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Changes in expression of splice cassettes of NMDA receptor GluN1 subunits within the frontal lobe and memory in mice during aging

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Abstract

Age-related decline in memory has been associated with changes in mRNA and protein expression of different NMDA receptor subunits. The NMDA receptor GluN1 subunit appears to be necessary and sufficient for receptor function. There is evidence that the mRNA expressions of some splice forms of the subunit are influenced by aging and/or behavioral testing experience in old mice. The present study explored the relationships between behavioral testing experience and protein expression of different GluN1 subunit isoforms in the prefrontal/frontal cortex of the brain during aging. Aged C57BL/6 mice with behavioral testing experience showed declines in performance in both spatial working and reference memory tasks. Protein expression of GluN1 C-terminal cassettes C2 and C2′, but not the C1 or N1 cassettes, was observed to decline with increasing age, regardless of experience. In middle-age animals, higher expressions of the GluN1 subunit and C2′ cassette proteins were associated with good reference memory on initial search. Aged animals with a higher protein expression of GluN1 subunits containing C1 cassettes and the whole population of GluN1 subunits exhibited a closer proximity to the former platform location within the final phase of probe trials. However, the old mice with high expression of the C1 cassette did not show an accurate search during this phase. The old mice with lower expression of the C1 cassette protein more closely mimicked the performances of the young and middle-aged mice. These results indicate that there was heterogeneity in the effect of aging on the expression of the GluN1 subunits containing different splice cassettes. It also suggests that the GluN1 subunit might be most important for good reference memory during middle age, but this relationship may not be maintained into old age.

Keywords

NMDA; aging; NR1; Zeta1; GluN1; splice form; splice variant; cassette; water maze; learning index; memory; cumulative proximity; prefrontal cortex

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1. Introduction

Aging has been associated with declines in various forms of memory in many different organisms. A type of memory that represents the ability of the organism to acquire and retain information in order to navigate properly through space, spatial memory, also declines with age (Kirasic and Bernicki, 1990; Moore et al., 1984). Rodents experience similar deficits in spatial memory abilities as humans and have been used as a model for age related memory decline (Barnes, 1988; Gage et al., 1984; Rapp et al., 1987). One type of excitatory glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor has been shown to be important for learning and memory, including spatial memory (see reviews: Magnusson, 1998b; Magnusson et al., 2010). These receptors are abundantly expressed in the frontal lobe and hippocampus (Jarvis et al., 1987; Kohama and Urbanski, 1997), regions responsible for encoding and retrieval of memory (see reviews: Fletcher and Henson, 2001; Martin and Clark, 2007). Antagonists of NMDA receptors inhibit memory performance (Alessandri et al., 1989; Mondadori et al., 1989; Morris, 1989; Morris et al., 1986) and block initiation of long-term potentiation (Bashir et al., 1991; Harris et al., 1984; Morris, 1989; Morris et al., 1986), indicating their importance in learning and memory. Binding of NMDA binding sites by agonists and antagonists changes with increasing age in both the prefrontal/frontal cortex and the hippocampus in various species of animals (Kito et al., 1990; Magnusson, 1995; 1997; 2000; Tamaru et al., 1991). Age-related changes in NMDA receptor expression within these regions have been correlated with declines in memory ability (Magnusson, 1998a; Magnusson, 2001). Although there may be other factors involved, the importance of NMDA receptors for memory, along with evidence of a significant association during aging, suggest that the age-related declines in the expression of NMDA receptors may contribute to memory declines.

NMDA receptors are excitatory receptors on neurons, which form heteromeric channels composed of two GluN1 (earlier names: NMDAR1, NR1, ζ1) subunits and two other subunits, either from the GluN2 family (GluN2A-D, formerly ε1–4 in mice) or from the GluN3 family (GluN3A-B) (Collingridge et al., 2009; Furukawa et al., 2005; Laube et al., 1998; Premkumar and Auerbach, 1997; Ulbrich and Isacoff, 2008). Eight different splice variants of the GluN1 subunit have been identified in the brain. These are generated by alternative splicing of one N-terminal (Exon 5) and two C-terminal (Exons 21 and 22) cassettes in the mRNA (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992). The C2 cassette contains a translational stop codon. In its absence, an additional sequence, which contains the next stop codon and is identified as the C2′ cassette, becomes part of the mature mRNA (Durand et al., 1992). The N1 cassette is present on the extracellular side of the receptor and C1, C2 and C2′ cassettes are present on the cytoplasmic side (Hollmann et al., 1993; Sugihara et al., 1992). These cassettes have been shown to be involved in various functional aspects of the NMDA channel, such as zinc modulation, altering affinity for agonists and antagonists, and spatio-temporal expression in the brain (Durand et al., 1993; Hollmann et al., 1993; Traynelis et al., 1995). We will indicate presence or absence of cassettes N1, C1 and C2 in individual splice variants throughout this article by a series of three subscripts following GluN1 with 0 indicating absence; 1 indicating presence and X indicating either presence or absence of the cassettes (Zukin and Bennett, 1995). For example, $GluN1_{X10}$ indicates presence or absence of the N1 cassette, presence of the C1 cassette, and absence of C2 cassette, but presence of the C2′ cassette.

