One of the first cognitive dysfunctions to arise with aging is memory loss, affecting an estimated 85% of elderly in the U.S. over the age of 80 with Age Associated Memory Impairment. A common feature in humans and animals experiencing memory loss with aging is the decline in N-methyl-D-aspartate (NMDA) receptor-binding densities in the brain. Variability in the effects of aging on the GluN1 subunit suggests that inflammation may play a role in NMDA receptor aging. The purpose of the present study was to determine the effects of an anti-inflammatory drug, ibuprofen, on spatial long-term & short-term memory and cognitive flexibility in male C57BL/6 mice from four different age groups (5, 14, 20, and 26 months of age at the end of testing). Mice were fed either Ibuprofen (375 ppm) in NIH31 diet or NIH31 diet alone for 6 weeks prior to testing. This dose had been shown to reduce pathology in an Alzheimer’s disease mouse model. Behavioral testing using the Morris Water Maze showed a significant effect of age on acquisition of spatial reference memory, the formation of spatial memory bias in probe trials, cognitive flexibility in reversal trials, and working memory, with 20 and 26 month old mice performing significantly worse than 5 month old mice. Ibuprofen enhanced overall performance in the working memory task when data was collapsed across naïve and test trials and ages. The oldest mice receiving ibuprofen showed significantly better delayed working memory, but near significantly ($p=0.08$) worse spatial reference memory, as compared to age-matched controls. 

In situ hybridization assays showed significant decreases over all ages in the mRNA densities for the GluN2B subunit, all GluN1 splice variants, and the GluN1-1 splice forms in the frontal lobes of mice consuming ibuprofen. The GluN1-3 splice form mRNA was significantly increased across ages in the frontal lobes of the ibuprofen-treated mice. There was no effect of ibuprofen on IL-1beta expression in the brains or spleens of these mice. These findings suggest that inflammation may play a role in working memory declines in aged
animals, but the reducing effects of ibuprofen across ages on several factors, without reducing age-related increases in cytokine expression suggests that ibuprofen has effects on mRNA for the NMDA receptor that are unrelated to aging or inflammation.

Key words:
N-Methyl-D-Aspartate receptor, memory, anti-inflammatory, aging
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Influences of an Anti-inflammatory Drug, Ibuprofen, on Spatial Memory and NMDA Receptor Subunit Expression During Aging

by

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This work is dedicated to my family for all their love and encouragement, especially to my parents, Norma Loza Pérez and Luis Márquez Cedillo PhD.
1. INTRODUCTION

Learning and memory decline are the first cognitive dysfunctions to arise with aging (Albert et al. 1992). Declines in spatial memory pose a significant problem for individuals in their interactions with the environment, in some cases rendering them dependent on others to navigate through daily tasks. Severity can range from Alzheimer’s Disease to Age Associated Memory Impairment (AAMI), a milder clinical state that affects the ability to process new and old information (Larrabee et al. 1994). AAMI is a common form of memory decline, experienced by an estimated 40% of individuals within the fifth decade and 85% of those over age 80 (Larrabee et al. 1994). As decreased fertility along with increased life expectancy in most areas of the world has generated a shift in relative populations from younger to older groups (UN, 2002), age-related memory loss will likely become more prevalent compared to previous years. Preventing or mitigating memory declines with aging will be a significant component to preserving quality of life through old age, especially in developed regions of the world where average life expectancy at age 80 is expected to increase by 27% over the next 50 years (UN, 2002).

In addition to humans, age-associated memory declines have been observed in nonhuman primates (Gallagher et al., 1993; Gallagher, 1997), dogs (Head et al., 1995), and rodents (Gage et al., 1984; Rapp et al., 1987; Barnes, 1988; Pelleymounter et al., 1990). In these animals, findings have consistently shown that aging negatively affects binding density of N-methyl-D-aspartate (NMDA) receptors in the cerebral cortex and hippocampus (Magnusson et al., 2010). NMDA receptors are one subtype of excitatory glutamate receptor that provide regulatory roles in neurotransmission and performance of memory tasks (Magnusson et al., 2010). These receptors are involved in initiating long-term potentiation (LTP), a cellular mechanism believed to underlie some types of memory formation (Mondadori et al., 1989; Morris and Davis, 1994; Lisman et al., 1998). Compared to other glutamate receptors, these appear to be more vulnerable to the aging process (Magnusson et al., 2010). The importance of the NMDA receptor in learning and memory suggests that its age-associated binding changes may contribute, among other factors, to memory decline with aging. Targeting the NMDA receptor complex may play a role in developing treatment options to prevent or improve age-
related changes in memory.

