricles (Fig. 2.6).

2.3.4 No effect of vectors on inflammation

Since adenoviral vectors have been shown to elicit an inflammatory response within brain tissue (Byrnes, Rusby et al. 1995), representative coronal sections from each age and treatment were stained with an isolectin specific for microglia (Streit 1990). Microglia are a marker of inflammation (Herber, Maloney et al. 2006), and are evident in tissue from the frontal lobe of a young mouse (positive control) that received bilateral injections of lipopolysaccharide from *Escherichia coli* into the frontal lobe (Fig. 2.7D). There was no appreciable difference in inflammation within the injection sites across treatments (Fig. 2.7A-C). Tissue from vector-treated animals exhibited inflammation comparable to vehicle-treated animals (Fig. 2.7A-C). There was no visible difference between aged or young vehicle-treated animals (data not shown).

2.4 Discussion

This study provides compelling evidence that increasing the expression of the GluN2B subunit of the NMDA receptor within the frontal lobe and caudate nucleus of aged mice is beneficial to memory. Aged mice with enhanced GluN2B subunit expression across these brains regions exhibited superior long-term spatial memory over their aged counterparts, and exhibited memory similar to young mice. This suggests that increasing expression of the GluN2B subunit within the aged brain can improve memory in aged mice.

Aged mice with enhanced GluN2B subunit expression across the frontal lobe and caudate nucleus improved learning in the second day of long-term memory training, as compared to aged controls, and the
improved performance was similar to that seen in young vehicle-treated mice. By the end of the second day of the long-term spatial memory task, these aged mice showed a stronger bias for the platform location compared to either aged control and, in fact, showed a similar bias for the platform location as compared to young vehicle-treated mice. Immunohistochemistry revealed increased GluN2B subunit expression within cells with either neuronal or glial characteristics across the frontal lobe and caudate of aged mice treated with the GluN2B vector compared to control treatments. Multiple neuronal processes showed enhanced GluN2B subunit expression, suggesting that it was enriched within dendrites, which is where excitatory synapses predominate. Together, these data suggest that the adenoviral vector effectively induced increased production of the GluN2B subunit and that aged animals with enhanced expression of the GluN2B subunit exhibited improved learning and retention in a long-term spatial memory task, comparable to young mice.

To confirm and expand these findings, a highly selective and activity-dependent GluN2B antagonist, Ro 25-6981 (Fischer, Mutel et al. 1997), was administered systemically to aged mice in order to determine whether the increased expression of the GluN2B subunit was responsible for the improved memory observed in aged mice with enhanced GluN2B subunit expression across the frontal lobe and caudate nucleus. Systemic delivery of Ro 25-6981 has previously been shown to impair memory performance in an object recognition task (Fontan-Lozano, Saez-Cassanelli et al. 2007). A dose of 5mg/kg of Ro 25-6981 was sufficient to impair spatial long-term memory in aged vehicle-treated mice compared to control by the second day of trials. However, an increased dose (10mg/kg) was required to impair spatial long-term memory in aged mice with enhanced GluN2B subunit expression in the frontal lobe compared to control, and to impair memory similarly to aged vehicle-treated mice that received only a 5mg/kg dose of Ro 25-6981. The lack of difference between the GluN2B vector- and vehicle-injected mice treated with DMSO appeared to be due to trends for a change in performance of both groups from the first experiment. This may be due to the administration of subcutaneous injections of DMSO, which can increase nociception.
(Colucci, Maione et al. 2008). Also, increased expression of the GluN2B subunit has been associated with increased sensitization to pain in GluN2B-enhanced animals (Wei, Wang et al. 2001), which may explain the worsening of performance in the GluN2B vector-injected mice. There was, however, an overall trend for better performance in the GluN2B vector-treated animals injected with the 5 mg/kg dose of Ro 25-6981 than the vehicle-treated animals receiving the same antagonist dose. The need for a higher dose of Ro 25-6981 to impair memory in aged mice with enhanced GluN2B subunit expression provides further evidence that it was the increased expression of the GluN2B subunit within the brains of aged mice that was responsible for superior memory.

The GluN2B subunit of the NMDA receptor imparts slower channel deactivation to the NMDA receptor, which is important for long-term potentiation (LTP), a cellular mechanism believed to underlie learning and memory (Tovar, Sprouffske et al. 2000). Transgenic mice designed to express higher levels of the GluN2B subunit from birth also possess superior memory across adulthood to middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). Region specific knockouts of the GluN2B subunit show that expression of the GluN2B subunit within the frontal lobe and the CA1 region of the hippocampus is necessary for long term potentiation and depression, and learning and memory in spatial and fear conditioning tasks (Brigman, Wright et al. 2010). Specifically, pharmacological and genetic blockade of the GluN2B subunit within the prefrontal cortex results in diminished long term potentiation and impaired fear conditioning (Zhao, Toyoda et al. 2005) Based on the above findings, the enhanced expression of GluN2B subunit within the frontal lobe and caudate nucleus could account for the superior memory observed in aged mice in this study.

Memory tasks in the Morris water maze involve acquisition, consolidation and/or retrieval of spatial memories (Morris and Davis 1994). The acquisition of long-term memories has been shown to occur early in training sessions in the Morris water maze and can be blocked by the application of protein
synthesis inhibitors prior to training sessions (Lattal and Abel 2001). In contrast, consolidation of long-term memories has been shown to occur within several hours to days after a training session for a memory task and can be extinguished by the application of protein synthesis inhibitors post-training sessions (Bourtchouladze, Abel et al. 1998). The NMDA receptor has been shown to be involved in both the acquisition and consolidation of spatial memories in the Morris water maze by blockade with the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (Liang, Hon et al. 1994). Lesions studies within the orbital cortex have been shown to impair consolidation of spatial memory within the Morris water maze (Vafaei and Rashidy-Pour 2004). Moreover, transgenic mice designed to express high levels of the GluN2B subunit throughout the brain, including the frontal lobe, exhibit improved learning on the second day of learning trials (Tang, Shimizu et al. 1999). These data may suggest that the enhanced learning observed in the second day of long-term spatial memory trials in aged mice with increased GluN2B subunit expression in the frontal lobe and caudate nucleus could have been the result of improved later learning (consolidation and/or later acquisition).

There was no significant effect of treatment on the performance of young mice in any memory task; and enhanced expression of the GluN2B subunit did not improve cognitive flexibility or working spatial memory in aged mice. However, transgenic mice designed to express high levels of the GluN2B subunit during development through adulthood possess superior memory compared to wild type (Tang, Shimizu et al. 1999). In addition, both the frontal lobe and hippocampus have been shown to be important for cognitive flexibility in rodents (de Bruin JP 1994; Watson and Stanton 2009) and both the NMDA receptor and the GluN2B subunit have been shown to be necessary for cognitive flexibility (Nicolle and Baxter 2003; Duffy, Labrie et al. 2008). Regions of the frontal lobe, such as the dorsomedial prefrontal cortex, have also been shown to be important to working spatial memory (Horst and Laubach 2009). Increased expression of the GluN2B subunit across brain regions has been implicated in improved short-term spatial memory (Wong, Setou et al. 2002; Cui, Jin et al. 2011). In light of these data, the present
results could suggest that the increase in GluN2B subunit expression presented here was not sufficient to improve memory in young animals or improve short-term spatial memory and cognitive flexibility in aged animals.

In conclusion, increasing the expression of the GluN2B subunit of the NMDA receptor in the frontal lobe appears to restore later long-term spatial memory of aged mice back to the level of young. This study further substantiates the important role of the GluN2B subunit in long-term memory. The present work also shows the importance of the decline of the GluN2B subunit to the memory loss that occurs with aging. Moreover, the evidence provided by this study suggests that a therapeutic aimed at enhancing the number or function of the GluN2B containing NMDA receptors in old age could potentially be used to effectively ameliorate memory loss.

2.5 Acknowledgements

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2.5.1 Disclosure statement

Yes, there is potential conflict of interest. R.H. has a financial interest in Viraquest, Inc., the company that provided the viral vector.
Figure 2.1
Fig. 2.1– The effects of enhanced GluN2B subunit expression in the frontal lobe on the performance of aged mice in memory tasks in the Morris water maze. Graphs A-F show the effects of the GluN2B vector or 2 control (control vector or vehicle) treatments on learning performance. In graphs A-D and F, lower proximity scores represent better performance. In graph E, greater differences between naïve and delayed trials represent better performance. The performance of young vehicle-treated mice is included for comparison. (A) Performance across blocks of four place trials for the two-day long-term spatial memory task. (B) Performance averaged across place trials for each individual day of the two-day long-term spatial memory task. (C) Performance within probe trials of the two-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Differences in performance between T naïve and T delay trials, averaged over working spatial memory sessions, over eight days. (F) Performance in 6 cued trials for the associative memory task. * p<0.05 for differences from aged GluN2B vector-treated mice. # p<0.05 for differences from young vehicle-treated mice. N=12-14. Bl = block of 4 place trials, Pr = probe trials, Pr0= naïve probe trials, R= reversal trials, C= cued trials. Error bars = standard error of the mean (SEM).
Figure 2.2
Fig. 2.2– The effects of the GluN2B antagonist, Ro 25-6981, on the performance of aged mice with enhanced GluN2B subunit expression in two memory tasks in the Morris water maze. Graphs A-C show the effects of Ro 25-6981 antagonism on the performance of aged mice receiving frontal lobe injections of GluN2B vector or vehicle, with lower proximity scores representing better performance. (A) Learning performance of aged mice across blocks of place trials for the two-day long-term spatial memory task. (B) Learning performance of aged mice averaged across place trials for day 2 of the two-day long-term spatial memory task. (C) Performance of aged mice in 6 cued trials for the associative memory task. * \( p \leq 0.05 \) for differences in performance between treatment groups indicated. N=4-16. Bl = blocks of 4 place trials, C= cued trials. Error bars = standard error of the mean (SEM).
Fig. 2.3- The expression of the GluN2B and control vectors *in vitro*. Representative images of rabbit skin cells treated with either GluN2B vector (A,B,C) or control vector (D,E) showing the protein expression of GFP (A,C,D) and GluN2B (B,C,E). Green= GFP (*in vivo*), Red= GluN2B subunit, Co-localized= yellow. Bar= 25 µm.
Fig. 2.4- Enhanced GluN2B subunit expression *in vivo* in the frontal lobe, approximately 21 days post-treatment. Representative images of coronal sections showing GluN2B subunit, GFP (vector reporter) and GFAP (glial marker) protein expression within the frontal lobe in different treatments: (A, D) vehicle, (B, E) control vector and (C, F) GluN2B vector within aged mice. Panels D-F are higher magnification images of areas shown in panels A-C, respectively. Green= GFP (*in vivo*), Red= GluN2B subunit, Blue= GFAP, Yellow= lipofuscin, co-localized GFP and GluN2B = orange, co-localized GFAP and GluN2B= purple, GluN2B subunit in neuronal-like cells (arrows), GluN2B subunit in astrocytes (arrowheads). Bar= 25 µm.
Fig. 2.5- Enhanced GluN2B subunit expression *in vivo* in neurons, approximately 21 days post-treatment. Representative image of a coronal section showing GluN2B subunit and neurofilament (neuronal marker) protein expression within the frontal lobe of an aged mouse treated with GluN2B vector. Panel A shows neurofilament only and Panel B shows GluN2B subunit only. Panel C shows GluN2B subunit with neurofilament. Red= GluN2B subunit, Green= neurofilament, co-localized neurofilament and GluN2B= orange, Bar= 5 µm.
Fig. 2.6– Brain regions with enhanced GluN2B subunit expression in the frontal lobe and caudate nucleus. Representative diagrams of coronal sections (adapted from Franklin & Paxinos’s Mouse Brain Atlas, 2007) show the locations of cells that were intensely labeled for the GluN2B subunit across the frontal lobe and caudate nucleus. cc= corpus callosum; Cg= cingulate cortex; CN= caudate nucleus; fmi= forceps minor of the corpus callosum; I=insular cortex; IL= infralimbic cortex; LO=lateral orbital cortex; LV= lateral ventricle; M1=primary motor cortex M2=secondary motor cortex; MO=medial orbital cortex; PrL= prelimbic cortex; S= somatosensory cortex; VO= ventral orbital cortex.
Fig.2.7– Inflammation in vector-treated mice was not increased over vehicle-treated mice in the frontal lobe, approximately 4 days post-treatment. Panels A-D show isolectin B₄ staining of microglia in representative coronal sections taken at the injection site in the frontal lobe. (D) Inflammation was visible in a lipopolysaccharide-treated brain (positive control) and, to a lesser extent, equally across the different treatments, within the injection sites only: (A) vehicle, (B) control vector and (C) GluN2B vector. Bar=50 µm.
References


CHAPTER III

MEMORY IN AGED MICE IS RESCUED BY ENHANCED EXPRESSION OF THE GluN2B (NR2B) SUBUNIT OF THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR IN THE DORSAL HIPPOCAMPUS
MEMORY IN AGED MICE IS RESCUED BY ENHANCED EXPRESSION OF THE GluN2B (NR2B) SUBUNIT OF THE \( N\)-METHYL-\( D\)-ASPARTATE (NMDA) RECEPTOR IN THE DORSAL HIPPOCAMPUS

Authors and affiliations

B. L. Brim\textsuperscript{a,b,c}, R. Haskell\textsuperscript{d}, R. Awedikian\textsuperscript{e}, N.M. Ellinwood\textsuperscript{e}, L. Jin\textsuperscript{a,b}, K. Magnusson\textsuperscript{a,b,c}

\textsuperscript{a}Molecular and Cellular Biosciences Program, Oregon State University, Corvallis, OR, 97331, U.S.A.
\textsuperscript{b}Department of Biomedical Sciences, Oregon State University, Corvallis, OR, 97331, U.S.A.
\textsuperscript{c}Healthy Aging Program, Linus Pauling Institute, Oregon State University, Corvallis, OR; 97331, U.S.A.
\textsuperscript{d}ViraQuest, Inc., North Liberty, IA; 52317, U.S.A.
\textsuperscript{e}Department of Animal Sciences, Iowa State University, Ames, IA, 50011, U.S.A.