Previous studies in our laboratory have shown that expression of some of the NMDA receptor subunits decline during aging in C57BL/6 mice (Magnusson, 2000; Magnusson et al., 2002). The GluN1 subunit has been shown to decline during aging (Magnusson et al., 2002) and to be associated with age-related memory decline in rodents (Magnusson et al.,

2007). In other studies, however, no significant decline in GluN1 subunit expression with aging in the same strain of mice was observed (Magnusson, 2001; Ontl et al., 2004; Zhao et al., 2009). We have hypothesized that this is the result of heterogeneity in the effects of aging and/or behavioral testing experience on different splice variants. In C57BL/6 mice, mRNA of the $GluN1_{X11}$ splice form (containing both C1 and C2 cassettes) of the GluN1 subunit is more susceptible to aging changes than other C-terminal splice forms (Das and Magnusson, 2008; Magnusson et al., 2005). Old animals that underwent behavioral testing also show increased mRNA expression of splice forms lacking the N1 cassette ($GluN1_{0XX}$) and an exacerbation of the effects of aging on mRNA expression of $GluN1_{X10}$, one of the splice forms lacking the C2 cassette in prefrontal/frontal cortex (Das and Magnusson, 2008). In the present paper, we have focused on examining the effects of aging and experience in a behavioral task on protein expression of the different splice variants of the GluN1 subunit in combined prefrontal and frontal cortical regions. Antibodies were not available for differentiating each of the GluN1 splice variants, so the investigation was confined to using antibodies raised against each of the four splice cassettes, N1, C1, C2, C2′ and all GluN1 splice variant proteins (GluN1). These were the same animals whose mRNA changes were reported previously (Das and Magnusson, 2008).

2. Materials and Methods

2.1 Animals

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

2.2 Behavioral testing

Spatial reference memory, working memory and cued control task abilities were tested using the Morris Water Maze. A 1.2 m diameter metal tank was covered with white contact paper and filled with water that was made opaque white with non-toxic paint. A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth. The cues were placed high on the walls of both the room and the tank. There were seven different platform positions available at five different distances from the tank wall. Trials were video taped using a 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 Pretraining—Pretraining was performed 2 days prior to reference memory training and consisted of each mouse swimming for 60 seconds in the tank without the platform. After all the mice 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 pretraining.

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

2.2.3 Spatial working memory—On days 15 through 24, mice were tested in a spatial working memory task (Magnusson et al., 2003). The task consisted of two sessions per day for 8 days. There was a two-day break between sessions eight and nine. The platform positions were changed between each session. Each session consisted of 4 trials. The first trial was a naïve trial (T_0) started by placing a mouse into an entry point and allowing it to search for the new platform position for a maximum of 60 seconds, after which the mouse was allowed to remain on the platform for 30 seconds, followed by cage rest for 10 minutes (delay period). In the second trial (T_{delay}) the mouse was placed in the water at a different entry point from the naïve trial and allowed to search for the platform for a maximum of 60 seconds. The mouse was again allowed to stay on the platform for 30 seconds and allowed to rest in the cage for 60 seconds. The mouse was placed into the water 2 more times at 2 different entry points and allowed to find the platform for 60 seconds. They spent 30 seconds on the platform and rested in the cage for 60 seconds between trials. Mice were then placed into their cages until the next session, which started about 3 hrs from the beginning of the first session. If the mouse failed to find the platform within the designated 60 seconds for any of the trials, it was led to the platform by the experimenter. The entry points within one session were randomly assigned for each trial. Working memory was assessed between T_0 and T_{delay}. The extra sessions were performed based on previous findings that mice need additional trials to show improvement between trials (Magnusson et al., 2003).