NMDA receptors are composed of a combination of the following subunit families: GluN1, GluN2A-D, or GluN3A-B (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). The GluN1 subunit appears to be necessary and sufficient for the formation of functional channels (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). Eight different splice variants of mRNA exist for the GluN1 subunit through alternative splicing of one N-terminal and two C-terminal cassettes, C1 and alternatively C2 or C2’ (Laurie and Seeburg, 1994; Zukin and Bennett, 1995) (Appendix I). Studies have found that mRNA for C-terminal splice forms, GluN1-1 (+ C1 and + C2 cassettes) and GluN1-3 (+ C1 and + C2’), show significant declines during aging in several brain regions even though overall GluN1 mRNA expression is not significantly affected by aging, suggesting these splice forms are more influenced by aging than the subunit as a whole (Das et al., 2008). The four members of the GluN2 family each enhance the activity of the receptor when coupled with GluN1 subunit, conferring different agonist/antagonist affinities and gating behaviors to the receptor (Kutsuwada et al., 1992; Yamazaki et al., 1992). Previous research has shown that of the NMDA receptor subunits, the GluN2B subunit is most affected by the aging process (Magnusson et al., 2000; 2002) and increasing GluN2B subunit expression throughout multiple brain regions from birth is beneficial to spatial long-term and delayed short-term memory (Tang et al. 1999; Cao et al. 2007). Although GluN3 subunits can be found in triheteromeric receptors with GluN1 and GluN2 subunits, it is not known how aging affects these subunits (Magnusson et al., 2010).

Increasing evidence suggests that some of the changes in NMDA receptor expression and memory during aging could be due to inflammation. These receptors have been shown to directly interact with inflammatory mediators such as stress-induced glucocorticoids (Nair et al., 2006). In old rats, redox sites of NMDA receptors appear to be in a more oxidized state, than seen in young (Bodhinathan et al., 2007), suggesting there is a more oxidized environment in the aged brain. Inflammation can be induced through infection, injury, or oxidative stress and aging has been noted to increase inflammation-associated molecules such as IL-1beta and TNFalpha (Blalock et al., 2003). A chronic inflammatory response has been described in brains of patients with
Alzheimer’s Disease (AD) (Lim et al., 2000). One study on a transgenic mouse model for AD showed chronic oral ibuprofen treatment was capable of suppressing plaque pathology, including amyloid deposition (Lim et al., 2000). An anti-inflammatory drug, sulindac, was able to improve spatial short-term memory and reverse the effects of normal aging on protein expression of the GluN1 and GluN2B subunits of the NMDA receptor in old rats (Mesches et al., 2004). It is not known, however, whether this effect is at the level of mRNA and/or protein and whether it would improve both long and short-term spatial memory and NMDA receptor expression at younger ages.

Based on evidence linking inflammation and NMDA receptors with memory decline, in the present study we analyzed the effects of an anti-inflammatory drug, ibuprofen, on spatial working (short-term) and reference (long-term) memory and NMDA receptor expression. We focused on analyzing the effects of ibuprofen on mRNA expression of the GluN1 subunit and its GluN1-1 and GluN1-3 splice forms and the GluN2B subunit, which studies have found to be selectively vulnerable to the effects of aging in C57BL/6 mice (Das et al., 2008; Magnusson, 2000, 2001; Ontl et al., 2004; Magnusson et al., 2006). We expected that chronic exposure to ibuprofen would reduce age-related increases in the inflammatory response mediated by cytokines. If inflammation is playing a role during aging on NMDA receptors, we also would expect to see ibuprofen increase mRNA expression of GluN2B and all GluN1 subunits as a whole and the GluN1-1 and GluN1-3 splice variants, but not affect GluN1-2 splice variants, which do not change significantly during aging (Magnusson et al, 2005) and as a result, improve cognitive memory at older ages. Determining the effects of an anti-inflammatory on memory and NMDA receptor subunits may contribute to the development of treatments aimed at ameliorating memory declines associated with aging.
2. MATERIALS & METHODS

2.1 Animals

A total of 48 male C57BL/6 mice (National Institute on Aging, NIH) from four different age groups (5, 14, 20 and 26 months of age at the end of the study) were used for the study. The animals were randomly assigned to two treatment groups consisting of 375 ppm of ibuprofen in NIH chow or NIH chow alone, for an N = 6 for each treatment and age group. The ibuprofen dose was obtained from a study by Lim and coworkers, which reduced pathology in an Alzheimer’s Disease mouse model (Lim et al., 2000). Animals were fed their corresponding diet ad libitum for two months total, both prior to and during behavioral testing, and housed under 12 hr light and 12 hr dark cycle. During the two-month total treatment administration, food mass prior to consumption and following consumption was tracked every 3-4 days along with animal weight. After behavioral testing, all animals were euthanized by exposure to CO₂ and decapitated. The brains were then harvested, frozen rapidly with dry ice and stored at -80°C until further processing.

2.2 Behavioral testing

Spatial reference memory, working memory, cognitive flexibility, and cued control task abilities were tested using the Morris Water Maze as previously described (Magnusson et al. 2003). A 1.2 m diameter plastic tank was filled with water (16–18°C) that was made opaque white with non-toxic paint. A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth (Appendix II). The cues were placed high on the walls of both the room and the tank. There were seven different platform positions available at five different distances from the tank wall. Trials were video taped using a CCD camera placed above the center of the tank on the ceiling of the room. Paths of the trials were analyzed by using the “SMART” video tracking system (San Diego Instruments, San Diego, CA, USA). There were different entry points for each trial and the mice were placed in the tank facing the wall.

2.2.1 Acclimation— Acclimation was performed 2 days prior to reference memory training and consisted of each mouse swimming for 60s in the tank without the platform. After all the mice completed the swimming
training, a platform was placed in a location not used for memory testing and the mice were trained to remain on the platform for 30 seconds. This procedure was repeated on the second day of acclimation.