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Abstract

The GluN2B subunit of the N-methyl-D-aspartate (NMDA) receptor shows age-related declines in expression across the hippocampus. This decline is strongly correlated to age-related memory decline. However, a subset of aged mice expressing higher levels of the GluN2B subunit within the synaptic membrane in the hippocampus than the other aged counterparts exhibit poorer long-term spatial memory. This study was designed to determine if increasing GluN2B subunit expression in the dorsal hippocampus would improve memory in aged mice. Mice were injected bilaterally with either the GluN2B vector, containing cDNA specific for the GluN2B subunit and enhanced Green Fluorescent Protein (eGFP); a control vector or vehicle. Spatial memory, cognitive flexibility and associative memory were assessed using the Morris water maze. Aged mice, with increased GluN2B subunit expression in the dorsal hippocampus, exhibited improved long-term spatial memory, comparable to young mice, early in training. Therefore, therapies that enhance GluN2B subunit expression across the aged brain could help ameliorate memory loss.

Keywords: Aging, spatial memory, long-term memory, reference memory, cognitive flexibility, associative memory, hippocampus, dentate gyrus, CA1, NMDA receptor, NR2B subunit, E2 subunit, Morris water maze, stereotaxic injection, C57BL/6 mice, adenoviral vector

3.1 Introduction

One subtype of glutamate receptors, the N-methyl-D-aspartate (NMDA) receptor, is a heteromeric tetramer composed of combinations of subunits from different families of proteins, the GluN1 (NR1), GluN2 (NR2) and GluN3 (NR3) subunit families (Dingledine, Borges et al. 1999). The NMDA receptor has been shown to be important for learning and memory (Collingridge 1987). It is highly expressed throughout the frontal lobe, caudate nucleus and hippocampus (Watanabe, Inoue et al. 1993; Rigby, Le
Bourdelles et al. (1996). These same brain regions are important for the acquisition, consolidation and retrieval of spatial memory (Spiers and Maguire 2007).

Aging causes declines in several cognitive functions (Albert 1994). One of the earliest cognitive functions to show decline with age is memory (Albert and Funkenstein 1992), and spatial memory, which is responsible for the navigation of organisms within their environment, is particularly vulnerable to the aging process (Barnes 1988). The NMDA receptor is similarly vulnerable to the aging process. Neurotransmitter binding to NMDA receptors, as well as the expression levels of its subunits, decline with increasing age throughout the frontal lobe, caudate nucleus and hippocampus and these declines in expression correlate with declines in spatial memory (Magnusson, Scruggs et al. 2007; Magnusson, Brim et al. 2010).

Of the NMDA receptor subunits, the expression of the GluN2B subunit exhibits the greatest declines in both protein and mRNA expression with age within the frontal lobe, caudate nucleus and sub-regions of the hippocampus (Magnusson 2000; Magnusson, Nelson et al. 2002; Bai, Hof et al. 2004). The GluN2B subunit is important for spatial memory and its decrease in the hippocampus, via experimental manipulation, has been shown to be sufficient to account for the degree of spatial memory impairment seen in aged rodents (Clayton, Mesches et al. 2002). Previous research has shown that increasing GluN2B subunit expression throughout multiple brain regions from birth is beneficial to memory and remains beneficial even into middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). However, a subset of aged mice expressing higher levels of the GluN2B subunit within the synaptic membrane in the hippocampus exhibit poorer long-term spatial memory than their aged counterparts (Zhao, Rosenke et al. 2009). It is currently not known whether increasing the expression of the GluN2B subunit within the hippocampus would be beneficial to memory or not.
The present paper explored the effects of increasing the expression of the GluN2B subunit within the dorsal hippocampus of aged mice using a replication deficient adenoviral vector to deliver cDNA specific to the GluN2B subunit. Adenoviral vectors have previously been used to successfully deliver genes to the central nervous system (CNS) (Benitez and Segovia 2003). The purpose of the present study was to determine whether the decline in spatial memory observed in aging could be improved by increasing the expression of the GluN2B subunit within the hippocampus of the aged brain. This would help determine whether a therapy aimed at enhancing the number of GluN2B containing NMDA receptors across the aged brain would be beneficial to memory.

3.2 Methods

3.2.1 Injection solutions

3.2.1.1 Adenoviral vectors

Custom adenoviral vectors were designed by Viraquest, Inc. (North Liberty, IA). A plasmid containing the cDNA for the GluN2B subunit derived from the mouse genome, a gift from M. Mishina, was subcloned into a human replication deficient type 5 adenoviral vector with a cytomegalovirus (CMV) promoter and reporter gene for enhanced green fluorescence protein (eGFP). The GluN2B vector contains the cDNA of the GluN2B subunit gene and eGFP. The control vector contains only the cDNA of eGFP. The GluN2B vector and control vector were diluted with the A195Viraquest buffer to give a final concentration of approximately 2.0 x10^8 PFU/mL. Animals meant for vehicle alone were injected with equivalent volumes of the A195Viraquest buffer.
3.2.1.2 Lipopolysaccharide

Lipopolysaccharide from *Escherichia coli* (Sigma Aldrich, St. Louis, Mo) was diluted in sterile water to give a final concentration of 1mg/mL and then stored at -80ºC until used.

3.2.2 Transfection of cells

A laboratory-generated variety of rabbit skin cells were transfected with a 1:10 dilution of either the control vector or the GluN2B vector, followed by an overnight incubation. GFP expression was monitored using a Leica DM LB microscope (Leica Microsystems, Wetzlar, Germany). To visualize GluN2B subunit expression, cells were fixed with methanol and then stained for the GluN2B subunit (see below).

3.2.3 Animals

A total of seventy-five male C57BL/6 mice (National Institute on Aging, NIH, Bethesda, MD and The Jackson Laboratories, Bar Harbor, ME) representing 2 different age groups (3 and 22-24 months of age) were used for this study. The animals were fed *ad libitum* and housed individually in micro-isolator cages on a 12/12 hour light/dark cycle. The animals within each age group were randomly divided into 3 vector treatment groups (each containing 7-15 animals) and bilaterally injected with either: vehicle, control vector, or GluN2B vector. Following behavioral testing (see below), approximately 14 days post-treatment, the mice were euthanized by exposure to CO₂, followed by decapitation. The brains were removed, frozen on dry ice, and stored at -80ºC.

3.2.4 Stereotaxic surgery

All mice underwent stereotaxic surgery and received bilateral injections centered on the dorsal hippocampus as described by Das et al with some modifications (Das, Jensen et al. 2012). Twenty-four
hours prior to surgery all animals were provided with acetaminophen (1.2 mg/mL) in the drinking water (Tylenol, McNeil-PPC Inc., Skillman, NJ). Each animal was induced with isoflurane and placed in the stereotaxic apparatus. 1-2 mm holes were drilled in the skull using the stereotaxic coordinates 1.70 mm caudal to bregma and ±1.0 mm lateral to the longitudinal suture for the hippocampus. Injections were delivered 2.5 mm ventral to the skull surface for the right and left hippocampi at a rate of 500 nL/minute with the use of an UltraMicroPump III with SYS-Micro4 controller (World Precision Instruments, Sarasota, FL). Injections consisted of equivalent volumes (hippocampi: 3µl per injection) and equivalent concentrations of GluN2B vector, control vector or vehicle. For the mouse receiving lipopolysaccharide, the volume per injection was 1µL (1µg lipopolysaccharide). Mice were injected subcutaneously with buprenorphine (0.1mg/kg) (Hospira, Lake Forest, IL) following recovery from anesthesia. Post-surgery, mice were provided with acetaminophen plus codeine phosphate solution (0.12 mg/mL) (Pharmaceutical Associates Inc., Greenville, SC) in their water for 72 hours followed by acetaminophen (1.2 mg/ ml) until they were euthanized. The mouse receiving lipopolysaccharide was treated with buprenorphine (0.1mg/kg) every 12 hours until euthanized 4 days post-injection.

3.2.5 Memory testing

3.2.5.1 Acclimation

Six to seven days post-surgery, mice were acclimated to the water maze for two consecutive days. Each session consisted of each mouse swimming for 60 seconds in the tank without the platform and then being placed on the platform in the tank and trained to remain there for 30 seconds. This platform position was different from those used for memory testing.

Spatial long-term memory, cognitive flexibility and associative memory were assessed using the Morris water maze. The water maze consisted of a 1.2 m diameter white tank filled with water made opaque by
non-toxic white paint (Prang, Dixon Ticonderoga Company, Heathrow, FL) with spatial cues placed around the tank. An escape platform was hidden 1 cm below water level when present, except in cued trials, in which it was 0.5 cm above the surface. The Atlantis platform (HVS Image Ltd, Twickenham, Middlesex, UK) was used for the escape platform. Trials were recorded and analyzed 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 tank wall.

3.2.5.2 Long-term spatial memory task

Following acclimation, mice underwent long-term spatial memory testing for 3 days. The task consisted of 8 place trials (platform present but hidden) per day and 1 probe trial (submerged 12 cm beneath the surface) at the end of each day. There was an hour rest period after every four trials. There was also a naïve probe trial at the beginning of the first day of spatial long-term memory testing. Probe trials were performed to assess any bias for the area of the tank in which the platform would be (naïve probe) or was localized during place trials (Gallagher, Burwell et al. 1993). During each probe trial the platform was inaccessible and the mouse was allowed to search in the water for 30 seconds. At the end of 30 seconds, the platform was brought to 1 cm below the surface of the water. 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 120 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 handler.

3.2.5.3 Cognitive flexibility task

Following the long-term spatial memory task, a reversal task was performed for 1 day to assess cognitive flexibility. This task consisted of 8 place trials followed by 1 probe trial. There was an hour rest period following every four trials. The escape platform was placed in the quadrant diagonally opposite to the
previous position for all reversal trials. Reversal trials consisted of 60 seconds maximum in the water searching for the platform and 30 seconds on the platform. A 30 second probe trial was performed at one hour after the last reversal trial.

3.2.5.4 Associative memory (control) task

Following the cognitive flexibility task, an associative (control) memory task was employed to test motivation, visual acuity, and physical ability for memory testing. The task consisted of 6 cued trials with the platform made visible with a 20.3 cm flag. For each trial, the platform was changed to a different position and each mouse 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.

3.2.6 Tissue sectioning

The brain of each animal was cut in half longitudinally and one half (alternating sides across animals) was sectioned coronally using a Leica CM1850 UV Cryostat (Leica Microsystems Inc., Bannockburn, IL). The 20 µm sections were cold-mounted onto plus slides (Thermo Fisher Scientific, Waltham, MA). Brain sections representing at least one animal from each experimental group (age by treatment) were placed on each slide and the order was determined by block design. Slides were kept at -80 degrees Celsius until further processing.

3.2.7 Immunohistochemistry

3.2.7.1 Peptide and antibodies

Representative coronal tissue sections from each animal were labeled with either 20 µg/mL dilution of biotin-conjugated isolectin B4 (Sigma Aldrich, St. Louis, MO) to visualize microglia (inflammation marker), a 1:100 dilution of an anti-NMDA ε2 (GluN2B) goat polyclonal antibody (Santa Cruz...
Biotechnologies, Santa Cruz, CA), and/or a 1:500 dilution of an anti-glial fibrillary acidic protein (GFAP) antibody (Abcam, Cambridge, MA) to visualize astrocytes or a 1:500 dilution of an anti-neurofilament antibody (Abcam, Cambridge, MA) to visualize neurons. Secondary antibodies used for visualization included rhodamine-conjugated donkey anti-goat antibody (Millipore, Billerica, MA) or Alexa Fluor 350 goat anti-rabbit antibody (Molecular Probes, Eugene, OR).

3.2.7.2 Staining protocol

Slides were fixed in 4% paraformaldehyde for 15 min. Slides were treated with 0.3% H$_2$O$_2$ solution for 15 min, followed by a 1 hour block in 0.1% BSA (isolectin B$_4$) or 5% serum (GluN2B or GFAP). Slides were then either incubated in primary antibody or isolectin B$_4$ for 48 hours. Slides were then treated with either 1:1000 dilution of secondary antibody and/or 1:800 avidin-biotin complex solution (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 1 hour each. Slides were visualized with 3,3'-Diaminobenzidine (DAB) (Sigma Fast DAB, Sigma Aldrich, St. Louis, MO) by incubation for either 5 min (isolectin B$_4$) or 30 min (GluN2B subunit). Nonspecific slides were incubated in either the absence of biotin-conjugated isolectin B$_4$ or primary antibody. Images were captured using a Leica DM LB microscope and SPOT camera (Diagnostic Instruments Incorporated, Sterling Heights, MI).

3.2.8 Injection site mapping

Representative coronal (rostral to caudal) sections from each animal were used to map the location of the injection site and the distribution of increased GluN2B subunit expression (or to verify its absence in control animals). All representative sections used for mapping were labeled for GluN2B and visualized with DAB staining.
3.2.9 Data Analysis

The data for each memory task was 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 seconds by the computer for the whole duration of the trial. Cumulative proximity was calculated by summing those distances. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle, WA). 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 the long-term spatial memory and cognitive flexibility tasks and all trials in working and associative memory (control) task. The difference between naïve and delayed trials was calculated from their respective corrected cumulative proximity measurements in the working memory tasks. For any probe trial, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score. Animals were removed from the study if there was considerable difference in injection location and protein expression from their counterparts (10 mice). These excluded animals were from both ages and most treatment groups.