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

2.3 Tissue subfractionation and protein isolation

Brains were hemisected along the plane of the longitudinal fissure. One half was dissected to obtain prefrontal/frontal cortices. Biochemical fractionation of the dissected tissue was performed as previously described (Dunah and Standaert, 2001) with a few modifications. Briefly, the tissue was homogenized in TE buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) plus 320 mM sucrose with the help of a Dounce homogenizer. The resulting homogenate was centrifuged at 1000 × g for 3 minutes using a Savant μSpeedFuge SFR13K refrigerated centrifuge with RSR20 rotor (Thermo Fisher Scientific, Waltham, MA, USA) and the pellet (P1) was discarded. The supernatant (S1) was centrifuged at 9000 × g for 11 minutes using the same centrifuge to produce the crude synaptosome pellet, P2. The P2 was

resuspended in TE buffer and sonicated. Protein determinations were made with Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.4 Western blotting

Sodium Dodecyl Sulfate - Poly Acrylamide Gel Electrophoresis (7.5%) was used for Western blotting and was performed as described previously (Magnusson et al., 2002). Each gel contained four different μg loads, viz. 1.5, 3, 6 and 12 μg/well, of standards obtained from crude synaptosomes prepared from combined caudal cortices from all the naïve young animals. Protein samples from different age/treatment groups were loaded on each gel to the left of standards and analyzed in triplicate. Gels were allowed to run for 2 hours at 125V. Proteins were transferred to Immobilon-FL Poly Vinylidene Flouride membranes (Millipore, Billerica, MA, USA) for 90 minutes at 100V at 4°C. Membranes were blocked for 1 hr at room temperature with shaking in 1:1 dilution of LI-COR Odyssey buffer (LI-COR Biosciences, Lincoln, NE, USA) and tris buffered saline (TBS). Membranes were then incubated overnight at 4°C in one of the following primary antibodies, NMDAR1 (Zymed Laboratories, San Francisco, CA, USA); N1, C2 and C2′ cassettes (Novus Biologicals, Littleton, CO, USA); C1 cassette (Sigma Aldrich, St. Louis, MO, USA) and GAPDH (Calbiochem, Merck KGaA, Darmstadt, Germany). Primary antibodies used were diluted to appropriate concentrations in the 1:1 dilution of LI-COR Odyssey buffer and TBS (1:500 for C1, 1:1000 for N1, C2, C2′, 1:3000 for GluN1 and 1:10000 for GAPDH). After rinsing for four times, five minutes each, in TBS + 0.1% Tween20, membranes were incubated in fluorescence-based secondary antibody (Alexa Fluor 680 (1:8000 dilution), LI-COR Biosciences; IR Dye 800 (1:4000 dilution), Rockland Immunochemicals, Gilbertsville, PA, USA) for one hour at room temperature. Bands were viewed by scanning in the LI-COR Odyssey imager.

2.5 Data analysis

Data for reference and working memory tests were analyzed as described earlier with a few modifications (Das and Magnusson, 2008). Briefly, the distance of the animal from the platform (proximity to platform) was measured every 0.2 seconds by the computer for the whole duration of the trial. These proximities were added together to generate a cumulative proximity for the trial. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle, WA, USA). A cumulative proximity measurement for the ideal path using the start position, average swim speed and platform position was calculated with the help of this macro. This cumulative proximity measure for the ideal path was subtracted from the cumulative proximity score for the whole track to obtain the corrected cumulative proximity scores for the place trials in reference memory tasks, all trials in working memory tasks and the cued control tasks. For the probe trials of the reference memory tasks, the corrected cumulative proximity score for the trial was divided by the corrected sample number to obtain a corrected average proximity score. Analysis of probe trials was done in two ways; first by calculating corrected cumulative proximity using data from full 30-second probe trials and then by dividing each probe trial into three intervals of 10 seconds each and calculating uncorrected cumulative proximities of each of the three intervals. Cumulative proximity measurements for the three intervals were not corrected because the second and third starting positions in the intervals were dependent on the animals' search pattern and correction for start position did not seem appropriate. The cumulative proximity measurements were averaged across the 30s trial or 10s trial intervals to give average proximity. Learning index was then calculated from the average proximity measurements generated by the two different analyses as described earlier (Gallagher et al., 1993). Briefly, a multiplier for each probe was obtained by dividing the mean average proximity measure of the young naïve animals from a given probe trial day by that of the first probe trial day. This factor was then multiplied by each animals average proximity

measure for that day. The resulting values for each individual animal were added across the probe trials to obtain learning index scores. For the working memory task, ratios of naïve to delayed trials were calculated from their respective corrected cumulative proximity measurements as described earlier (Das & Magnusson 2008).