2.2.2 Spatial long-term memory—On days 3 through 5, mice underwent long-term memory testing. The task consisted of 8 place trials per day for 3 days with one probe trial at the end of each day separated by an hour from the last place trial. The place trials were performed in two 4 trial blocks, with a 90 minute interblock interval. The platform was kept in the same quadrant (SE) for each place trial and the start positions were randomly assigned (NW, NE and SW). Place trials consisted of 60 seconds maximum in the water searching for the platform, 30 seconds on the platform and 2 mins of cage rest. If a mouse failed to find the platform within the designated 60 seconds, it was led to the platform by the experimenter. While the place trials were used as an assessment of the animal’s overall spatial reference memory, the probe trial at the end of each day was used as an assessment of the animal’s ability to show a bias for the platform location (Gallagher et al., 1993). A naive probe trial was performed prior to the first place trial, in order to detect a bias for the target quadrant and to allow us to see improvement in bias development on the first day, if present. During the probe trial, the platform was removed and the mice were allowed to search in the water for 30 seconds from a randomly assigned start position.

2.2.3 Cognitive Flexibility— On the sixth day, mice underwent reversal trials to test cognitive flexibility. During the two blocks of four reversal trials each, with a 90 minute interblock interval, the platform was placed in the opposite quadrant to that which had been used in the previous days for reference memory testing. The platform was kept in the same quadrant (NW) for each place trial and the start positions were randomly assigned (SE, NE and SW). Mice were allowed to search for the platform for 60 sec followed by a 30 sec sit in the platform and a 2 min cage rest. A final probe trial consisting of a 30 sec swim with no platform took place following a 1 hr cage-rest period.

2.2.4 Spatial short-term memory—On days 7 through 13, mice were tested in a spatial working memory task (Magnusson et al., 2003). The task consisted of two sessions with a 3 hr period in between. The platform
positions were changed between each session. Each session consisted of 4 trials. The first trial was a naïve trial ($T_{\text{naïve}}$) initiated by placing a mouse into an entry point and allowing it to search for the new platform position for a maximum of 60 seconds, after which the mouse was allowed to remain on the platform for 30 seconds, followed by cage rest for 10 minutes (delay period). In the second trial ($T_{\text{delay}}$) the mouse was placed in the water at a different entry point from the naïve trial and allowed to search for the platform for a maximum of 60 seconds. The mouse was again allowed to stay on the platform for 30 seconds and allowed to rest in the cage for 2 min. The mouse was placed into the water 2 more times at 2 different entry points and allowed to search the platform for 60 seconds. They spent 30 seconds on the platform and rested in the cage for 2 min between trials. Mice were then placed into their cages until the next session, which started about 3 hrs from the beginning of the first session. If the mouse failed to find the platform within the designated 60 seconds for any of the trials, it was led to the platform by the experimenter. The entry points within one session were randomly assigned for each trial. Working memory was analyzed for differences in performance between $T_{\text{naïve}}$ and $T_{\text{delay}}$. The extra trials were performed based on previous findings that mice need additional trials to show improvement between trials (Magnusson et al., 2003), but these trials were not included in the analysis.

2.2.5 Cued control task—— On day 14, mice underwent 6 cued trials. Cued trials were designed to test motivation, visual acuity, and physical ability for the task. The platform was visible and was marked by a 20.3 cm support with a flag. For each cued trial, the platform was changed to a different position and the mouse was placed into the tank facing the wall at one of the entry points and was allowed to search for the platform for 60 seconds. All mice were tested at one platform position before the platform was moved to a new position.

2.3 Brain Sectioning

The frozen brain from each animal was cut sagittally into two halves. One half was used for sectioning for in situ hybridization. Use of the left and right half of the brain was varied between individuals. Slides used for brain tissue sections were coated with 0.5% gelatin solution and air-dried beforehand. Horizontal brain sections of 12 $\mu$m thickness, representing animals from each age and behavioral group were placed on each slide and kept frozen at $-80^\circ$C until used. Brains were divided into 3 cutting groups, each group containing 2
representative animals from each age and treatment group. Positions of age/treatment representative brain sections on slides were varied in a randomized block design between each cutting group to control for the variability while washing during \textit{in situ} hybridization.

2.4 \textit{In situ} hybridization

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

- \textbf{GluN2B}, 5\textquotesingle CACTG TAGCGGTCACTCTTTGAAAGAGA A CTTG GCCGTACAG GTCGC 3\textquotesingle
- \textbf{GluN1-1}, TCCACCCCCCGTGCTCGCTTTGGAGGACCTACGTCTC;
- \textbf{GluN1-3}, GATATCAGTGGGATGGTACTGCGTGTCTTTGGAGGA- CCTA;
- \textbf{GluN1-2}, TCCACCCCCCGTGCTCTGCAGGTTCTTCCTCCACACGTTC; and
- \textbf{GluN1-pan}, GCACAGCGGGCCTGGTTCTGGGTTGCGCGAGCGC-GACCACCTCGC (Watanabe et al., 1993; Laurie and Seeburg, 1994).

Oligonucleotides were labeled with $^{33}$P-dATP (Perkin Elmer, Waltham, MA; reference date specific activity: 3000 Ci/mM) using terminal deoxyribonucleotidyl transferase (Invitrogen Corp., Carlsbad, CA) and purified in Microspin G-25 columns (Amersham Bioscience, Piscataway, NJ).