3.2.10 Statistical Analysis

The data for memory tasks were statistically analyzed by Analysis of Variance (ANOVA) using Statview software. Performance in long-term spatial memory, cognitive flexibility, and associative memory tasks were analyzed separately by repeated measures ANOVA and two-way ANOVA followed by Fisher’s protected least significant difference post-hoc analysis as indicated. Analysis of place trials within days was planned because of previous work showing greater age differences on day 2 (Magnusson, Scruggs et
The response variable was performance in behavioral trials while the independent variables were age and/or treatment. For all comparisons, only $p \leq 0.05$ were considered statistically significant.

### 3.3 Results

#### 3.3.1 Effect of the GluN2B vector on spatial and associative memory

There was an overall effect of age on cumulative proximity in the long-term spatial memory task, with young animals spending more time closer to the platform than aged animals ($F_{(1,59)}=28.9$, $p=0.0001$; young=$4703 \pm 738$, aged=$6864 \pm 836$). For the cognitive flexibility task, there was an overall significant effect of age on cumulative proximity ($F_{(1,59)}=27.2$, $p<0.0001$; young=$3983+1036$, aged=$6448+1044$), with young animals having lower proximity scores than aged animals. Within the associative memory (control) task, all spatial cues were removed and the platform position was cued with a flag. There was no overall significant effect of age ($F_{(1,58)}=0.77$, $p=0.38$; young=$3057+880$, aged=$2663+753$) or treatment ($F_{(2,58)}=0.33$, $p=0.72$) across associative memory trials.

There were significant interactions between age or treatment and blocks of place trials in the spatial long-term memory trials ($p=0.0003-0.004$). In order to make comparison between treatments, each age group was analyzed separately. Comparisons between aged treatments and young vehicle treatment were included to assess the degree of improvement. There were no significant effects of treatment on the young mice in any of the behavioral tasks (Appendix Fig. 2, $p=0.11-0.97$).

Within aged mice, there was no overall effect of treatment across blocks of four place trials (Fig. 3.1A-B, $F_{(2,33)}=0.66$, $p=0.52$) but there was a significant interaction of treatment by blocks of four place trials (Fig.3.1A-B, $F_{(10,165)}= 3.1$, $p=0.0012$). Aged mice treated with the GluN2B vector had lower cumulative proximity scores than both of the aged control groups in the first day of long-term spatial memory trials (Fig. 3.1B, $p=0.002-0.05$). The cumulative proximity scores of aged mice treated with the GluN2B vector
for the first day were not significantly different from those of young vehicle-treated mice (Fig. 3.1B, \(F_{(1,26)}=1.2, p=0.29\)). However, for aged mice treated with the GluN2B vector there was a trend for higher cumulative proximity scores in later trials compared to aged controls (Fig. 3.1A). For day 2 and 3, aged GluN2B vector-treated mice had higher cumulative proximity scores, as compared to young vehicle-treated animals (Fig. 3.1A-B, \(p=0.004-0.03\)). For probe trials, there was no overall effect of treatment on average proximity scores for aged animals (Fig. 3.1C, \(p=0.06-0.77\)). In the flexibility task, there was no significant effect of treatment on cumulative proximity scores within aged animals (Fig. 3.1D, \(F_{(2,33)}=0.68, p=0.52\)). There was no significant effect of treatment on cumulative proximity scores within aged (Fig. 3.1E, \(F_{(2,33)}=0.29, p=0.75\)) in the associative memory (control) task.

### 3.3.2 Effect of vectors on GluN2B protein expression

The efficacy of the adenoviral vectors used was first confirmed *in vitro*. Rabbit skin cells were transfected with the GluN2B vector and expressed both the GluN2B subunit and GFP, which co-localized (Fig. 3.2A-C). Rabbit skin cells transfected with the control vector only expressed GFP only (Fig. 3.2D-E). *In vivo*, there was increased GluN2B immunoreactivity within the hippocampus and corpus callosum for the hippocampal injections in mice treated with the GluN2B vector as compared to endogenous expression (Fig. 3.3C, F, I, J, K). Neither control treatments, vehicle (Fig. 3.3A, D, G) or control vector (Fig. 3.3B, E, H) elicited a change in GluN2B immunoreactivity from endogenous expression. Increased GluN2B subunit expression was visible in, but was not restricted to neurons and neuronal processes (Fig. 3.3C, F, I, J, K; Fig. 3.4). GFAP-positive cells also showed enhanced GluN2B immunoreactivity (Fig. 3.3I, J, K). There was lipofuscin (yellow profiles) present in the images of aged brains (Fig. 3.3A-I). Injection site mapping of strongly labeled cells following the hippocampal injections indicated that increased GluN2B subunit expression was restricted to the corpus callosum, sub-regions of the hippocampus, including the
dentate gyrus and CA1 region, and in the ependymal cells lining the dorsal 3rd ventricle and lateral ventricles (Fig. 3.4).

3.3.3 No effect of vectors on inflammation

Since adenoviral vectors have been shown to elicit an inflammatory response within brain tissue (Byrnes, Rusby et al. 1995), representative coronal sections from each age and treatment were stained with an isolectin specific for microglia (Streit 1990). Microglia are a marker of inflammation (Herber, Maloney et al. 2006), and is evident in tissue from the frontal lobe of a young mouse (positive control) that received bilateral injections of lipopolysaccharide from Escherichia coli into the frontal lobe (Fig. 3.5D). There was no appreciable difference in inflammation within the injection sites across treatments (Supplemental Fig. 3.5A-C). Tissue from vector-treated animals exhibited inflammation comparable to vehicle-treated animals (Fig. 3.5A-C). There was no visible difference between aged or young vehicle-treated animals (data not shown).

3.4 Discussion

This study provides compelling evidence that increasing the expression of the GluN2B subunit of the NMDA receptor within the dorsal hippocampus of aged mice is beneficial to memory. Aged mice with enhanced GluN2B subunit expression across the dorsal hippocampus exhibited superior early long-term spatial memory over their aged counterparts, and exhibited memory similar to young mice. This suggests that increasing expression of the GluN2B subunit within the aged brain can improve memory in aged mice.

Aged mice with increased GluN2B subunit expression in the dorsal hippocampus exhibited improved early learning in the first day of the long-term spatial memory task compared to aged controls, and performed similarly to young vehicle-treated mice only on the first day. However, enhanced expression of
the GluN2B subunit within the hippocampus of the aged brain did not improve spatial bias development. This could be due to the fact that memory was enhanced early in learning trials but plateaued by the end of the second day. By the end of the second day, the performance of aged mice with enhanced GluN2B subunit expression had plateaued and their performance was significantly poorer than young vehicle-treated mice throughout the long-term spatial memory trials. This could suggest that either the enhanced expression was either not sufficient to rescue memory during this phase of the long-term spatial memory task or that enhanced GluN2B subunit expression was having a detrimental effect in the aged animals. Immunohistochemistry revealed increased GluN2B subunit expression within cells with either neuronal or glial characteristics across the hippocampus of aged mice treated with the GluN2B vector compared to control treatments. In addition, multiple neuronal processes showed enhanced GluN2B subunit expression, suggesting that it was enriched within dendrites, which is where excitatory synapses predominate. Together, these data suggest that the adenoviral vector effectively induced increased production of the GluN2B subunit and that aged animals with enhanced expression of the GluN2B subunit exhibited improved early learning and retention in a long-term spatial memory task, comparable to young mice. However, these data also suggest that there is the potential for enhanced GluN2B subunit expression to negatively impact later learning and memory in the long-term spatial memory task.

The NMDA receptor has been shown to be involved in both the acquisition and consolidation of spatial memories in the Morris water maze by studies involving receptor blockade with the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (Liang, Hon et al. 1994). Specifically, NMDA receptors within the CA1 region of the hippocampus have been shown to be necessary to the acquisition of spatial memories (Tsien, Huerta et al. 1996). The acquisition of long-term memories has been shown to occur early in training sessions in the Morris water maze and can be blocked by the application of protein synthesis inhibitors prior to training sessions (Lattal and Abel 2001). This may suggest that the improvement in learning seen within the first day of long-term spatial memory trials (2 hour period) in
aged mice with enhanced GluN2B subunit expression in the hippocampus was the result of improved acquisition. Our results suggest that enhancing GluN2B subunit expression within the hippocampus of aged mice might improve early learning (acquisition). A more global enhancement of this subunit throughout the brain may improve overall learning and memory. However, given the trend for poorer performance of aged mice with enhanced GluN2B subunit expression later in learning trials, there is the potential for more detrimental effects with a more global enhancement.

The GluN2B subunit of the NMDA receptor imparts slower channel deactivation to the NMDA receptor, which is important for long-term potentiation (LTP), the cellular mechanism believed to underlie learning and memory, and has been shown to enhance memory and learning (Tang, Shimizu et al. 1999; Tovar, Sprouffske et al. 2000; Akashi, Kakizaki et al. 2009). Specifically, region specific knockouts of the GluN2B subunit show that expression of the GluN2B subunit from the frontal lobe to the CA1 region of the hippocampus is necessary for long term potentiation and depression, and learning and memory in spatial and fear conditioning tasks (Brigman, Wright et al. 2010). Moreover, transgenic mice designed to express higher levels of the GluN2B subunit from birth also possess both enhanced LTP and superior memory across adulthood to middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). Based on the above findings, the enhanced expression of GluN2B subunit within the dorsal hippocampus could account for the superior memory observed in aged mice in this study.

There was no significant effect of treatment on the performance of young mice in any memory task; and enhanced expression of the GluN2B subunit did not improve cognitive flexibility in aged mice. However, transgenic mice designed to express high levels of the GluN2B subunit during development through adulthood possess superior memory compared to wild type (Tang, Shimizu et al. 1999). In addition, both the frontal lobe and hippocampus have been shown to be important for cognitive flexibility in rodents (de Bruin JP 1994; Watson and Stanton 2009) and both the NMDA receptor and the GluN2B subunit have
been shown to be necessary for cognitive flexibility. In light of these data, the present results could suggest that the increase in GluN2B subunit expression was not sufficient to improve memory in young mice or improve cognitive flexibility in aged animals.

In conclusion, increasing the expression of the GluN2B subunit of the NMDA receptor in the dorsal hippocampus appears to restore early long-term spatial memory of aged mice back to the level of young. This study further substantiates the important role of the GluN2B subunit in long-term memory and its potential region-specific role in learning and memory. The present work also shows the importance of the decline of the GluN2B subunit to the memory loss that occurs with aging. Moreover, the evidence provided by this study suggests that a therapeutic aimed at enhancing the number or function of the GluN2B containing NMDA receptors across the aged brain could potentially be used to effectively ameliorate memory loss. However, caution should be exercised since aged mice with enhanced GluN2B subunit expression had poorer performance compared to young vehicle-treated mice later in the long-term spatial memory trials. This could suggest that the enhancement actually impaired the memory of these aged mice. Therefore, the potential for negative effects of enhanced GluN2B subunit expression in the aged brain should be investigated further.

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3.5.1 Disclosure statement

Yes, there is potential conflict of interest. R.H. has a financial interest in Viraquest, Inc., the company that provided the viral vector.
Figure 3.1
Fig. 3.1– The effects of enhanced GluN2B subunit expression in the hippocampus on the performance of aged mice in memory tasks in the Morris water maze. Graphs A-E show the effects of the GluN2B vector or 2 control (control vector or vehicle) treatments on learning performance. In graphs A-E, lower proximity scores represent better performance. (A) Performance within blocks of four place trials for the three-day long-term spatial memory task. (B) Performance averaged across place trials for individual days for the three-day long-term spatial memory task. (C) Performance within probe trials of the three-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Performance in 6 cued trials for the associative memory task. * $p \leq 0.05$ for differences from aged GluN2B vector-treated mice. # $p \leq 0.05$ for differences from young vehicle-treated mice. N=7-15. Bl = blocks of 4 place trials or 2 reversal trials, Pr = probe trials, Pr0= naïve probe trials, C= cued trials. Error bars = standard error of the mean (SEM).
Fig. 3.2- The expression of the GluN2B and control vectors *in vitro*. Representative images of rabbit skin cells treated with either GluN2B vector (A,B,C) or control vector (D,E) showing the protein expression of GFP (A,C,D) and GluN2B (B,C,E). Green= GFP (*in vivo*), Red= GluN2B subunit, Co-localized= yellow. Bar= 25 µm.
Fig. 3.3- Enhanced GluN2B subunit expression *in vivo* in the hippocampus, approximately 14 days post-treatment. Representative images of coronal sections showing GluN2B subunit, GFP (vector reporter) and GFAP (glial marker) protein expression within the hippocampus in different treatments: (A, D, G) vehicle, (B, E, H) control vector and (C, F, I, J, K) GluN2B vector in aged mice. Panels D-K represent higher magnification images of areas shown in panels A-C, respectively. Green= GFP (*in vivo*), Red= GluN2B subunit, Blue= GFAP, Yellow= lipofuscin, co-localized GFP and GluN2B = orange, co-localized GFAP and GluN2B= purple, GluN2B subunit in neuronal-like cells (arrows), GluN2B subunit in astrocytes (arrowheads). I) CA1 stratum radiatum. J) CA1 stratum pyramidale. K) dentate gyrus molecular layer, upper blade. Bar= 25 µm.
Fig. 3.4- Enhanced GluN2B subunit expression in vivo in neurons, approximately 21 days post-treatment. Representative image of a coronal section showing GluN2B subunit and neurofilament (neuronal marker) protein expression within the frontal lobe of an aged mouse treated with GluN2B vector. Panel A shows neurofilament only and Panel B shows GluN2B subunit only. Panel C shows GluN2B subunit with neurofilament. Red= GluN2B subunit, Green= neurofilament, co-localized neurofilament and GluN2B= orange, Bar= 5 µm.
Fig. 3.5- Brain regions with enhanced GluN2B subunit expression in the hippocampus. Representative diagrams of coronal sections (adapted from Franklin & Paxinos’s Mouse Brain Atlas, 2007) (Paxinos and Franklin 2007) show the location of cells intensely labeled for the GluN2B subunit across the hippocampus. CA1= Cornu Ammonis 1; CA2= Cornu Ammonis 2; CA3= Cornu Ammonis 3; cc= corpus callosum; D3V= dorsal 3rd ventricle; LV= lateral ventricle; RSA= retrosplenial agranular cortex, RSG= retrosplenial granular cortex; S= somatosensory cortex; V= visual cortex.
Fig. 3.6– Inflammation in vector-treated mice was not increased over vehicle-treated mice in the hippocampus, approximately 14 days post-treatment. Panels A-D show isolectin B₄ staining of microglia in representative coronal sections taken at the injection site in the hippocampus. (D) Inflammation was visible in a lipopolysaccharide-treated brain (positive control) and, to a lesser extent, equally across the different treatments, within the injection sites only: (A) vehicle, (B) control vector and (C) GluN2B vector. Bar=50 µm.
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CHAPTER IV

A PEPTIDE-CONJUGATED MORPHOLINO DESIGNED TO INHIBIT THE TRANSLATION OF THE CYCLIN DEPENDENT KINASE 5 (CDK5) PROTEIN ENHANCES GLUN2B (NR2B) SUBUNIT EXPRESSION AND MAY IMPROVE LONG-TERM SPATIAL MEMORY IN AGED MICE.
A PEPTIDE-CONJUGATED MORPHOLINO DESIGNED TO INHIBIT THE TRANSLATION OF THE CYCLIN DEPENDENT KINASE 5 (CDK5) PROTEIN ENHANCES GLUN2B (NR2B) SUBUNIT EXPRESSION AND MAY IMPROVE LONG-TERM SPATIAL MEMORY IN AGED MICE.