Protein blots were analyzed using Li-Cor Odyssey software version 1.1. Integrated intensity measures were recorded with the help of the software using median background subtraction method. A standard curve was obtained using a linear fit with Prism software version 4.0 (GraphPad Software Inc., La Jolla, CA, USA) from the observed integrated intensity values for known loads of caudal cortex. Sample values were interpolated from the standard curve as caudal cortex equivalents. GAPDH was used as a loading control and caudal cortex equivalents for each GluN1 protein were divided by the caudal cortex equivalent of GAPDH expressed within the same lane. Statistical analyses for both behavioral trials and protein expression were done with analysis of variance (ANOVA) followed by Fisher's protected least significant difference using Statview software version 5.0.1 (SAS Institute, Cary, NC, USA). If no significant effect of behavioral testing was observed on protein expression of the individual splice cassettes, results from the naïve and behaviorally-tested groups were averaged together for further analysis.

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

Based on the correlational analysis, there appeared to be two distinct populations of twenty-six month old animals with respect to C1 cassette/GAPDH expression and learning index in the third 10-second interval of the probe trials. Differences in the means of these two populations for C1/GAPDH expression were analyzed by one-way ANOVA and repeated measures ANOVA was performed on C1/GAPDH expression × 10-second intervals. In order to better interpret the correlational results, performance differences between 26 month old mice with high (High C1) and low (Low C1) expression of the C1/GAPDH were further examined across probe trials. The learning index scores for High C1 and Low C1 were compared between the three different 10-second intervals of the probe trials. In order to determine whether there was a delay in accurate search for the High C1 group, an index for the time of initial entry into the platform zone across the 6 probe trials was calculated similar to that described above for the learning index. A value of 30 was assigned to any probe trials in which an animal did not ever enter the platform location. In order to assess perseveration and delay of accurate search within the third 10-second interval, the number of platform crossings per second was calculated by counting the number of times an animal entered into the platform location during the first 20 seconds and the last 10 seconds of the probe trials and dividing each by the amount of time analyzed. This was averaged across probe trials.

3. Results

3.1 Spatial memory characterization

A significant main effect of age was observed in the place trials of the reference memory task ($F_{(2,30)} = 7.1$, $p = .003$). Twenty-six month old mice had significantly higher cumulative proximity scores averaged across all trials than both younger ages (Fig. 1A). There was a significant main effect of age on the learning index scores for the full 30-second probe trials, which were calculated from all probe trials ($F_{(2,30)} = 4.95$, $p = .01$). Both the eleven month old ($p = .05$) and twenty-six month old ($p = .004$) mice had significantly higher learning index scores as compared to the 4 month olds in the full 30-second probe trials (Fig. 1B).

For analyzing persistence and flexibility, probe trials were divided into 3 intervals of 10 seconds each and analyzed separately. A significant main effect of age ($p = .001$) was observed only in the first 10-second interval of probe trials but not on the second or third 10-second intervals (Fig. 1B). Learning index scores in the four month olds were significantly lower than those of the eleven ($p = .003$) and twenty-six ($p < .001$) month olds in the first 10-second interval of probe trials (Fig. 1B). There was a significant main effect of age on the learning index scores in probe trials collapsed across the different intervals ($F_{(2,95)} = 4.84$, $p = .01$) and a significant main effect of intervals collapsed across different ages ($F_{(2,95)} = 8.25$, $p < .001$). There was no significant interaction between age and intervals, but since the comparisons between individual intervals was a part of the experimental plan, performance of each age group between the intervals was analyzed individually. Four month old mice did not have a significant change in learning index scores between first and second interval, but had significantly higher scores in the third as compared to the second probe trial interval (Fig. 1B; $p = .025$). Learning index scores in the 11-month old animals were significantly lower in the second interval as compared to the first ($p = .04$) and third ($p = .03$) intervals (Fig. 1B). The twenty-six month olds had significantly lower learning index scores in the second interval ($p = .04$) as compared to the first, but did not change significantly in the third interval ($p = .08$; Fig. 1B).