\textit{In situ} hybridization was performed as described by Watanabe et al. (1993) and a previous study in our lab (Magnusson et al., 2005). Briefly, each solution step was performed with gentle rotation on a rotating table except for the hybridization steps. Slides with sections were thawed, air-dried, fixed in 4\% paraformaldehyde–PBS, pH 7.2 (25 $^\circ$C) for 15 min, placed in 2 mg/ml glycine in PBS, pH 7.2 (25 $^\circ$C) for 20 min, and placed in 0.25\% acetic anhydride–0.1 M triethanolamine, pH 8.0 (25 $^\circ$C) for 10 min. Slides were placed in coplin jars (25 $^\circ$C) for 2 h in a prehybridization solution consisting of 50\% formamide, 0.1 M Tris–HCl, pH 7.5, 4X SSC (1X SSC=150 mM NaCl and 15 mM sodium citrate), 0.02\% Ficoll, 0.02\% polyvinylpyrrollidone, 0.02\% bovine serum albumin, 2\% sarkosyl, and 250 $\mu$g/ml salmon testes DNA. Slides were then successively washed for 5 min each in 2X SSC, 70 and 100\% ethanol, and air-dried for 15 min. Hybridization was performed by placing 150 $\mu$l of prehybridization solution containing 10\% dextran sulfate and 0.33 pmol of $^{33}$P-labeled oligonucleotide probe onto the slides, covering the slides with parafilm, and
incubating them for 18 h in a 42 °C oven, humidified with 5X SSC. After incubation, coverslips were removed; slides were rinsed for 40 min in 2X SSC and 0.1% sarkosyl (25 °C) and for 2×40 min in 0.1X SSC and 0.1% sarkosyl (55 °C) and air-dried. Nonspecific hybridization was determined by addition of 50-fold excess non-radiolabeled oligonucleotide to the hybridization solution on some slides. Slides were exposed to Kodak Biomax films for 4-14 days depending on the splice form or subunit, along with a slide containing 14C standards.

Brain and standard images were captured using a Macintosh G4 computer with a Powerlook 2100 XL scanner (UMAX, Taiwan) and NIH ImageJ software. Quantitative densitometry was performed on the images from four sections for total hybridization and two sections for nonspecific hybridization from each animal with the use of NIH ImageJ software. The different brain regions analyzed for mRNA expression were deep (cortical layers IV–VI) layers of medial prefrontal cortex (areas containing cingulate cortex, infralimbic cortex and prelimbic cortex) and lateral prefrontal cortex (deep and superficial (cortical layers II–III) layers of ventral orbital cortex, lateral orbital cortex, insular cortex (areas containing both granular and agranular insular cortex), secondary motor cortex, primary motor cortex and the somatosensory cortex (areas containing both primary and secondary somatosensory cortex). Specific signal was determined by subtracting nonspecific hybridization from total hybridization. The 14C standards were used to convert optical density to fmol of labeled 33P-dATP/mm² tissue (Eakin et al., 1994).

2.5 Inflammatory response
Collaborator C. Wong carried out experimentation as previously described by Wong et al. (2013). Levels of cytokine IL-1beta in the brain and spleen were used as measures of inflammatory response. Samples from each treatment and age group were analyzed (N=5-6). Total RNA from spleen and brain tissues were isolated using TRIzol (Invitrogen). Total RNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for quantitative real-time polymerase chain reaction (qRT-PCR) (Invitrogen). Real-time PCR was performed using the following PCR primers: mouse IL-1beta (forward: 5’-AAGATGAAGGGCTGCTTCAA-3’, reverse: 5’-TGAAGGAAAAGAAGGTGCT- CATG-3’) and mouse
18S ribosomal RNA (18S) (forward: 5′-CCGCAGCTAGGAATAATGGAAT-3′, reverse: 5′-CGAACCTCCGACTTTCGTTCT-3′). Real-time PCR reactions were performed using Fast SYBR Green Mastermix (Invitrogen) on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene copies were determined using the standard curve method. A standard curve was generated from serial dilutions of purified plasmid DNA that encoded for each gene of interest. Data represent the copy number of the gene of interest normalized to the copy number of 18S housekeeping genes.

2.6 Data analysis

Data for behavioral testing were analyzed as described earlier (Magnusson et al., 2003). Cumulative proximity was used to measure performance in the place, reversal, working memory and cued trials. Cumulative proximity was obtained from the Smart tracking program according to the method of Gallagher et al. (1993), and was manually corrected for start position. The computer measured, every 0.2s, the animal’s distance from the platform, or proximity measure, for the duration of the animal's swim. These proximity measures were then added together to give a cumulative proximity. The proximity measures were corrected for start position by calculating the cumulative proximity for the ideal path, based on swim speed and starting point, and subtracting this from the cumulative proximity measurement from the tracking system. Average proximity to the platform was used to assess performance in the probe trials (Gallagher et al., 1993). Proximity measures were used to assess performance in these studies because they are less influenced by swim speed differences than more traditional measures such as latency to reach the platform (Gallagher et al., 1993; Magnusson, 1998a). The proximity measures are also more sensitive to some of the alternative strategies that animals can use to find the platform that may not involve place learning (Gallagher et al., 1993).