Authors & affiliations:

B. L. Brim\textsuperscript{a,b,c}, H. Moulton\textsuperscript{b}, V. Elias\textsuperscript{b,c}, K. Magnusson\textsuperscript{a,b,c}

\textsuperscript{a}Molecular and Cellular Biosciences Program, Oregon State University, Corvallis, OR, 97331, U.S.A.

\textsuperscript{b}Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR, 97331, U.S.A.

\textsuperscript{c}Healthy Aging Program, Linus Pauling Institute, Oregon State University, Corvallis, OR, 97331, U.S.A.
Abstract:

Declines in memory with increased age appear to be influenced by N-methyl-D-aspartate (NMDA) receptor expression. There are declines in both the mRNA and protein expression of the GluN2B (NR2B) subunit of the NMDA receptor throughout the frontal lobe, caudate nucleus and hippocampus during aging. Declines in GluN2B subunit expression correlate with impaired memory function. The cyclin dependent kinase 5 (Cdk5) enhances endocytosis of the GluN2B subunit-containing NMDA receptors from the synapse. Inhibiting Cdk5 increases the number of GluN2B subunits at the synapse and within the whole cell. The current study was designed to determine if using antisense phosphorodiamidate morpholino oligomers (Morpholinos) to decrease the expression of Cdk5 protein within the brain would improve memory in aged mice. Morpholinos were conjugated to a cell penetrating peptide, which enhances cellular uptake, and delivered bilaterally to the lateral ventricles of both young and aged, male C57BL/6 mice via acute stereotaxic injection. Treatments consisted of equivalent volumes and concentrations of either vehicle, control Morpholino or a Morpholino targeting the mRNA of Cdk5 (Cdk5 Morpholino). Memory was evaluated in the Morris water maze and with the use of a novel object recognition task. Aged mice treated with the Cdk5 Morpholino exhibited improved early acquisition and spatial bias in the long-term spatial memory trials, as well as improved performance overall, compared to control Morpholino-treated animals. However, aged mice treated with the Cdk5 Morpholino performed similarly to vehicle-treated aged animals. The presence of the peptide-conjugated Morpholinos within the brain may have impaired performance in the Morris water maze task since control Morpholino-treated animals performed significantly worse than vehicle-treated animals.

There was significantly greater gliosis in peptide-conjugated Morpholino-treated animals over vehicle-treated brains, suggesting it was neurotoxic. In contrast, young mice treated with the Cdk5 Morpholino showed impaired early acquisition and spatial bias but a trend for improved later learning in the long-term
spatial memory task compared to control Morpholino-treated animals. Treatment with the Cdk5 Morpholino had no significant effect on cognitive flexibility, associative memory or novel object recognition for young or aged animals. Immunohistochemistry revealed increased GluN2B subunit expression within cells with characteristics of neurons and astroglia in regions of the frontal lobe, caudate nucleus and hippocampus of aged mice who received the Cdk5 Morpholino compared to control treatments. However, the increased GluN2B subunit expression appeared to be greater within the hippocampus. These results suggest that inhibiting the translation of Cdk5 using Morpholinos increased GluN2B subunit expression in both young and aged brains and may have improved long-term spatial memory or, at least, mitigated the neurotoxic effects of the petide-conjugated Morpholinos in aged mice. An effective but non-neurotoxic dosage of peptide conjugated Cdk5 Morpholino still needs to be determined.

4.1 Introduction

One of the earliest cognitive functions to show decline with age is memory (Albert and Funkenstein 1992) and spatial memory, which is responsible for the navigation of organisms within their environment, is particularly affected by the aging process (Barnes 1988). The N-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors, are especially important to spatial memory (Morris 1989) and also appear to be especially vulnerable to the aging process (Magnusson, Brim et al. 2010).

NMDA receptors are heteromeric tetramers composed of combinations of subunits from different families of proteins; the GluN1 (NR1), GluN2 (NR2) and GluN3 (NR3) subunit families (Dingledine, Borges et al. 1999). NMDA receptors are highly expressed throughout the frontal lobe, caudate nucleus and hippocampus (Watanabe, Inoue et al. 1993; Rigby, Le Bourdelles et al. 1996), The expression of several subunits of the NMDA receptors decline with age (Magnusson, Brim et al. 2010) and these declines in expression correlate with impaired spatial memory (Magnusson, Scruggs et al. 2007; Magnusson, Brim et
al. 2010). However, the GluN2B subunit exhibits the greatest decline in expression with age (Magnusson 2000; Magnusson, Nelson et al. 2002; Bai, Hof et al. 2004; Magnusson, Kresge et al. 2006) and these declines in GluN2B subunit expression are associated with impaired spatial memory (Clayton, Mesches et al. 2002; Magnusson, Scruggs et al. 2007; Zhao, Rosenke et al. 2009).

Previous research has shown that increasing GluN2B subunit expression throughout multiple brain regions from birth is beneficial to memory and remains beneficial even into middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). Recent research has also shown that increasing GluN2B subunit expression in the frontal lobe or hippocampus, using a viral vector, rescued long-term spatial memory in age mice, restoring it back to the level of young mice but at different phases of learning (refer to Chapters 2 and 3). However, the viral vectors used provided limited distribution and duration of enhanced GluN2B subunit expression, a common limitation of viral vectors (Breakefield 1993; Tomanin and Scarpa 2004). Also, the enhanced expression of the GluN2B subunit was not specific to neurons, or the synapse.

Cyclin dependent kinase 5 (Cdk5) is predominately expressed within the brain and, unlike the GluN2B subunit, its expression levels do not significantly decline with age (Wu et al. 2000). Its expression has actually been shown to be elevated in multiple neurodegenerative diseases, including Alzheimer’s disease (Pei, Grundke-Iqbal et al. 1998; Town, Zolton et al. 2002). Cdk5 promotes the removal of GluN2B subunit-containing NMDA receptors from the synapse by phosphorylating post-synaptic density protein 95 (PSD-95), a scaffolding protein localized to post-synaptic membranes. This phosphorylation of PSD-95 inhibits Src family kinases from binding to PSD-95 and phosphorylating the 1472 tyrosine residue at the c-terminus of the GluN2B subunit, which inhibits clathrin-mediated endocytosis of GluN2B containing NMDA receptors (Zhang, Edelmann et al. 2008). Previous research in cell culture and in vivo has shown that inhibiting Cdk5 increases the number of GluN2B subunits at the synapse (Zhang, Edelmann et al. 2008). Studies in young adult transgenic mice have also shown that, in the absence of
Cdk5, there is an increase in both mRNA and protein levels of the GluN2B subunit at the synapse and in the whole cell. Inhibition of Cdk5 in both studies also resulted in increased phosphorylation of the GluN2B tyrosine 1472 (Hawasli, Benavides et al. 2007; Zhang, Edelmann et al. 2008). The transgenic Cdk5 knockout mice also exhibit superior spatial memory and enhanced NMDA-dependent LTP (Hawasli, Benavides et al. 2007). In addition, an aging intervention with dietary supplementation of blueberry extract improved NMDA-dependent LTP in aged rats and resulted in increased phosphorylation of the 1472 tyrosine residue of the GluN2B subunit, which maintains GLuN2B-containing receptors at the synapse (Coultrap, Bickford et al. 2008).

Phosphorodiamidate morpholino oligomers (Morpholinos) were chosen to target Cdk5 within the aged brain because they do not trigger an immune response like siRNA or viral vectors, do not interact strongly with proteins like the negatively-charged antisense oligonucleotides, and are not rapidly degraded in cell cultures or in animals (Amantana, Moulton et al. 2007; Youngblood, Hatlevig et al. 2007). Morpholinos are synthetic uncharged P-chiral analogs of nucleic acids that act as antisense oligomers and inhibit the interactions of macromolecules with mRNA by base pairing with the targeted mRNA in a complimentary manner (Moulton 2008). Morpholinos have been used systemically to reduce expression of inhibitory factors or enhance splicing of genes long-term (Alter, Lou et al. 2006; Summerton 2007; Moulton and Moulton 2010). Peptide-conjugated Morpholinos were chosen over unmodified Morpholinos because they are far more effective at entering cells at lower doses and have an increased half-life over unmodified Morpholinos (Moulton, Hase et al. 2003; Amantana, Moulton et al. 2007; Moulton and Moulton 2010).

The present paper explored the benefit of enhancing the expression of the GluN2B subunit within the brains of aged mice using antisense phosphorodiamidate morpholino oligomers (Morpholinos), conjugated to a peptide that enhances cellular uptake, in order to decrease the expression of Cdk5 protein.
The purpose of the present study was to determine whether the decline in spatial memory observed in aging could be improved by enhancing the expression of the GluN2B subunit at the synapse throughout the aged brain. This would help determine whether a therapy aimed at enhancing the number of GluN2B containing NMDA receptors at the synapse throughout the aged brain would be beneficial to memory.

4.2 Methods

4.2.1 Injection solutions

4.2.1.1 Morpholinos

Custom antisense phosphorodiamidate morpholino oligomers (Morpholinos) were designed by Gene Tools (Philomath, OR). Morpholinos were conjugated to a cell penetrating peptide, an arginine rich peptide, which enhances cellular uptake, as well as fluorescein for visualization in vivo. The cell penetrating peptide conjugate, P7, has the structure (RXR)$_4$XB, where R=arginine, X=6-aminohexanoic acid and B=beta-alanine. The Cdk5 Morpholino contains a sequence complimentary to the mRNA of the Cdk5 gene (CCAGTTTCTCGTATTTCTGCATTGC), which targets the ATG start codon region of the mRNA of Cdk5. The standard control Morpholino contains a sequence (CCTTTACCTCAGTTACAATTATA) that is not complimentary to any known gene in mice, but is complimentary to a splice-generating mutation at position 705 in beta-globin pre-mRNA in reticulocytes from thallasemic humans. In those cells, this Morpholino will correct a splicing error and thereby generate a correctly-spliced mRNA which codes for normal beta-globin chains. The Cdk5 Morpholino and the control Morpholino were diluted with sterile saline (Mediatech Inc., Manassas, VA) to give a final concentration of approximately 25 micromolar. Animals meant for vehicle alone were injected with equivalent volumes of sterile saline. In the Morpholino toxicity study, the control Morpholino was diluted to either 30, 10, 3, 0.15 or 0.03 micromolar concentration.
4.2.2 Animals

A total of seventy-six male C57BL/6 mice (National Institute on Aging, NIH, Bethesda, MD and The Jackson Laboratories, Bar Harbor, ME) representing 2 different age groups (3 and 24 months of age) were used for this study. The animals were fed ad libitum and housed individually in ventilated cages on a 12/12 hour light/dark cycle. The animals within each age group were randomly divided into 3 treatment groups and treated with either: vehicle, fluorescein-tagged peptide-conjugated standard control Morpholino (control Morpholino), or fluorescein-tagged peptide-conjugated Cdk5 Morpholino (CdK5 Morpholino). A separate group of young mice were randomly divided into 6 treatment groups and treated with either: vehicle or 30, 10, 3, 0.15 or 0.03 micromolar concentration of fluorescein-tagged peptide-conjugated standard control Morpholino (control Morpholino). Following behavioral testing (see below), approximately 14 days post-treatment, the mice were euthanized by exposure to CO₂, followed by decapitation. The brains were removed, frozen on dry ice, and stored at -80°C.