A significant main effect of age was observed in both the naïve and delayed trials of working memory tasks ($F_{(2,63)} = 11.36$, $p < .001$). The 11 and 26 month old mice had significantly higher cumulative proximity scores than the 4 month olds in naïve trials of working memory tasks (Fig. 1C). In the delayed trials of working memory tasks, the 26 month old mice had significantly higher cumulative proximity scores than both the 4 and 11 month old mice (Fig. 1C). Significantly lower cumulative proximity scores in the delayed trials, as compared to the naïve trial, in the working memory task were observed in 4 and 11 month old mice, but not in the 26 month olds (Fig. 1C). There was a larger ratio of the naïve over delayed trials in 11 month old mice as compared to the 26 month olds (Fig. 1D).

The cued control task was designed to test motivation, motor ability and visual acuity among animals used in the study. Cumulative proximity scores of three 26 month old mice in the cued trials were two standard deviations higher than the mean of the animals included in behavioral testing and so were excluded from all the analysis reported in this study (not shown). Remaining mice showed no significant difference in the cumulative proximity scores in the cued trials between the different age groups of animals included in the study (Fig. 1E). Performance of mice in all the platform locations, except for the north, was lower than the cumulative proximity scores observed during both reference and working memory trials (Fig. 1E).

The young and middle-aged mice were observed to have significantly faster swim speeds than the old mice in the place trials ($p < 0.001$, Table 1) of the reference memory task. In the naïve trials for working memory, swim speeds of the 11 month old mice were significantly

faster than the 26 month old mice and that of the 4 month old mice were significantly faster than both 11 and 26 month old mice in naïve trials across the sessions ($p < .001$, Table 1). Swim speeds of the 4 and 11 month old mice across all the platform positions in cued tasks were significantly faster ($p < .001$, Table 1) than the 26 month olds.

3.2 Protein expression

3.2.1 GluN1 subunits, N1 and C1 cassettes—Analysis of expression of a protein sequence that is common to all the splice variants (GluN1) showed no main effect of behavioral testing experience ($F_{(1,62)} = .001$, $p = .98$; Fig. 2A). There was also no effect of age on the protein expression of GluN1/GAPDH when the data were collapsed across the two behavioral groups ($F_{(2,65)} = .7$, $p = .49$; Fig. 2A). No significant main effect of behavioral treatment was observed on the protein expression of the N1 cassette/GAPDH ($F_{(1,62)} = 1.0$, $p = .32$; Fig. 2B), which is present on the extracellular side of the plasma membrane, or the C1 cassette/GAPDH ($F_{(1,62)} = 1.1$, $p = .29$; Fig. 2C), which is present on the intracellular side of the plasma membrane. There was no significant main effect of age on protein expression of N1 cassette/GAPDH ($F_{(2,65)} = 1.4$, $p = .26$; Fig. 2B) or C1 cassette/GAPDH ($F_{(2,65)} = 1.7$, $p = .19$; Fig. 2C) when the data were collapsed across the two behavioral groups.

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

3.3 Relationships between protein expression and reference memory

Pearson's correlation coefficients were calculated for the learning index scores of the full 30-second probe intervals and the three 10-second intervals of probe trials for reference memory and the T_0/T_{delay} ratios for working memory with the protein expression of the different cassettes of the GluN1 subunit in different age groups of mice (Fig. 3,4; Table 2). Two significant correlations each for 11 and 26 month old mice were found between expression of GluN1 subunit proteins and learning index within intervals of the probe trials for reference memory (Fig. 3B, E, 4C, F; Table 2). The 11 month old animals showed significant negative correlations between the learning index for the first 10-second interval of the probe trials and protein expression of all GluN1 subunits (Fig. 3B, Table 2) and GluN1 subunits containing the C2′ cassette (Fig. 3E, Table 2). Because a lower learning index score indicated more time spent near the platform location, this negative correlation indicated that high expression of the proteins was associated with better proximity to the platform location within the first 10-second interval. The 4 (Fig. 3A, D) and 26 (Fig. 3C, F) month old mice showed no similar significant correlations between these same proteins and learning index in the first interval (Table 2). Significant negative correlations were observed in the 26 month old animals between learning index in the third interval of the probe trials for reference memory and protein expression of GluN1 subunit as a whole (Fig. 4C, Table 2) and GluN1 subunits containing the C1 cassette (Fig. 4F, Table 2). This same comparison was not significant in the 4 (Fig. 4A, Table 2) or 11 (Fig. 4B, Table 2) month old animals for GluN1 subunits as a whole/GAPDH (Fig. 4A, B) or the C1 cassette/GAPDH (not shown). A comparison across intervals for the 26 month olds showed a non-significant trend for an opposite relationship (i.e., positive correlation between learning index and C1 cassette/

GAPDH) in the second 10-second interval (Fig. 4E), as compared to the third (Fig. 4E, Table 2). No other significant correlations were found (Table 2).