Age-related differences in performance in working and reference memory and cued tasks were analyzed separately by repeated measures ANOVA and three-way ANOVA (Age X Treatment X Trial (T naïve vs. T delay)) for working memory and two-way ANOVA (Age X Treatment) for all other behavioral measures, followed by Fisher's protected post-hoc analysis using Statview software (SAS Institute Inc., Cary, NC). Age-related differences in mRNA were analyzed separately for each brain region by two-way ANOVA followed by
Fisher's protected post-hoc analysis. Food consumption and weight differences were also analyzed with repeated measures ANOVA.
3. RESULTS

3.1 Behavioral cognitive testing

3.1.1 Effect of ibuprofen on spatial long-term memory

There was a near significant main effect of treatment on cumulative proximity scores in place trials for spatial long-term memory ($F_{(1,35)} = 3.99, p=0.054$). There was an overall effect of age on cumulative proximity scores in place trials for spatial long-term memory ($F_{(3,35)} = 3.75, p=0.02$), with young animals spending more time closer to the platform than aged animals. The youngest (5-month old) group of animals showed a significantly lower cumulative proximity (cm) to the platform compared to the two oldest groups (20-month olds ($p=0.03$) and 26-month olds ($p=0.01$)) during place trials (Figure 1a), when data was collapsed across treatments. There was also a significantly smaller cumulative proximity (cm) in the 14-month old group compared to the oldest group ($p=0.03$) in place trials (Figure 1a), with treatment groups combined. An age by treatment interaction was observed in place trials ($F_{(3,35)} = 4.04; p=0.01$). Given this significant interaction, the individual age groups were analyzed separately for treatment effects. The oldest mice treated with ibuprofen showed near significantly ($p=0.08$) greater cumulative proximity scores in the spatial reference memory place trials, compared to age-matched controls (Figure 1a).

There was no significant main effect of treatment ($F_{(3,35)} = 0.51, p=0.48$) or age ($F_{(1,35)} = 2.48, p=0.07$) for average proximity scores in probe trials for long-term spatial memory bias. These results included only animals that completed all the place trials. Some older animals required more rest and were not able to complete all place trials. Given that there was a significant interaction ($F_{(4,24)} = 5.29, p<0.01$) between probe trial performance in animals with missing place trials compared to those that completed all place trials, only animals that completed all place trials were included in the probe trial analysis.
3.1.2 Effect of ibuprofen on delayed short-term memory

There was a significant main effect of treatment \(F_{(1,74)} = 6.54, p=0.01\) on performance in short-term memory sessions, with ibuprofen-treated animals exhibiting lower cumulative proximity scores when data was collapsed across both T_{naive} and T_{delay} trials and age groups. There were significant main effects of age \(F_{(3,74)} = 16.33, p<0.01\) on performance across trials T_{naive} and T_{delay} and sessions on cumulative proximity in the working memory task with young animals showing lower cumulative proximities, as compared to old. Because these analyses include T_{naive} trials, these results were not reflective of working memory alone. Spatial short-term memory was assessed by comparing cumulative proximity in T_{naive} versus T_{delay} trials over 8 sessions with a 10 min delay. There was a significant interaction between age and treatment in working memory trials \(F_{(3,74)}=3, p=0.04\), when data were collapsed across sessions, so we analyzed each age and treatment group separately for performance in T_{naive} versus T_{delay}. The 26-month old mice treated with ibuprofen, but not the controls, showed significantly lower cumulative proximity scores (cm) in the T_{delay} trials, compared to the T_{naive} \(p=0.049\). The 14-month-old mice treated with ibuprofen \(p<0.01\), as well as the age-matched control-treated mice \(p=0.02\), both showed significantly lower cumulative proximity (cm) in delayed trials compared to naive (Figure 2).

3.1.3 Effect of ibuprofen on cognitive flexibility and associative memory

There were no significant effects of treatment on cumulative proximity in reversal trials for cognitive flexibility testing \(F_{(3,38)}=0.28; p=0.84\). There was an overall effect of age on cumulative proximity scores in reversal trials within the cognitive flexibility task \(F_{(3,38)} = 3.53; p=0.02\). The 5-month old group showed significantly smaller cumulative proximity scores than the 20-month \(p<0.01\) and 26-month old groups \(p=0.02\) (Figure 3), when the data were collapsed across treatments and reversal trials. Within the associative memory (control) task, which was used as an assessment of motivation and sensory and motor skills, there was no overall significant main effect of age \(F_{(3,38)}= 1.75; p=0.17\) or treatment \(F_{(3,38)} = 2.74; p=0.06\) (Figure 4).
3.2 In Situ Hybridization

3.2.1 Effect of ibuprofen on mRNA densities for NMDA subunits

*In situ* hybridization assays showed an overall significant effect of treatment ($F_{(1,195)}=4.98; p=0.03$), with ibuprofen reducing mRNA densities (fmole $^{33}$P-dATP/mm$^2$) for the GluN2B subunit across all brain regions analyzed (Table 1). There was also a significant interaction between brain regions, age and treatment ($F_{(5,195)}=13.25; p<0.01$) on mRNA densities for GluN2B subunit. The lateral frontal superficial region showed an effect of treatment ($F_{(1,39)}=43.91; p<0.01$), and age by treatment interaction ($F_{(3,39)}=3.56; p=0.02$). Ibuprofen treatment significantly reduced GluN2B expression in the lateral frontal superficial brain region of 5-month ($p=0.02$), 14-month ($p<0.01$), and 20-month groups ($p=0.01$), but did not have an effect on the oldest 26-month group ($p=0.41$) (Figure 5a, Table 1).