4.2.3 Stereotaxic surgery

All mice underwent stereotaxic surgery and received bilateral injections centered on the lateral ventricle as described by Das et al. with some modifications (Das, Jensen et al. 2012). Each animal was anesthetized with isoflurane and placed in the stereotaxic apparatus. 1-2 mm holes were drilled in the skull using the stereotaxic coordinates 0.22-0.58 mm caudal to bregma and ±1.0-1.5 mm lateral to the longitudinal suture. Injections were delivered 2.2-2.5 mm ventral to the skull at a rate of 500 nL/minute with the use of an UltraMicroPump III with SYS-Micro4 controller (World Precision Instruments, Sarasota, FL). Injections consisted of equivalent volumes (5µl per injection) and equivalent concentrations of GluN2B vector, control vector or vehicle. Post-injection, bilateral cannulae were inserted into the lateral ventricles. Mice were injected subcutaneously with buprenorphine (0.1mg/kg) (Hospira, Lake Forest, IL) following recovery from anesthesia. Twenty-four hours post-surgery, mice
were provided Mexloxicam (5 mg/kg) (Hospira, Lake Forest, IL). Three to four days post-surgery, mice received a second bilateral infusion into the lateral ventricles (5 µL per injection).

4.2.4 Memory testing

4.2.4.1 Acclimation

Four to five days post-surgery, mice were acclimated to the water maze for two consecutive days. Each session consisted of each mouse swimming for 60 seconds in the tank without the platform and then being placed on the platform in the tank and trained to remain there for 30 seconds. This platform position was different from those used for memory testing.

Spatial long-term memory, cognitive flexibility and associative memory were assessed using the Morris water maze. The water maze consisted of a 1.2 m diameter white tank filled with water made opaque by non-toxic white paint (Prang, Dixon Ticonderoga Company, Heathrow, FL) with spatial cues placed around the tank. An escape platform was hidden 1 cm below water level when present, except in cued trials, in which it was 0.5 cm above the surface. Trials were recorded and analyzed 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 tank wall.

4.2.4.2 Long-term spatial memory task

Following acclimation, mice underwent long-term spatial memory testing. The task consisted of 8 place trials (platform present but hidden) per day and 1 probe trial (platform absent) at the end of each day. There was an hour rest period after every four trials. There was also a naïve probe trial at the beginning of the first day of spatial long-term memory testing. Probe trials were performed to assess any bias for the area of the tank in which the platform would be ( naïve probe) or was localized during place trials.
(Gallagher, Burwell et al. 1993). During each probe trial the platform was removed and the mouse was allowed to search in the water for 30 seconds. 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 120 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 handler.

4.2.4.3 Cognitive flexibility task

Following the long-term spatial memory task, a reversal task was performed to assess cognitive flexibility. This task consisted of 8 place trials followed by 1 probe trial. There was an hour rest period following every four trials. The escape platform was placed in the quadrant diagonally opposite to the previous position for all reversal trials. Reversal trials consisted of 60 seconds maximum in the water searching for the platform and 30 seconds on the platform. A 30 second probe trial was performed at one hour after the last reversal trial.

4.2.4.4 Associative memory (control) task

Following the cognitive flexibility task, an associative (control) memory task was employed to test motivation, visual acuity, and physical ability for memory testing. The task consisted of 6 cued trials with the platform made visible with a 20.3 cm flag. For each trial, the platform was changed to a different position and each mouse 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.
4.2.4.5 Object recognition task

4.2.4.5.1 Habituation

Four to five days post-surgery, mice were acclimated to the object recognition arena for 5 consecutive days. Each session consisted of each mouse exploring the empty arena for 5 minutes. The arena was identical for each mouse each day and identical to the one used for the object recognition task.

Both 1 and 24 hour retention of object recognition memory was assessed using the object recognition task. The arena consisted of a 30 x 30 cm opaque open box. Different objects were placed in the arena depending on the task, but in the same location, with the exception of habituation, where the arena remained empty. Trials were recorded using the ‘SMART’ video tracking system (San Diego Instruments, San Diego, CA) and manually timed. Mice were always placed in the arena facing the arena wall. The arena was cleaned between sessions with 70% ethanol.

4.2.4.5.2 Rehabituation

Nine to 10 days post-surgery, mice were re-acclimated to the object recognition arena prior to familiarization. Each session consisted of each mouse exploring the empty arena for 10 minutes. The arena was identical for each mouse and identical to the one used for all phases of the object recognition task.

4.2.4.5.3 Familiarization

Nine to ten days post-surgery, mice were placed in the same arena now containing to two identical objects (termed familiar objects). Mice were given 10 minutes to explore these objects. The length of time spent exploring each object was manually recorded by two observers.
4.2.4.5.4 Novel object recognition

4.2.4.5.4 1 hour retention

One hour after familiarization, mice were placed in the same arena now containing one familiar object and one novel object. Mice were given 5 minutes to explore these objects. The length of time spent exploring each object was manually recorded by two observers.

4.2.4.5.5 24 hour retention

Twenty-four hours post-familiarization, mice were placed in the same arena now containing one familiar object and one new novel object. Mice were given 5 minutes to explore these objects. The length of time spent exploring each object was manually recorded by two observers.

4.2.5 Tissue sectioning

The brain of each animal was cut in half longitudinally and one half (alternating sides across animals) was sectioned horizontally using a Leica CM1850 UV Cryostat (Leica Microsystems Inc., Bannockburn, IL). The 12 μm sections were cold-mounted onto gelled slides (Thermo Fisher Scientific, Waltham, MA). Brain sections representing at least one animal from each experimental group (age by treatment) were placed on each slide and the order was determined by block design. Slides were kept at -80 degrees Celsius until further processing.
4.2.6 Immunohistochemistry

4.2.6.1 Antibodies

Representative coronal tissue sections from each animal were labeled with either a 1:100 dilution of an anti-NMDA ε2 (GluN2B) goat polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), and/or a 1:500 dilution of an anti-glial fibrillary acidic protein (GFAP) antibody (Abcam, Cambridge, MA) to visualize astrocytes or a 1:500 dilution of an anti-neurofilament antibody (Abcam, Cambridge, MA) to visualize neurons. Secondary antibodies used for visualization included rhodamine-conjugated donkey anti-goat antibody (Millipore, Billerica, MA), a fluorescein-conjugated goat anti-rabbit antibody (Millipore, Billerica, MA) or an Alexa Fluor 350 goat anti-rabbit antibody (Molecular Probes, Eugene, OR).

4.2.6.2 Staining protocol

Slides were fixed in 4% paraformaldehyde for 15 min. Slides were treated with 0.3% H$_2$O$_2$ solution for 15 min, followed by a 1 hour block 5% serum (GluN2B or GFAP). Slides were then either incubated in primary antibody or isolectin B$_4$ for 48 hours. Slides were then treated with either 1:1000 dilution of secondary antibody and/or 1:800 avidin-biotin complex solution (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 1 hour each. Slides were visualized with 3,3'-Diaminobenzidine (DAB) (Sigma Fast DAB, Sigma Aldrich, St. Louis, MO) by incubation for either 15 min (GFAP) or 30 min (GluN2B subunit). Nonspecific slides were incubated in either the absence of biotin-conjugated isolectin B$_4$ or primary antibody. Images were captured using a Leica DM LB microscope and SPOT camera (Diagnostic Instruments Incorporated, Sterling Heights, MI).
4.2.8 Injection site mapping

Representative horizontal sections from each animal were used to map the location of the injection site and the distribution of increased GluN2B subunit expression (or to verify its absence in control animals). All representative sections used for mapping were labeled for GluN2B and visualized with DAB staining.

4.2.9 Data Analysis

Data for each spatial memory task was 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 seconds by the computer for the whole duration of the trial. Cumulative proximity was calculated by summing those distances. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle, WA). 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 the long-term spatial memory and cognitive flexibility tasks and associative memory (control) task. For any probe trial, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score.

Data for each object recognition task was analyzed as described previously (Ennaceur and Delacour 1988), with a few modifications. Videos recorded by the ‘SMART’ video tracking system (San Diego Instruments, San Diego, CA) were used to measure locomotor activity (by the distance traveled) and the duration of object exploration. Exploration was defined as instances in which the animals approached and faced an object or had active physical contact with it. The object exploration ratio was calculated for each object separately and was defined as the time exploring a specific object/the total time of exploration of
both objects. The percentage of time spent exploring the familiar object was defined as the time spent exploring the familiar object on the same side as the novel object over the total time of exploration of both objects. The percentage of time spent exploring each novel object was defined as the time spent exploring the novel object over the total time of exploration of both objects. Values above fifty percent indicate that the animals spent more time exploring one object over another.

Animals were removed from the study if they performed significantly worse in cued trials from the rest of the animals (greater than the mean plus two standard deviations; 5 mice) or if there was considerable differences in locations of the injection from their counterparts (15 mice). These excluded animals were from both ages and most treatment groups.

4.2.10 Statistical Analysis

Data for memory tasks were statistically analyzed by Analysis of Variance (ANOVA) using Statview software. Performance in long-term spatial memory, cognitive flexibility, and associative memory and object recognition tasks were analyzed separately by repeated measures ANOVA and two-way ANOVA followed by Fisher’s protected least significant difference post-hoc analysis as indicated. Analysis of place trials within days was planned because of previous work showing greater age differences on day 2 (Magnusson, Scruggs et al. 2007). The response variable was performance in behavioral trials while the independent variables were age and/or treatment. For all comparisons, only $p<0.05$ were considered statistically significant.
4.3 Results

4.3.1 Effect of the Cdk5 Morpholino on spatial and associative memory

There was an overall effect of age on cumulative proximity scores in the long-term spatial memory task, with young animals spending more time closer to the platform than aged animals ($F_{(1,39)}=30.7, p=0.0001$; young=7161±790 cm, aged=10357±614 cm). There was also an overall effect of treatment on cumulative proximity scores in the long-term spatial memory task ($F_{(2,39)}=8.1, p=0.0011$), with control Morpholino-treated animals having higher scores than Cdk5 Morpholino or vehicle-treated animals. For the cognitive flexibility task, where the location of the platform was moved to the opposite quadrant from that used in the long-term spatial memory task, there was an overall significant age by treatment interaction on cumulative proximity scores ($F_{(1,39)}=3.7, p=0.04$; young vehicle= 5274±1151 cm, young control Morpholino= 8746±1541 cm, young Cdk5 Morpholino= 7508±3120 cm, aged vehicle=9522±1089 cm, aged control Morpholino=11828±933 cm, aged Cdk5 Morpholino=9743±1124 cm), with young control Morpholino-treated animals spending more time closer to the platform. Within the associative memory (control) task, all spatial cues were removed and the platform position was made visible with a flag as a cue to its position for the cued trials. For this control task, there was no overall significant effect of age ($F_{(1,39)}=1.9, p=0.2$; young= 780±383 cm, aged=587±187 cm).

4.3.1.1 Effects of the Cdk5 Morpholino on spatial and associative memory in aged animals

For aged mice, there was an overall significant effect of treatment across blocks of four place trials (Fig. 4.1A, $F_{(2,22)}=4.4, p=0.02$) and a significant interaction between treatments and blocks of 4 place trials (Fig. 4.1A, $F_{(10,110)}=2.6, p=0.007$). Aged mice treated with the Cdk5 Morpholino had significantly lower cumulative proximity scores than the aged control Morpholino group on the first day of long-term spatial memory trials (Fig. 4.1B, $p=0.004$) and overall (Fig. 4.1B, $p=0.03$). However, aged mice treated with the
Cdk5 Morpholino had similar cumulative proximity scores to vehicle-treated aged animals on day 1 of long-term spatial memory trials (Fig. 4.1B, \( p=0.3 \)) and overall (Fig. 4.1B, \( p=0.8 \)). The presence of the peptide-conjugated Morpholinos within the brain may have worsened performance in the Morris water maze task since control Morpholino-treated animals had significantly higher cumulative proximity scores than vehicle-treated animals on day 1 (Fig. 4.1B, \( p=0.05 \)) and overall (Figure 4.1B, \( p=0.02 \)).

Within probe trials, aged mice treated with the Cdk5 Morpholino exhibited lower average proximity to the platform location compared to the aged control Morpholino group by the end of the first day (Fig. 4.1C, \( p=0.02 \)) and second day (Fig. 4.1C, \( p=0.04 \)). However, aged mice treated with the Cdk5 Morpholino had similar average proximity to the platform location compared to the aged vehicle group by the end of the first day (Fig. 4.1C, \( p=0.7 \)) and second day (Fig. 4.1C, \( p=0.4 \)). The presence of the peptide-conjugated Morpholinos within the brain may have worsened performance in the Morris water maze task since control Morpholino-treated animals had significantly higher average proximity scores than vehicle-treated animals by the end of day 1 (Fig. 4.11C, \( p=0.05 \)) and overall (Figure 4.1C, \( p=0.003 \)). There was no significant effect of treatment on cumulative proximity in reversal trials in the aged animals (Fig. 4.1D, \( F_{(2,22)}=0.8, p=0.5 \)). Within the associative memory (control) task, there was a near significant effect of treatment within aged animals (Fig. 4.1F, \( E_{(2,22)}=3.0, p=0.07 \)), with Cdk5 Morpholino-treated aged mice showing a trend for performing better than either aged control.

### 4.3.1.2 Effects of the Cdk5 Morpholino on spatial and associative memory in young animals

For young mice, there was an overall significant effect of treatment across blocks of four place trials (Fig. 4.2A, \( F_{(2,17)}=4.7, p=0.02 \)). Young mice treated with the Cdk5 Morpholino had significantly higher cumulative proximity scores than the young vehicle group on the first day of long-term spatial memory trials (Fig. 4.21B, \( p=0.02 \)) and overall (Fig. 4.21B, \( p=0.04 \)). However, young mice treated with the Cdk5 Morpholino had similar cumulative proximity scores to control Morpholino treated aged animals by day 1.
of long-term spatial memory trials (Fig. 4.2B, \( p=0.4 \)) and overall (Fig. 4.2B, \( p=0.1 \)). However, the presence of the peptide-conjugated Morpholinos within the brain may have worsened performance in the Morris water maze task since young mice treated with the control Morpholino had significantly higher cumulative proximity scores than the vehicle-treated animals by the second (Fig. 4.2B, \( p=0.02 \)) and third day (Fig. 4.2B, \( p=0.01 \)) of long-term spatial memory trials and overall (Fig. 4.2B, \( p=0.008 \)).