Based on the correlational analysis, there appeared to be two distinct populations of twenty-six month old animals with respect to C1 cassette/GAPDH expression and learning index in the third 10-second interval of the probe trials. The means for C1 cassette/GAPDH for these two populations (High C1 group = 1.5 ± .06, Low C1 group = .68 ± .07 (means ± SEM, units = μg caudal cortex equivalents C1 cassette/ GAPDH)) differed significantly ($F(1,7) = 100$, $p < .0001$) from each other by more than two standard deviations. The Low C1 group also had significantly lower C1 cassette/GAPDH than young (1.5 ± .2; $p = .02$), but there was no difference between High C1 and young ($p = .93$). There was no significant main effect of the level of C1 cassette expression when data were collapsed across the different intervals ($F_{(1,20)} = 1.752$, $p = .2$) but there was a significant interaction between the intervals and C1 cassette/GAPDH expression level ($F_{(2,20)} = 12.71$, $p = .003$). Aged mice in the Low C1 group had significantly lower learning index scores in the second 10-second interval as compared to the first and third intervals (Fig 5A). Animals in the High C1 group had a significantly lower learning index score in the third 10-second interval than in the first 10-second interval (Fig. 5A). There was no difference in learning index scores between Low C1 and High C1 animals within the first interval. The High C1 group showed a higher learning index score than the Low C1 group in the second 10-second interval, but a lower learning index score than the Low C1 animals in the third interval (Fig. 5A).

In order to examine whether the lower learning index, i.e., more time spent close to the platform, within the third interval for the High C1 animals may have represented perseveration or a delayed accurate search, time to first entry into the platform zone over the whole probe trial and the average number of platform crossings/second per probe trial within the first two 10-second intervals combined versus the third interval were examined. Results indicated that the 11 month olds and both the High C1 and Low C1 26 month old mice took a significantly longer time to enter the platform zone for the first time, as compared to the four month old mice (Fig. 5B). Significantly lower average numbers of platform crossings/second were observed in 11 month olds and both the High C1 and Low C1 26 month olds, as compared to the four month olds during the first 20 seconds of the probe trials (Fig. 5C). During the third interval of the probe trial only the 26 month olds in the High C1 group had significantly lower platform crossings/second than the four month olds (Fig. 5C). Significantly lower average numbers of platform crossings/second in the last interval, as compared to the first 20 seconds of probe trials was observed in the four month olds and 26 month olds in the High C1 group (Fig. 5C). There was no significant difference in swim speed between the High C1 group in the first 20 seconds versus the last 10 seconds of the probe trials or as compared to the Low C1 group (Fig. 5D)

4. Discussion

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

relationship was not maintained in the aged mice. Correlational analysis showed that high expressions of all GluN1 subunits and those containing the C1 cassettes were associated with closer proximity to the platform in the final phase of the probe trials for reference memory in the old mice. This significant relationship appeared to be due more to a loss of perseveration, exemplified by a widening of the search pattern, in the old mice with lower expression of subunits containing the C1 cassette, than a more accurate search by those with higher C1 cassette expression.

4.1 Age-related changes in memory

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

Our lab has observed a progression of behaviors in mice during probe trials, consisting of an initial phase of approaching the platform location, followed by repeated search on or around the former platform location and then a widening of search for the platform in other parts of the tank (unpublished observation). Similar observations were made by Maei and coworkers, who described a peak in search accuracy in mice by 10–15 seconds during a probe trial and decline thereafter (Maei et al., 2009). They reasoned the decline as an inclination of mice to search elsewhere when the platform is absent from the original location. In an effort to separate these different phases and to determine whether perseverance in mice can be attributed to NMDA receptors, we divided the full 30-second probe trials into three intervals of 10 seconds each. Our data from the young mice suggested that the first phase included the initial approach to the learned platform position, the second phase may have reflected persistence and the third phase may have indicated a loss of perseverance in young mice. The data on platform crossings indicated that they did still enter the platform location during the third interval, suggesting that the learning index score in this interval may reflect a widening of the search area, as opposed to abandoning the search in that area entirely.