*In situ* hybridization assays showed there was no main effect of treatment ($p_{\text{range}}=0.18-0.71$) or age ($p_{\text{range}}=0.53-0.72$) on mRNA density measurements for GluN1-1, GluN1-2, and GluN1-3 splice forms and all GluN1 splice variants (GluN1-pan) subunit across all brain regions. There was a significant interaction between brain region and treatment for all GluN1 splice variants (GluN1-pan; $F_{(6,234)}=6.61; p<0.01$) and GluN1-1 ($F_{(6,234)}=8.04; p<0.01$) and GluN1-3 splice forms ($F_{(6,233)}=7.03; p<0.01$), but not for the GluN1-2 splice form ($F_{(6,234)}=1.89; p=0.08$). Ibuprofen significantly reduced mRNA densities for all GluN1 splice variants ($p=0.01$) and the GluN1-1 splice form ($p=0.01$), but increased mRNA density for the GluN1-3 splice form ($p=0.01$) across all ages in the superficial layers of the lateral frontal cortex (Figure 5b-e). In the cerebellum, ibuprofen showed a main effect of treatment ($p=0.0004$); raising mRNA densities for all GluN1 splice variants combined in all ages (Figure 5f).

3.3 Effect of ibuprofen on brain and spleen IL-1beta cytokine mRNA

There was no significant effect of treatment on IL-1beta protein expression in the brain ($F_{(1,38)}=0.12; p=0.73$) or spleen ($F_{(1,38)}=0.06; p=0.81$). There was a significant main effect of age on IL-1beta cytokine expression in the brain ($F_{(3,38)}=12.22; p<0.01$), as well as the spleen ($F_{(3,38)}=3.93; p<0.02$), measured as fold change from 5-month old controls, with older animals having greater expression that younger animals (Figure 6).
3.4 Weight differences for test subjects

There was no significant main effect of treatment on individual mouse weights when averaged across age groups and different weighing days \( F_{(1,39)} = 2.57; p=0.12 \) (Figure 7). There was a significant main effect of age \( F_{(3,39)} = 15.83; p<0.01 \), with young animals having lower weights compared to older animals, when averaged across treatments and weighing days. There was a significant interaction between weighing days and treatment \( F_{(3,39)} = 2.34; p=0.04 \), but this showed no significant effects of treatment when each day was analyzed individually.
4. DISCUSSION

This study showed that an anti-inflammatory drug, ibuprofen, improved short-term memory performance in the oldest animal group (26-month old), as compared to age-matched control groups, but had no significant effects on long-term memory or cognitive flexibility. Equal performance across age groups and treatments in the cued control task, suggests that motivation, sensory and motor skills were not factors that could account for differences observed in memory performance. At the mRNA level, ibuprofen showed significant effects in the superficial layers of the lateral frontal cortex. In this region, ibuprofen significantly reduced expression for the GluN2B subunit in all but the oldest animal group, where it had no effect. Ibuprofen also significantly reduced mRNA expression for all GluN1 splice variants and the GluN1-1 splice form, and enhanced mRNA density for the GluN1-3 splice form across all ages, but had no effect on the GluN1-2 splice form. Ibuprofen had no effect on inflammatory response measured by IL-1beta cytokine levels in the brain and spleen. These findings suggest that inflammation may play a role in working memory declines in aged animals, but the reducing effects of ibuprofen across ages on several factors, without reducing age-related increases in cytokine expression suggests that ibuprofen had effects on mRNA for the NMDA receptor that were unrelated to aging or inflammation.

Using the Morris water maze, spatial short-term memory was assessed by comparing cumulative proximity to the platform in T naïve versus T delay trials over 8 sessions with a 10 min delay. An observed interaction between age and treatment in working memory trials when data were collapsed across sessions allowed us to analyze each age and treatment group separately for performance in T naïve versus T delay. Improvement in the T delay compared to T naïve trials was observed in the 14-month old groups of both ibuprofen and control treatment, as well as in the 26-month old group treated with ibuprofen, but not in the age-matched control group. Similar results were obtained for the control group in previous studies (Magnusson et al. 2003). Our findings indicate that ibuprofen was capable of improving short-term spatial memory in old mice.
In the spatial long-term memory task, we observed that there were signs of reduction in learning ability between the ages of 20 and 26 months, as evidenced by the place learning trials. Similar age-related differences have been observed in previous studies (Magnusson, 1998a; Magnusson, 2001). Although there was no significant overall effect of ibuprofen, an age by treatment interaction allowed further comparison between differences in treatment between individual age groups. The near significantly ($p=0.08$) worse performance of the oldest ibuprofen treated animals, compared to control groups, suggests that ibuprofen may have had effects that were not beneficial in all types of spatial memory. These results may be of interest for future studies with an increased number of animals.