Within probe trials, young mice treated with the Cdk5 Morpholino exhibited higher average proximity to the platform location compared to the vehicle-treated animals by the end of the first day (Fig. 4.2C, \( p=0.009 \)). However, young mice treated with the Cdk5 Morpholino had similar average proximity to the platform location compared to the young control Morpholino group by the end of the first day (Fig. 4.2C, \( p=0.3 \)). There was a near significant effect of treatment on cumulative proximity in reversal trials in the young animals (Fig. 4.2D, \( F_{(2,17)}=3.0, p=0.08 \)), with control Morpholino-treated animals performing better than either control group. Within the associative memory (control) task, there was no significant effect of treatment within young animals (Fig. 4.2E, \( F_{(2,17)}=0.8, p=0.5 \)).

### 4.3.2 Effect of the Cdk5 Morpholino on object recognition memory

There was no significant overall effect of age (Fig. 4.3A-F, \( F_{(1,34)}=0.2, p=0.7 \); young=58\(\pm\)3\%, aged=60\(\pm\)3\%) or overall effect of treatment on percentage of time spent exploring objects in the object recognition task (Fig. 4.3A-F, \( F_{(2,34)}=0.6, p=0.5 \)). For the familiarization task, where two identical objects were presented, there was no overall significant effect of age (Fig. 4.3A, D, \( F_{(1,34)}=1.0, p=0.3 \); young=50\(\pm\)2\%, aged=48\(\pm\)3\%) or treatment (Fig. 4.3A, D, \( F_{(2,34)}=1.0, p=0.4 \)) on percentage of time spent exploring the familiar object on the same side as the novel object. For the 1hr retention task, where one familiar and one novel object were presented, there was no overall significant effect of age (Fig. 4.3B, E, \( F_{(1,34)}=0.06, p=0.8 \); young=59.7\(\pm\)4.0\%, aged=62\(\pm\)3\%) or treatment (Fig. 4.3B, E, \( F_{(2,34)}=0.7, p=0.5 \)) on percentage of time spent exploring the novel object. For the twenty-four hr retention task, where one
familiar object and one new novel object were presented, there was no overall significant effect of age (Fig. 4.3C, F, $F_{(1,34)}=1.5$, $p=0.2$; young = 64±3%, aged = 70±3%) or treatment (Fig. 4.3C, F, $F_{(2,34)}=0.8$, $p=0.5$) on percentage of time spent exploring the novel object.

4.3.3 Concentration of peptide-conjugated Morpholino that does not impair memory

There was no significant overall effect of treatment (Fig. 4.4A, $F_{(5,15)}=1.2$, $p=0.3$) or significant interaction between treatment and blocks of four place trials (Fig. 4.4A, $F_{(5,25)}=1.1$, $p=0.4$). Young mice treated with doses 0.03-30 micromolar of control Morpholino had cumulative proximity scores similar to vehicle-treated animals unlike the original study. However, there was a trend in long-term spatial memory trials for higher cumulative proximity scores in young mice treated with the 0.03 and 3-30 micromolar dose of control Morpholino compared to vehicle-treated animals (Fig. 4.4A-B), especially during the first day of trials.

Across probe trials, there was a near-significant overall effect of treatment with young mice treated with the 0.03 micromolar control Morpholino exhibiting higher average proximity to the platform location compared to all other treatments (Figure 4.4C, $F_{(5,14)}=2.8$, $p=0.06$). There was a no overall significant effect of treatment on cumulative proximity in reversal trials in these animals (Fig. 1D, $F_{(5,15)}=0.4$, $p=0.9$). Within the associative memory (control) task, there was a near significant effect of treatment within these young animals (Fig. 4.4E, $F_{(5,15)}=2.8$, $p=0.54$), with 30 micromolar control Morpholino animals having higher cumulative proximity scores compared to other treatments.

4.3.4 Effect of the Cdk5 Morpholino on GluN2B protein expression

In vivo, there was increased GluN2B immunoreactivity within the brains of mice treated with the Cdk5 Morpholino as compared to endogenous expression, especially in the hippocampus, which is shown here (Fig. 4.5A-K, 4.6). Neither control treatments, vehicle or control Morpholino, (Fig.4.5A-B, D-E, G-H)
elicited a change in GluN2B immunoreactivity from endogenous expression. Increased GluN2B subunit expression was visible in neuronal cells and processes (Fig. 4.5C, F, I-K, 4.6). Injection site mapping of strongly labeled cells indicated that increased GluN2B subunit expression was predominately localized to the deep cortical layers, caudate nucleus, corpus callosum, and regions of the hippocampus, including the dentate gyrus and CA1, CA2 and CA3 regions (Fig. 4.6).

4.3.5 Peptide-conjugated Morpholinos caused CNS injury greater than vehicle

Since the control Morpholino-treated mice performed worse than vehicle-treated mice in the original study, representative horizontal sections from young mice from each treatment from the original study, as well as from the toxicity study, were stained with an antibody against astrocytes. These mice had survived for approximately 14 days post-treatment. Gliosis, which is a proliferation of astrocytes in damaged areas of the central nervous system (CNS), is a histopathological sign of neurotoxicity and is evident in tissue from the frontal lobe of a young mouse (positive control) that received bilateral injections of lipopolysaccharide from *Escherichia coli* into the frontal lobe (O'Callaghan 1991) (Fig. 4.7Q). There was significantly greater gliosis in brains of animals receiving 3-30 micromolar doses of the peptide conjugated control or Cdk5 Morpholino treatment (Fig. 4.7D-H, L-P, U-Y) compared to vehicle (Fig. 4.7A, I, R). However, brains from animals receiving 0.03-0.15 micromolar doses of the peptide-conjugated control Morpholino (Figure 4.7B-C, J-K, S-T) exhibited no evidence of gliosis greater than vehicle (Fig. 4.7A, I, R). Gliosis in aged animals was comparable to young (data not shown).

4.4 Discussion

The present study provides suggestive evidence that using a cell penetrating peptide-conjugated Morpholino designed to target Cdk5 may increase the expression of the GluN2B subunit of the NMDA receptor within the deep layers of the frontal cortex, caudate nucleus and hippocampus of aged mice and
this enhanced expression may be beneficial to memory. Aged mice treated with the Cdk5 Morpholino exhibited enhanced GluN2B subunit expression across these brains regions and superior long-term spatial memory over aged mice receiving the control Morpholino. However, the peptide-conjugated Morpholinos caused significant gliosis over vehicle-treatment and appeared to have impaired memory. Several lower concentrations of peptide conjugated Morpholino were tested and one was found that caused gliosis similar to vehicle and exhibited similar memory to vehicle across memory tasks. Together, these results suggest that increasing expression of the GluN2B subunit within the aged brain may improve memory in aged mice and Morpholinos could be an effective tool to accomplish this goal, provided a nontoxic but effective dose can be achieved.

Aged mice treated with the Cdk5 Morpholino showed improved learning on the first day of long-term spatial memory training and overall, as compared to control Morpholino-treated aged animals. By the end of the first and second day of the long-term spatial memory task, aged mice treated with the Cdk5 Morpholino showed a stronger bias, as measured by probe trials, for the platform location compared to the aged mice treated with the control Morpholino. However, aged mice treated with the Cdk5 Morpholino performed similarly to vehicle-treated aged animals. The presence of the peptide-conjugated Morpholinos within the brain appears to have worsened performance in the Morris water maze task, since control Morpholino-treated animals performed significantly worse than vehicle-treated animals. Immunohistochemistry for GFAP, a marker for neurotoxic damage, revealed increased gliosis in brains treated with either Morpholino treatment over vehicle-treated brains.

In contrast, young mice treated with the Cdk5 Morpholino performed significantly worse than the vehicle-treated young animals on the first day of long-term memory trials and overall. However, young mice treated with the Cdk5 Morpholino performed similarly to control Morpholino treated young animals, suggesting that the presence of the peptide-conjugated Morpholinos within the brain may have
worsened performance in the Morris water maze task. Immunohistochemistry for GFAP, a marker for neurotoxic damage, again revealed increased gliosis in brains injected with either Morpholino treatment over vehicle-treated brains. Moreover, young mice treated with the control Morpholino performed significantly worse than the vehicle-treated animals by the second and third day of long-term memory trials. By the end of the first day, Cdk5 Morpholino-treated young animals exhibited a weaker bias, as measured by probe trials, for the platform location compared to the vehicle-treated animals. However, young mice treated with the Cdk5 Morpholino had a similar bias for the platform location compared to the young control Morpholino group by the end of the first day. Young mice treated with the Cdk5 Morpholino showed impaired early acquisition and spatial bias but a trend for improved later learning in the long-term spatial memory task compared to control Morpholino-treated animals.

Immunohistochemistry revealed increased GluN2B subunit expression within cells with characteristics of neurons and astroglia in regions of the frontal lobe, caudate nucleus and hippocampus of aged mice who received the Cdk5 Morpholino compared to control treatments. However, the increased GluN2B subunit expression appeared to be greater within the hippocampus. These data suggest that the Cdk5 Morpholino effectively enhanced GluN2B subunit expression and in a more global fashion than previous studies. However, immunohistochemistry also revealed evidence of increased gliosis within both control and Cdk5 Morpholino brains compared to vehicle across ages, suggesting that the peptide-conjugated Morpholino, in the dose used, was neurotoxic. This could account for the poorer performance of mice treated with the control Morpholino compared to vehicle and similar performance of mice treated with the Cdk5 Morpholino to vehicle-treated animals. However, it should be taken into consideration that improved long-term spatial memory experienced by mice treated with the Cdk5 Morpholino, over those treated with the control-Morpholino, was not necessarily due to the observed enhancement in GluN2B subunit expression in the brains of these mice. It is possible that the Cdk5 Morpholino simply somehow counteracted the toxic effects caused by the control Morpholino. However, since both the control and
Cdk5 Morpholino-treated mice both showed similar levels of gliosis, it seems unlikely that anti-toxic effects of the Cdk5 Morpholino was the cause of the memory improvement seen Cdk5 Morpholino-treated animals.

However, it should also be taken into consideration that Cdk5 is not simply involved in promoting clathrin-mediated endocytosis of GluN2B-containing NMDA receptors (Zhang, Edelmann et al. 2008). Therefore, it is possible that inhibiting Cdk5 expression could have off-target effects. As mentioned previously, Cdk5 has been implicated in the hyperphosphorylation of tau proteins in Alzheimer’s disease, a hallmark of the progression of its pathology (Pei, Grundke-Iqbal et al. 1998; Town, Zolton et al. 2002).

Outside of the central nervous system, Cdk5 has been implicated in the progression of many forms of cancer, including pancreatic, prostate and breast cancer (Strock, Park et al. 2006; Goodyear and Sharma 2007; Eggers, Grandgenett et al. 2011). In fact, a current treatment for these cancers is a Cdk5 inhibitor, roscovitine (Iseki, Ko et al. 1998; Edamatsu, Gau et al. 2000; Mohapatra, Chu et al. 2005; Goodyear and Sharma 2007). In each of these cases, antagonizing the function of Cdk5 could be presumed to be generally positive, given that Cdk5 is promoting disease progression. However, Cdk5 is also implicated in normal mitochondrial function. Specifically, Cdk5 function has been shown to phosphorylate HtrA2, a mitochondrial serine protease, that allows for the maintenance of mitochondrial membrane potential under stressful conditions (Fitzgerald, Camprubi et al. 2012). Disruption of Cdk5 function in neurons has been shown to result in apoptosis through the mitochondrial death pathway (Meuer, Suppanz et al. 2007).

In order to determine a non-toxic dose of peptide-conjugated Morpholino for future studies, a separate group of young mice received bilateral acute injections of either vehicle or a range of lower doses of control Morpholino (0.03-30 micromolar). Immunohistochemistry against glial fibrillary acidic protein (GFAP), an astrocytic marker, revealed that there was enhanced gliosis in the highest dose (30 micromolar, similar to the original study) over vehicle-treated mice. Gliosis, which is a proliferation of
astrocytes in damaged areas of the central nervous system (CNS), is a histopathological sign of neurotoxicity (O Callahagn 1991). Mice that received the lowest doses of control Morpholino (0.15 and 0.03 micromolar) showed no evidence of gliosis greater than vehicle. However, animals treated with 0.03 micromolar dose showed trends for impaired memory over vehicle-treated animals across memory tasks. In contrast, animals treated with the 0.15 micromolar dose showed trends for memory more similar to vehicle-treated animals across memory tasks. Future studies will determine if the 0.15 micromolar dose is effective at enhancing the GluN2B subunit using the Cdk5 Morpholino.

The GluN2B subunit of the NMDA receptor imparts slower channel deactivation to the NMDA receptor, which is important for LTP, the cellular mechanism believed to underlie learning and memory, and has been shown to enhance memory and learning (Tang, Shimizu et al. 1999; Tovar, Sprouffske et al. 2000; Akashi, Kakizaki et al. 2009). Specifically, phosphorylation of tyrosine 1472 of the GluN2B subunit has been shown to be necessary for LTP maintenance (Nakazawa, Komai et al. 2001). The phosphorylation of the tyrosine 1472 of the GluN2B subunit maintains GluN2B subunit-containing receptors at the synapse by inhibiting clathrin-mediated endocytosis (Zhang, Edelmann et al. 2008). Cdk5 promotes clathrin-mediated endocytosis of GluN2B containing receptors (Zhang, Edelmann et al. 2008). Transgenic studies in adult mice have shown that removing Cdk5 results in increased levels of GluN2B mRNA and protein expression and increased levels of GluN2B phosphorylated at tyrosine 1472, as well as enhanced memory (Hawasli, Benavides et al. 2007). These data suggest that enhanced GluN2B subunit expression and its associated memory improvement could have been due to blocking the translation of Cdk5 within the brain.