Relationships between different ages in learning index scores for the first 10-second interval of the probe trials were similar to the full probe trial showing higher scores in the old mice than the younger ones. Twenty-six month old mice in the first 10-second interval spent more time away from the platform compared to the young mice, suggesting that there was more difficulty remembering the platform location for the old than the young. The old mice did spend significantly more time near the platform location during the second 10-second

interval, as compared to the first 10-second interval. This could indicate some persistence of memory in the second interval or may be related to the slower swim speed impacting the first interval more than the second. The young and middle-aged mice showed a loss in persistence by changing the strategy to search further away from the platform, as evidenced by a significant increase in learning index scores in the third 10-second interval as compared to the second 10-second interval. Dean and coworkers have described a deficit in memory ability of aged mice and attributed it to increased perseveration in old mice (Dean et al., 1981). Prefrontal cortex has been identified as a brain region responsible for increases in perseveration in humans, primates and other rodents (Clarke et al., 2004; Hampshire et al., 2008; Head et al., 2009; Schwabe et al., 2004). Although the old mice did not show a significant worsening of performance in the third versus the second 10-second interval, there was not strong evidence of performance perseveration in the aged mice as a group in this study.

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

4.2 Age-related changes in GluN1 subunit protein expression

The whole population of GluN1 subunits in the present study did not show any change in protein expression pattern with age or behavioral testing experience. Protein expression of the GluN1 subunit in some of our other studies also showed no effects of the aging process (Ontl et al., 2004; Zhao et al., 2009). Major differences between the current study and other studies showing changes in GluN1 subunit expression with age (Magnusson et al., 2002, 2007) are the ages of the oldest mouse group (30 mo. in Magnusson et al. (2002) vs. 26 months in the present study) and use of different methods for normalization for loading in the protein gels (μg protein loaded in Magnusson et al. (2007) vs. division by GAPDH within the same lane in the current study). The N-terminal cassette, N1, and C-terminal cassette, C1, also did not show any change in protein expression with age in the current study, although they showed trends for declines across ages. There was, however, evidence that some old mice maintained C1 cassette expression, while others showed decreased expression, indicating a heterogeneous effect of aging between individuals with respect to this splice cassette.

GluN1 splice variants containing C-terminal cassettes C2 or C2′ in the current study showed declines with increasing age across the two behavioral groups. A similar decline in protein expression of NR1 subunits containing C2 cassette, but not the C2′ cassette, was observed in hippocampus of 24 month old rats as compared to the 6 month olds (Clayton and Browning, 2001). The C-terminal tail of the GluN1 subunit of the NMDA receptor has been suggested to be important for several functions including retention and export from the endoplasmic reticulum (Mu et al., 2003; Scott et al., 2001; Standley et al., 2000), assembly and trafficking (Mu et al., 2003; Standley et al., 2000), long-term stabilization of synapses and spines (Alvarez et al., 2007) and phosphorylation of the subunit by PKA and PKC (Durand

et al., 1992; Tingley et al., 1997). The C2′ cassette contains a signal responsible for export of the GluN1 subunit from the endoplasmic reticulum (Mu et al., 2003; Standley et al., 2000). Maintenance of normal spine density and stabilization of synapses in rat pyramidal neurons depends on the expression of C2 cassettes (Alvarez et al., 2007). Potentiation due to phosphorylation of GluN1 subunits of NMDA receptors depends on the presence or absence of C1 (Tingley et al., 1997) or C2 cassettes (Durand et al., 1992), respectively. Clustering of receptors depends on the interaction of proteins yotiao and neurofilament L with C1 cassette (Ehlers et al., 1998; Lin et al., 1998). Splicing out of the C2 cassette results in seven-fold increase of potentiation of NMDA receptors by PKC (Durand et al., 1992). Thus, reduction in expression of the GluN1 subunits containing the C2 cassette may result in altered phosphorylation state of the receptor due to the loss of phosphorylation sites. It may also result in instability of the synapses dominated by the NMDA receptor in the prefrontal/frontal cortex. These studies suggest that the C-terminal tail of the NMDA receptor subunits, specifically expression levels of the C2 and C2′ cassettes, are important regulators of the GluN1 subunit of the NMDA receptor. Changes in expression of these cassettes during aging could alter plasticity and morphology of neurons.

Since the whole population of GluN1 subunits did not show any change in expression with age and the presence of C2′ cassette on a GluN1 subunit excludes the presence of C2 cassette and vice versa, an opposite trend in expression was expected between the GluN1 subunits expressing C2 and C2′ cassettes. However, both C2 and C2′ cassettes exhibited reduced expression with increasing age. One possible explanation for this could be a change or damage to the C-terminal cassettes in the aged brain that does not result in removal of the whole subunit protein.