In probe trials, used to discriminate spatial and non-spatial strategies with the removal of the platform, we observed there were no differences of performance in treatment or age. These results suggested that ibuprofen did not affect the development of search bias in the mice and that the search biases were equivalent across ages. Probe trials take place an hour following the last place trial with the platform removed. Not all animals carried out all of the last place trials as some of the older mice required more rest. Statistical analysis showed a significant difference in probe trial performance between mice with missing place trials and those with no missing place trials. It may be possible that mice that did not complete place trials were poor learners leading them to tire and not complete the trials, but it may also be possible that these same animals may have preformed poorly on probe trials as a result of not receiving as many place learning trials. We therefore only used data for probe trials including animals that completed all place trials. Given there was no effect of missing place trials on working memory or cued trials, all animals were included in these analyses.

Cognitive flexibility testing, used to assess the ability of mice to learn a new platform position, showed that ibuprofen had no effect on the performance of animals compared to the control group. We observed that there were signs of reduction in cognitive flexibility between the ages of 20 and 26 months, as evidenced by the reversal trials. Equivalent performance of mice across treatment and age groups in the cued control task, suggested that motivation, sensory and motor skills were not factors that could account for differences observed in memory performance.
In situ hybridization assays showed that ibuprofen treatment significantly reduced GluN2B mRNA expression in the lateral frontal superficial brain region of all but the oldest 26-month group. It also reduced mRNA expression across all ages in the lateral frontal superficial brain region for all GluN1 splice variants (GluN1-pan) and GluN1-1 splice forms, but increased expression for GluN1-3 splice forms. Ibuprofen did not have an effect on GluN1-2 splice form. In situ hybridization assays also showed that ibuprofen had effects in the cerebellum. In this brain region, ibuprofen had the opposite effect compared to the superficial layers of the lateral frontal cortex by enhancing mRNA expression for all the GluN1 splice variants. Interestingly, these results show that ibuprofen differentially affects mRNA expression in different brain regions. As studies show, the GluN2B subunits (Magnusson et al., 2010) and GluN1-1 and GluN1-3 splice variants (Das et al. 2008; Magnusson et al., 2005) appear to be selectively vulnerable to the effect of aging in C57BL/6 mice. If inflammation was playing a role during aging on NMDA receptors, we expected to see ibuprofen improve the expression of GluN2B and all GluN1 subunits as a whole and the GluN1-1 and GluN1-3 splice variants, but not affect GluN1-2 splice variants. These results, which deviate from the expected effects of ibuprofen on NMDA subunits and splice variants, suggest ibuprofen has effects on mRNA for the NMDA receptor that are unrelated to aging.

The effects of ibuprofen on IL-1beta cytokine levels in the brain and spleen provide the most compelling results for analyzing the mechanism of ibuprofen on memory and mRNA expression of NMDA receptor subunits. IL-1beta cytokines are one of several types of small signaling molecules involved in mediating inflammatory responses (Dinarello, 1994). As a measure of the effects of ibuprofen on inflammation, we analyzed IL-1beta cytokines mRNA levels in the brain and spleen. We found that ibuprofen had no effect on reducing IL-1beta cytokine levels compared to age-matched control groups. These results suggested that the observed effects of ibuprofen on memory and mRNA expression of NMDA subunits were not through an anti-inflammatory mechanism. There were no significant differences between the two treatment groups on individual mouse weight across ages, suggesting that the self-administered diet gave equivalent doses of treatment. Although food consumption was also tracked, this data was not used as it was ultimately determined
not to be a reliable measure of comparing individual animals, given several animals were caged together and ate from the same food source.

A recent study on the postmortem frontal cortices of Alzheimer disease (AD) and bipolar disorder (BD) patients has shown that increased cyclooxygenase-2 (COX-2) expression related to increased neuroinflammatory markers like IL-1beta, previously reported in the same AD and BD frontal cortex samples, may partly be due to the hypomethylated state of the COX-2 CpG promoter region (Rao et al., 2012). This study proposes that chronic treatment of mood stabilizer and antipsychotic drugs for AD and BD acting at the cellular level may provide transient protection by correcting neuroinflammatory and synaptic remodeling, but may not provide full recovery by not targeting epigenetic regulation (Rao et al., 2012). This study also suggests that attenuation of COX-2 expression at transcription or posttranscriptional levels (as in the ibuprofen mechanism) may not be enough to override the epigenetic mechanism at the COX-2 promoter region (Rao et al., 2012). These findings may explain why cytokine levels may not be altered by ibuprofen, which acts as a prostaglandin inhibitor by targeting COX, if additional epigenetic mechanisms are playing a role in upregulating COX-2 expression through hypomethylation of the COX-2 promoter region.

In this study, ibuprofen was shown to have effects at improving working memory in the oldest animal group as well as reducing mRNA expression of most NMDA subunits across ages. These effects appeared to be independent of inflammation and aging. Inflammation may account for changes in NMDA subunit expression through various mechanisms including changes in receptor activity that could lead to alterations in mRNA expression and/or synaptic localization or direct damage to proteins. It may be possible that epigenetic mechanisms may play a role in altering inflammatory responses that override the effects of drugs acting at the cellular level. Further studies in the continuation of this project analyzing NMDA protein subunit levels may reveal more comprehensive interpretations for the current findings.
**Figure 1.** Effects of age and ibuprofen on performance (measured as proximity) in place (a) and probe (b) trials to assess spatial long-term memory. a) The 20 and 26 month old mice showed significantly higher proximities than the 5 month olds. High proximity indicates poorer memory for the platform location. The oldest ibuprofen group in place trials showed near significantly (p=0.08) greater cumulative proximity compared to aged-matched control. b) There was no significant effect of age or ibuprofen treatment on performance in probe trials *p <0.05 for difference from 5 month old mice. Bracket indicates significant age differences were collapsed across treatments. Mean ± SEM, N = 6.