Pharmacological and genetic blockade of the GluN2B subunit within the prefrontal cortex results in diminished LTP and impaired fear conditioning (Zhao, Toyoda et al. 2005). Region specific knockouts of the GluN2B subunit show that expression of the GluN2B subunit within the frontal lobe and CA1 region
of the hippocampus is necessary to synaptic plasticity and learning and memory (Brigman, Wright et al. 2010). Moreover, transgenic mice designed to express higher levels of the GluN2B subunit from birth also possess enhanced LTP and superior memory, both spatial and object recognition memory, across adulthood to middle-age (Tang, Shimizu et al. 1999; Cao, Cui et al. 2007). Based on the above findings, the enhanced expression of GluN2B subunit seen in Cdk5 Morpholino-treated animals could account for the superior memory observed.

Memory tasks in the Morris water maze involve the acquisition, consolidation and/or retrieval of a memory (Morris and Davis 1994). NMDA receptors have been shown to be involved in both the acquisition and consolidation of spatial memory in the Morris water maze by blockade with the competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) (Liang, Hon et al. 1994). Specifically, NMDA receptors within the CA1 region of the hippocampus have been shown to be necessary to the acquisition of spatial memories (Tsien, Huerta et al. 1996). GluN2B-containing NMDA receptors in the frontal cortex have been shown to be necessary to memory and learning as well as LTP (Cui, Jin et al. 2011). Moreover, transgenic mice designed to express high levels of the GluN2B subunit throughout the brain, including the frontal lobe, exhibit improved learning on the second day of learning trials (Tang, Shimizu et al. 1999). Therefore, it seems feasible that an increase in the expression of the GluN2B subunit across regions of the frontal lobe, caudate nucleus and hippocampus might be responsible for improved acquisition and/or consolidation of long-term memories observed in young and aged mice.

Enhanced expression of the GluN2B subunit within the frontal lobe and caudate nucleus or hippocampus did not improve cognitive flexibility or novel object recognition memory. There was no overall significant difference in the performance of aged or young mice with increased expression of the GluN2B subunit compared to controls in reversal trials, in which the escape platform was moved to the opposite
quadrant, or object recognition. However, the frontal lobe and the hippocampus have both been shown to be important for cognitive flexibility in rodents (de Bruin JP 1994; Watson and Stanton 2009) and both the NMDA receptor and the GluN2B subunit have been shown to be necessary for cognitive flexibility (Nicolle and Baxter 2003; Duffy, Labrie et al. 2008). Moreover, novel object recognition has been shown to be improved in transgenic mice with enhanced GluN2B subunit expression across the brain (Tang, Shimizu et al. 1999). This could suggest that the increase in GluN2B subunit expression was not sufficient to improve cognitive flexibility or novel object recognition in animals.

In conclusion, the Cdk5 Morpholino increased the expression of the GluN2B subunit of the NMDA receptor in the frontal lobe, caudate nucleus and, especially, the hippocampus, and this enhancement appears to improve long-term spatial memory in aged mice. The evidence provided by this study suggests that cell penetrating peptide-conjugated Morpholinos could be effectively used to manipulate gene expression more globally in the brain and that inhibiting translation of Cdk5 could enhance GluN2B subunit expression and memory in aged mice. Moreover, a therapeutic aimed at enhancing the number or function of the GluN2B containing NMDA receptors in old age could potentially be used to effectively ameliorate memory loss. However, future studies are still needed to determine an efficacious but nontoxic dose of the Cdk5 Morpholino.

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4.5.1 Disclosure statement

There is no potential conflict of interest.
Figure 4.1
Fig. 4.1 – The effects of the Cdk5 Morpholino on the performance of aged mice in memory tasks in the Morris water maze. Graphs A-E show the effects of the Cdk5 Morpholino or 2 control (control Morpholino or vehicle) treatments on learning performance. Lower proximity scores represent better performance. (A) Performance across blocks of four place trials for the three-day long-term spatial memory task. (B) Performance averaged across place trials for each individual day of the three-day long-term spatial memory task. (C) Performance within probe trials of the three-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Performance in 6 cued trials for the associative memory task. * $p<0.05$ for differences from aged vehicle-treated mice. ^ $p<0.05$ for differences from aged Cdk5 Morpholino-treated mice. N=8-10. Bl = block of 4 place trials, Pr = probe trials, Pr0= naïve probe trials, R= reversal trials, C= cued trials. Error bars = standard error of the mean (SEM).
Figure 4.2
Fig. 4.2 – The effects of the Cdk5 Morpholino on the performance of young mice in memory tasks in the Morris water maze. Graphs A-E show the effects of the Cdk5 Morpholino or 2 control (control Morpholiono or vehicle) treatments on learning performance. Lower proximity scores represent better performance. (A) Performance within blocks of four place trials for the three-day long-term spatial memory task. (B) Performance averaged across place trials for individual days for the three-day long-term spatial memory task. (C) Performance within probe trials of the three-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Performance in 6 cued trials for the associative memory task. * p<0.05 for differences from young vehicle-treated mice. ^ p<0.05 for differences from young Cdk5 Morpholino-treated mice. N=5-8. Bl = block of 4 place trials, Pr = probe trials, Pr0= naïve probe trials, R= reversal trials, C= cued trials. Error bars = standard error of the mean (SEM).
Figure 4.3

A. Percentage exploration (%)

B. Percentage exploration (%)

C. Percentage exploration (%)

D. Percentage exploration (%)

E. Percentage exploration (%)

F. Percentage exploration (%)

Legend:
- young vehicle
- young control Morpholino
- young Cdk5 Morpholino

Legend:
- aged vehicle
- aged control Morpholino
- aged Cdk5 Morpholino

Familiarization

Object recognition

1 hr retention

24 hr retention
Fig. 4.3-The effects of the Cdk5 Morpholino on the performance of young and aged mice in the novel object recognition task. Graphs A-F show the effects of the Cdk5 Morpholino or 2 control (control Morpholiono or vehicle) treatments on learning performance in the object recognition task. Higher percentages of time in the retention tasks represent better performance, but in the familiarization task may indicate a bias. (A, B) Performance within the familiarization task. (C, D) Performance within the 1-hour retention task. (E, F) Performance within the 24-hour retention task. N=5-10. Error bars = standard error of the mean (SEM).
Figure 4.4
Figure 4.4- The effects of different doses of the control Morpholino on the performance of young mice in memory tasks in the Morris water maze. Graphs A-E show the effects of different doses of the control Morpholino or vehicle on learning performance. Lower proximity scores represent better performance. (A) Performance within blocks of four place trials for the three-day long-term spatial memory task. (B) Performance averaged across place trials for individual days for the three-day long-term spatial memory task. (C) Performance within probe trials of the three-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Performance in 6 cued trials for the associative memory task. N=3-6. Bl = block of 4 place trials, Pr = probe trials, Pr0= naïve probe trials, R= reversal trials, C= cued trials. Error bars = standard error of the mean (SEM).
Fig. 4.5 Enhanced GluN2B subunit expression with the Cdk5 Morpholino in the aged brain, approximately 14 days post-treatment. Representative images of horizontal sections showing GluN2B subunit and neurofilament (neuronal marker) protein expression within the aged hippocampus in different treatments: (A, D, G) vehicle, (B, E, H) control Morpholino and (C, F, I, J, K) Cdk5 Morpholino aged mice. Panels D-K represent higher magnification images of areas shown in panels A-C, respectively. I) dentate gyrus molecular layer, upper blade J) CA1 stratum pyramidale . J) CA3 stratum radiatum. Blue= neurofilament, Red= GluN2B subunit, co-localized neurofilament and GluN2B= pink, GluN2B subunit in neurons (arrows). Bar= 25 µm.
Fig. 4.6- Brain regions with enhanced GluN2B subunit expression in the deep cortex, caudate nucleus, corpus callosum and hippocampus. Representative diagram of a horizontal section (adapted from Franklin & Paxinos’s Mouse Brain Atlas, 2007)(Paxinos and Franklin 2007) shows the location of cells intensely labeled for the GluN2B subunit across these regions. CA1= Cornu Ammonis 1; CA2= Cornu Ammonis 2; CA3= Cornu Ammonis 3; DG= dentage gyrus, cc= corpus callosum; LV= lateral ventricle; DC=deep cortical layers V-VI in frontal lobe.
Fig. 4.7—Peptide-conjugated Morpholinos caused CNS injury greater than vehicle, as shown approximately 14 days post-treatment. Gliosis, an important histopathological sign of CNS injury, was increased over vehicle-treated mice in mice treated with 3–30 micromolar doses of control or Cdk5 Morpholino across ages in the frontal lobe, caudate nucleus and hippocampus. Panels A-Y show glial fibrillary acidic protein (GFAP) staining of astrocytes in representative horizontal sections of brains from young animals. Panels A-H represent the frontal cortex. Panels I-P represent the caudate nucleus. Panels R-Y represent the hippocampus. (Q) Gliosis was visible in a lipopolysaccharide-treated brain (positive control) and, to a lesser extent, within the 3-30 micromolar control or Cdk5 Morpholino-treated animals over vehicle-treated animals (D-H, L-P, U-Y). There was not visible gliosis in vehicle-treated animals (A, I, R) or .03-.15 micromolar doses control Morpholino-treated animals (B-C, J-K, S-T). Gliosis was similar in aged animals (data not shown). Bar=25 µm.
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CHAPTER V
CONCLUSION
Aging leads to multiple functional declines, often resulting in a loss of independence both financially and in terms of quality of life. Declines are observed in memory, cognitive flexibility, strength, sensation, balance and motor coordination (Timiras 2003). Of these, memory deterioration arises first, usually evident by the fifth decade in humans (Albert and Funkenstein 1992). Memory decline poses a serious risk to the quality of life to elderly individuals and can predispose them to entering care facilities early, creating a huge economic burden (Glisky 2007; Okura and Langa 2011). Given the importance of age-related memory decline, it is crucial to understand its molecular basis and determine possible interventions. This current research attempted to further understand the molecular mechanisms of memory decline during normal aging and the potential for possible interventions.

The present work attempted to increase the expression of the GluN2B subunit within the aged brain, regionally, and then globally, to determine if enhancement of the GluN2B subunit could potentially alleviate age-related declines in memory. The first two studies were designed to determine if increasing GluN2B subunit expression in the frontal lobe or hippocampus would improve memory in aged mice, since the expression of the GluN2B subunit appears to be differentially affected by aging in these regions. Both studies showed that enhancing GluN2B subunit expression in old age can ameliorate some of the age-related memory declines by restoring certain components of memory back to the level of young animals. Specifically, enhancement of the GluN2B subunit within the frontal lobe enhanced later learning (later acquisition and/or consolidation) in the long-term spatial memory task of the Morris water maze; however, enhancement of this subunit in the dorsal hippocampus improved early learning in the same task. These findings could be due to the fact that both regions can play differential but complimentary roles in memory formation. The frontal lobe, including medial prefrontal cortex, has been shown to contribute to long-term memory by maintaining the learned information which requires consolidation (Granon, Vidal et al. 1994; Kessels, Postma et al. 2000). However, the hippocampus has been shown to play a role in the consolidation of less stable short-term memories into more stable long-term memories,
which requires acquisition (Takehara, Kawahara et al. 2003; Frankland, Bontempi et al. 2004; Maviel, Durkin et al. 2004). Both studies suggest that it could be beneficial to memory to enhance GluN2B subunit expression throughout the aged brain, particularly in regions key to memory and learning. However, there are three caveats. First, enhancement of the GluN2B subunit within the dorsal hippocampus impaired later long-term spatial memory in aged mice compared to young. This result could suggest that the over expression of the GluN2B subunit could be having a negative effect on memory in these aged animals, similar to the effect seen in that subpopulation of normal aged mice that expressed higher levels of GluN2B subunit in the hippocampus. Secondly, over-expression of the GluN2B subunit could result in excitotoxicity (refer to discussion on excitotoxicity below). However, no detrimental effects were seen in young mice that received the same treatment as the aged, suggesting excitotoxicity was not an issue in these studies. Moreover, given the improvement on day 2 of the spatial long-term memory task in aged mice with enhanced expression within the frontal lobe, a more global enhancement could counteract the poorer performance observed in aged mice with enhanced GluN2B subunit within the hippocampus. Yet, a more global expression of the GluN2B subunit within the aged brain could be detrimental if over-expression results in excitotoxicity. This is a real possibility given that the enhanced expression was not strictly synaptic and extrasynaptic NMDA receptors have been implicated in glutamate excitotoxicity (Hardingham, Fukunaga et al. 2002). However, it is possible that more global enhancement of the GluN2B subunit, strictly at the synapse, could be more beneficial to memory, and reduce the risk for excitotoxicity.

The third study explored the benefits of a more global and synapse specific increase in GluN2B subunit expression within the aged brain. An antisense phosphorodiamidate morpholino oligomer (Morpholinos) conjugated to a cell-penetrating peptide was used to decrease the expression of Cdk5 protein, which enhances endocytosis of the GluN2B subunit-containing NMDA receptors from the synapse (Zhang, Edelmann et al. 2008). The study was flawed in that the control Morpholino-treated mice performed
worse than vehicle-treated mice. This was the result of neurotoxicity, suggesting that a more extensive toxicity study should have been performed first. However, though memory was improved in aged mice treated with the Cdk5 Morpholino, it is not certain whether this was due to the enhancement of GluN2B subunit expression observed in the Cdk5 mice, or if the Cdk5 Morpholino had other effects that lessened the toxicity of the peptide-conjugated Morpholino in the brain. Since gliosis was as great in Cdk5 Morpholino-treated brains, as in control Morpholino-treated brains, it is likely that the observed improvement in memory was the effect of the enhanced GluN2B subunit expression. In this context, the results of the third study suggest that the Cdk5 Morpholino increased GluN2B subunit expression in the aged brain and this enhancement may have improved long-term spatial memory in aged mice. Moreover, it suggests that a global enhancement of the GluN2B subunit within the aged brain could be beneficial to memory. However, future studies are needed to determine the efficacy of the Cdk5 Morpholino at a lower dosage and to show enhancement of GluN2B-containing NMDA receptors at the synapse to solidify this hypothesis.