**Figure 2.** Performances in T_{naive} and 10 minute T_{delay} trials for working memory averaged across sessions. The oldest mice treated with ibuprofen, but not control diet, showed significantly better performance in the delayed trial, as compared to the naive, as did both treatment groups at 14 months of age. * p < 0.05 for difference between the T_{naive} trial and the T_{delay} trial for the same age and treatment. Mean ± SEM. N = 6.
Figure 3. Effects of age and ibuprofen on performance (measured as proximity) in reversal trials to assess cognitive flexibility when the data was averaged across trials. The 20 and 26-month old mice showed significantly higher proximities than the 5 month olds. There were no significant effects of ibuprofen treatment on reversal trials. *p < 0.05 for difference from 5 month old mice. Bracket indicates significant differences collapsed across treatments. Mean ± SEM, N = 6.

Figure 4. Performances in cued control task averaged across trials. There were no significant effects of age or treatment on performances in the cued control task, an assessment of sensory/motor skills and motivation. Mean ± SEM. N = 6.
Figure 5. mRNA densities (fmole $^{33}$P-dATP/mm$^2$) for all GluN2B and GluN1 (GluN1-pan) subunits and 3 splice forms of the GluN1 subunit in the superficial layers (I-III) of the lateral frontal cortex (a-d) and cerebellum (f) for different ages and treatments. *p<0.05 for main effect of treatment, #p<0.05 for difference between treatments within age groups. Brackets indicate that the treatment difference was collapsed across all age groups. Mean ± SEM, N = 6.
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### Table 1

Average mRNA density (fmole $^{33}$P-dATP/mm$^2$) with standard error of the mean for all GluN2B and GluN1 (GluN1-pan) subunits and 3 splice forms of the GluN1 subunit for seven brain regions. *Indicates main effect of treatment.

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</tr>
<tr>
<td>Medial frontal deep</td>
<td>398 ± 12</td>
<td>326 ± 13</td>
<td>357 ± 15</td>
<td>324 ± 7</td>
<td>364 ± 20</td>
<td>307 ± 22</td>
<td>355 ± 17</td>
<td>317 ± 12</td>
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<tr>
<td>Lateral frontal deep</td>
<td>339 ± 6</td>
<td>291 ± 16</td>
<td>327 ± 16</td>
<td>278 ± 15</td>
<td>324 ± 17</td>
<td>282 ± 21</td>
<td>290 ± 12</td>
<td>290 ± 8</td>
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</tr>
<tr>
<td>Lateral frontal superficial</td>
<td>524 ± 24</td>
<td>440 ± 20</td>
<td>554 ± 19</td>
<td>398 ± 16</td>
<td>527 ± 7</td>
<td>428 ± 20</td>
<td>459 ± 28</td>
<td>432 ± 11</td>
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<tr>
<td>Caudate</td>
<td>227 ± 8</td>
<td>214 ± 11</td>
<td>216 ± 16</td>
<td>200 ± 16</td>
<td>210 ± 11</td>
<td>202 ± 16</td>
<td>188 ± 8</td>
<td>190 ± 9</td>
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<tr>
<td>Dentate granule cell layer-upper</td>
<td>910 ± 29</td>
<td>922 ± 31</td>
<td>905 ± 27</td>
<td>931 ± 25</td>
<td>916 ± 21</td>
<td>929 ± 34</td>
<td>861 ± 45</td>
<td>923 ± 18</td>
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<tr>
<td>CA3</td>
<td>873 ± 31</td>
<td>881 ± 19</td>
<td>870 ± 41</td>
<td>880 ± 29</td>
<td>867 ± 28</td>
<td>890 ± 29</td>
<td>840 ± 35</td>
<td>884 ± 5</td>
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</table>

*Showed main effect of treatment
Figure 6. IL-1beta levels, as a fold change from 5-month-old controls, in the brain (a) and spleen (b) from different ages and treatments. There was a significant overall effect of age ($p<0.02$), but no effect of ibuprofen in IL-1beta levels in the brain or spleen. $N = 5-6$. Mean ± SEM.

Figure 7. Average C57BL/6 mouse weight (g) from different ages and treatments averaged across weighing sessions. There was a significant overall effect of age ($p<0.01$), but no significant main effect of treatment on individual mouse weights when averaged across age groups and different weighing days ($p=0.12$). $N = 5-6$. Mean ± SEM.
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(A) Schematic representation of an NMDA receptor (Chaffey H. et al.) and (B) C-terminal splice variants of the GluN1 subunit (Magnusson).
(A) Morris Water Maze diagram depicting the hidden platform underwater and visual cues surrounding the tank (Mallick et al.) (B) Representative examples of paths to target in early and later sessions.