As mentioned previously (refer to Chapter 1, section 1.9.3), many different diet and drug interventions have been used previously to attenuate the effects of aging. These aging interventions have also indirectly affected the expression levels or phosphorylation state of the GluN2B subunit. These interventions include the long-term intermittent fasting diet (L-IFD), a variant of caloric restriction, supplementation with omega-3 polyunsaturated fatty acids (n-3 PUFA) or blueberry extract, and drug-based treatments with nicotine, Sulindac, and insulin-like growth factor 1 (IGF-1). All of these interventions enhanced the expression of the GluN2B subunit within the aged brain, with the exception of supplementation with blueberry extract, which enhanced phosphorylation of the GluN2B subunit at tyrosine residue 1472 (Mesches, Gemma et al. 2004; Delibas, Doguc et al. 2005; Sonntag, Ramsey et al. 2005; Dyall, Michael et al. 2007; Fontan-Lozano, Saez-Casanelli et al. 2007; Coultrap, Bickford et al. 2008). Several of these interventions improved NMDA-dependent LTP, a cellular mechanism believed to underlie learning and
memory, including the L-IF diet and blueberry extract supplementation (Fontan-Lozano, Saez-Cassanelli et al. 2007; Coultrap, Bickford et al. 2008). Likewise, a few of these interventions resulted in improved memory in aged animals including the L-IF diet as well as treatment with nicotine or Sulindac (Mesches, Gemma et al. 2004; Delibas, Doguc et al. 2005; Fontan-Lozano, Saez-Cassanelli et al. 2007). However, only the L-IF diet intervention directly correlated enhanced expression of the GluN2B subunit to enhanced memory and LTP in aged animals (Fontan-Lozano, Saez-Cassanelli et al. 2007).

Though these aging intervention studies do suggest that increased GluN2B subunit expression within the aged brain can be beneficial to memory and LTP, it is not clear whether these interventions up-regulated the GluN2B subunit or simply prevented age-related declines. However, our studies do show that up-regulating the expression of the GluN2B subunit within aged mice is beneficial. More importantly, none of these aging interventions are specific for the GluN2B subunit or have consistent effects on the GluN2B subunit. However, our studies directly target the GluN2B subunit. Also, while one aging intervention did show the memory improvements seen are due to enhancement of the GluN2B subunit, many do not. That leaves uncertainty as to whether the effects of these aging interventions on memory are due to the enhancement of the GluN2B subunit or a combination of other factors that were not assessed. Some studies do not even address whether their aging intervention improved memory, or how its effects compared to young animals. In contrast, our studies directly addressed whether or not enhanced expression of the GluN2B subunit within the aged brain improves memory. They also demonstrate how memory is affected compared to young animals, which is important for an aging intervention. Furthermore, our studies also attempted to address whether the enhanced GluN2B subunit expression is functionally responsible for the improved memory by using a GluN2B specific antagonist. However, as mentioned previously, our studies do have limitations. Two key limitations of our studies include not currently knowing the degree to which GluN2B subunit expression needs to be enhanced in the aged brain or whether a more global, synapse specific enhancement would be beneficial to memory. Moreover, there
is currently no viable or noninvasive way to enhance GluN2B subunit expression within the aging human brain. However, our studies do suggest that enhancing expression in the aged brain could help alleviate age-associated memory decline.

Still, restoring aging NMDA receptors back to a young NMDA receptor, by enhancing GluN2B subunit expression, could have potential disadvantages that need to be seriously considered. The primary concern with restoring or preventing NMDA receptor changes during aging is whether this could make individuals more prone to glutamate excitotoxicity. Glutamate excitotoxicity is a pathological process by which cells within the CNS are damaged and/or undergo apoptosis by excessive stimulation (Olney 1969; Olney and Sharpe 1969). Glutamate excitotoxicity can occur when glutamate receptors, like NMDA receptors, are over-stimulated (Choi, Koh et al. 1988; Choi 1992).

Pathologically high levels of glutamate can cause excitotoxicity by allowing high levels of calcium ions (Ca$^{2+}$) to enter the cell (Choi 1985; Choi 1987). It is possible that an overabundance of GluN2B-containing NMDA receptors could have a similar effect because they have slower deactivation kinetics of the channel, and so allow greater Ca$^{2+}$ influx into cells (Grant, Bacskaia et al. 1998; Tovar, Sprouffske et al. 2000). Excess calcium in the cytosol can lead to cell damage and apoptosis. Specifically, excess cytosolic calcium could activate a caspase cascade, or trigger mitochondrial release of reactive oxygen species (ROS), both leading to apoptosis (Leist, Volbracht et al. 1997; Urushitani, Nakamizo et al. 2001).

NMDA receptor-mediated excitotoxicity has been implicated in playing a role in ischemia and in neurodegenerative diseases of the central nervous system (CNS), including Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease and Huntington's disease (Shaw and Ince 1997; Sonsalla, Albers et al. 1998; Urushitani, Nakamizo et al. 2001; Heng, Detloff et al. 2009; Okamoto, Pouladi et al. 2009; Li, Jin et al. 2011). However, NMDA receptor hyperfunction has primarily been considered a contributing factor to the pathogenesis of Alzheimer’s disease. Memantine, one of the
current treatments for Alzheimer’s disease, is actually an NMDA receptor antagonist (Fleischhacker, Buchgeher et al. 1986; Bormann 1989). In mouse models of Alzheimer’s like disease, memantine has been shown to reduce amyloid plaque burden and even enhance object recognition memory (Dong, Yuede et al. 2008; Scholtzova, Wadghiri et al. 2008). It has also been shown to reduce neurodegeneration by inhibiting beta amyloid-induced excitotoxicity (Miguel-Hidalgo, Alvarez et al. 2002). These data suggest that blocking NMDA receptor function during aging would be more beneficial than restoring the aging NMDA receptor to that of young by enhancing GluN2B subunit expression. However, memantine mainly antagonizes abnormal hyperactivity at extrasynaptic NMDA receptors and not normal activity at synaptic NMDA receptors (Xia, Chen et al. 2010). In addition, extrasynaptic NMDA receptor activation leads to increased beta amyloid production and apoptosis, while activation of synaptic NMDA receptors is thought to be neuroprotective (Hardingham, Fukunaga et al. 2002; Bordji, Becerril-Ortega et al. 2010). There is also evidence that normal, physiological levels of NMDA receptor activity can enhance neuronal survival (Gould, Cameron et al. 1994; Ikonomidou, Bosch et al. 1999). This could suggest that it may be beneficial, or even neuroprotective, to repair the changes in NMDA receptors that occur during aging by enhancing the expression of the GluN2B subunit. However, measures would have to be taken to avoid increasing the susceptibility to excitotoxicity or Alzheimer’s disease. This could require determining exactly how much as of an enhancement of the GluN2B subunit is beneficial before it becomes harmful, as well as trying to target that enhancement strictly to the synapse.

Given the results of the studies presented here, several follow up studies would be needed to further prove the therapeutic potential of enhancing GluN2B-containing receptors at the synapse in the aged brain. One critical study would be to enhance the number of GluN2B-containing NMDA receptors strictly in neurons, preferably at synapses, throughout the aged brain, and determine how memory is impacted compared to age-matched controls. This might be accomplished using either a viral vector with a neuron
specific promoter, or by inhibiting Cdk5 expression with a Cdk5 specific antagonist, such as roscovitine, or nontoxic dose of the Cdk5 Morpholino within the aged brain.

An adenoviral vector with a neuron-specific promoter could be designed, similar to the GluN2B vector, to enhance GluN2B subunit expression specifically in neurons. However, it likely would require multiple acute injections bilaterally to achieve a more global enhancement within the aged brain. Also, this enhancement would not be strictly synaptic. However, treatment with memantine could be used to block any enhancement of extrasynaptic GluN2B-containing receptors.

Another alternative would be to target Cdk5 in order to maintain remaining GluN2B-containing receptors at synapses within the aged brain. This could be an equally viable intervention since an aging intervention already showed that an increase in existing GluN2B-containing receptors at the synapse improved NMDA-dependent LTP in aged animals (Coultrap, Bickford et al. 2008). This could be accomplished by either inhibiting the function of Cdk5 within the aged brain using a Cdk5 antagonist, such as roscovitine, or using a nontoxic but efficacious dose of the Cdk5 Morpholino. Cannulae with osmotic pumps centered on the lateral ventricles would have to be used to achieve a global effect because neither treatment will bypass the blood brain barrier if delivered systemically. However, due to the cumbersome nature of bilateral osmotic pumps, these animals would not be able to swim in the Morris water maze. Therefore, memory would have to be characterized using another task such as the novel object recognition task (refer to Chapter1, section 1.4), and/or a variant of the novel object recognition task designed to assess spatial memory.

Of course prior to these studies, preliminary work would be required to determine the most effective dose as well as the duration and localization of enhanced GluN2B subunit expression and/or GluN2B-containing receptors. Either of the proposed studies could also be used to better understand the molecular mechanism that underlies the enhancement in memory in aged animals as a result of enhanced of GluN2B subunit expression. The brains of these animals could be fractionated into synaptic and extrasynaptic
membranes in order to determine the localization and degree of enhanced GluN2B subunit expression by western blot. They could also be used to electrophysiologically measure any enhancements in NMDA and LTP currents due to enhanced GluN2B-containing receptors.

However, further research should also address any negative consequences of enhancing GluN2B subunit expression within the aged brain, especially whether such an enhancement would increase susceptibility to developing excitotoxicity or to developing neurodegenerative diseases, such as Alzheimer’s disease. In order to address whether enhancement of GluN2B-containing receptors at synapses increases susceptibility to NMDA receptor-mediated excitotoxicity in aged mice, excitotoxicity could be induced using NMDA or an NMDA specific agonist, in GluN2B-enhanced and normal aged mice. Their brains could then be examined to see if any biomarkers for NMDA-receptor excitotoxicity, such as increased neuronal death, are elevated in aged mice with enhanced GluN2B subunit expression compared to age-matched controls. If this study was done in aged mice treated with the adenoviral vector, memantine could also be applied to differentiate between the excitotoxic effects of extrasynaptic versus synaptic GluN2B-containing receptors. It would be more difficult to assess whether having an enhancement of GluN2B-containing receptors at synapses would increase susceptibility to Alzheimer’s disease, given that the exact cause of the disease is unknown. However, it might be possible to use a mouse model of Alzheimer’s disease to determine whether enhancing GluN2B subunit expression and/or GluN2B-containing receptors at the synapse will further exacerbate the progression of Alzheimer’s disease. Transgenic mice over-expressing a human Familial Alzheimer’s disease-linked Presenilin 1 (PS1) variant (L286V mutation) have been shown to have an increased vulnerability to excitotoxic damage (Grilli, Diodato et al. 2000). These animals could be enhanced for GluN2B subunit expression at a young age, before pathology is evident, and compared to both aged-matched controls and wild-type mice, to determine if there is an increase in amyloid plaque load in the Alzheimer’s mice with enhanced GluN2B subunit expression.
Studies of this nature would help address whether age-associated memory impairment can be ameliorated by enhancing the number of GluN2B-containing receptors in the aged brain as proposed by the work presented here. They would also help alleviate any concerns over the detrimental effects of a therapeutic aimed at enhancing GluN2B-containing receptors in the aged brain. These studies could also provide impetus for the development of a GluN2B-specific agonist that could be used, possibly in conjunction with memantine, to enhance memory in aged individuals while reducing the risk for excitotoxicity or neurodegenerative diseases like Alzheimer’s disease.
Bibliography


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APPENDIX
Figure 1
Fig. 1— The effects of enhanced GluN2B subunit expression in the frontal lobe on the performance of young mice in memory tasks in the Morris water maze. Graphs A-F show the effects of the GluN2B vector or 2 control (control vector or vehicle) treatments on learning performance. In graphs A-D and F, lower proximity scores represent better performance. In graph E, greater differences between naïve and delayed trials represent better performance. (A) Performance across blocks of four place trials for the two-day long-term spatial memory task. (B) Performance averaged across place trials for each individual day of the two-day long-term spatial memory task. (C) Performance within probe trials of the two-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Differences in performance between $T_{\text{naïve}}$ and $T_{\text{delay}}$ trials, averaged over working spatial memory sessions, over eight days. (F) Performance in 6 cued trials for the associative memory task. * $p<0.05$ for differences from young GluN2B vector-treated mice. N=12-14. Bl = block of 4 place trials, Pr = probe trials, Pr0= naïve probe trials, R= reversal trials, C= cued trials. Error bars = standard error of the mean (SEM).
Figure 2
Fig. 2 – The effects of enhanced GluN2B subunit expression in the hippocampus on the performance of young mice in memory tasks in the Morris water maze. Graphs A-E show the effects of the GluN2B vector or 2 control (control vector or vehicle) treatments on learning performance. In graphs A-E, lower proximity scores represent better performance. (A) Performance within blocks of four place trials for the three-day long-term spatial memory task. (B) Performance averaged across place trials for individual days for the three-day long-term spatial memory task. (C) Performance within probe trials of the three-day long-term spatial memory task. (D) Performance across blocks of two reversal trials for the flexibility task. (E) Performance in 6 cued trials for the associative memory task. N=7-15. Bl = blocks of 4 place trials or 2 reversal trials, Pr = probe trials, Pr0 = naïve probe trials, C = cued trials. Error bars = standard error of the mean (SEM).