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

4.3 Relationships between memory and GluN1 subunit protein expression

Significant relationships were found between GluN1 subunit expression and performance within intervals of probe trials for reference memory, but there were no significant relationships between either performance in a working memory task or in full probe trials for reference memory and protein expression of the GluN1 subunit splice cassettes or all GluN1 subunits in any age groups of mice. When individual 10-second probe trial intervals were analyzed, higher expression of all GluN1 subunits and GluN1 subunits containing C2′ cassettes ($GluN1_{X10}$ and $GluN1_{X00}$) were observed to be associated with closer proximity to the former platform location in the first interval of the probe trials in the middle aged animals. This relationship between specific GluN1 subunit proteins and memory performance in middle-aged animals was not maintained in the other age groups. This

suggests that the GluN1 subunit in the prefrontal/frontal cortex played a role in enhancing accuracy of the initial approach to the platform. This role appeared to be most important in the middle-aged mice. These correlational results suggest that the NMDA receptors in aged individuals did not function the same as in the middle-aged.

Aged mice were the only age group that showed a significant relationship in the final interval. In examining the significant correlations in the final interval alone, it appeared that the old mice with high protein expression of GluN1 subunits as a whole and those with the C1 cassette in the prefrontal/frontal cortex showed more perseverance than those with lower expression levels. However, examination of the aged mice within all three intervals showed that the mice with higher expression of all GluN1 subunit or C1 cassette proteins were the worst performers within the second interval, the interval in which the aged mice as a group performed the best. This suggested that high expression of GluN1 subunits or C1 cassettes may have been associated with a delay in applying an accurate search strategy. However, there was no significant difference in time taken to first reach the platform among the aged animals with High or Low C1 cassette expression, suggesting that the initial approach to the platform was not affected by C1 cassette expression. The aged mice with lower expression levels of the C1 cassette appeared to show a loss of perseveration between the second and third interval, similar to younger mice, but did not significantly decrease the number of platform crossings in the third interval. This appeared to be due to a widening of the search pattern, but included returns to the platform location. Interestingly, although they searched closer to the platform during the third interval of the probe trials than the Low C1 group, aged mice with higher expression of the C1 cassette went through the platform location less number of times on average during the last 10 seconds of the probe trial as compared to the first 20 seconds. It appeared that they remained slightly closer to the correct area than during the second interval, but did not accurately identify the exact location. This suggests that the GluN1 subunit, particularly subunits containing the C1 cassette, within the prefrontal/frontal cortex of older individuals may contribute to a less accurate search strategy when the platform is absent. It also suggests that splice variants $GluN1_{X01}$ and $GluN1_{X00}$, both of which lack the C1 cassette, may be more conducive to maintaining a young phenotype with respect to loss of perseveration.

There is other evidence of a negative relationship between NMDA receptors and memory in aged animals (Magnusson et al., 2010). Within the group of aged C57BL/6 mice, higher expression of the GluN1 subunit within the synaptic membrane of the hippocampus was found in the mice with the worst spatial reference memory (Zhao et al, 2009). High densities of NMDA receptor binding within old rats in regions of the hippocampus have also been shown to be associated with poor long-term memory retention in the water maze (Topic et al., 2007) and a non-spatial complex maze task (Ingram et al., 1992) and in the striatum are related to poor set shifting (Nicolle and Baxter, 2003) and poor spatial learning (Nicolle et al., 1996). Aged rats that were unimpaired in a spatial memory task showed greater age-related declines in MK801 binding in the cortex and hippocampus than those that were impaired (Le Jeune et al., 1996). These studies suggest that NMDA receptors within the aged brain may not function the same as they did in younger mice. The heterogeneity that is seen in the effects of aging on different NMDA receptor subunits (Magnusson 2000, Das and Magnusson, 2008) may contribute to this by changing the subunit compositions of the remaining receptors from that seen in younger animals.

In conclusion, this study provides evidence for heterogeneity in the effects of aging on expression of the GluN1 subunits with different splice cassettes, both across aging and within the group of aged mice. Expression of C-terminal cassettes C2 and C2′ were reduced with increasing age, but were not affected by behavioral testing experience. Middle-aged mice benefited more from the GluN1 subunit for good reference memory than both younger

and older mice. The GluN1 subunits of the NMDA receptors in the prefrontal/frontal cortex of old mice appeared to be important for performance in the final phase of searching in a spatial reference memory task. However, lower expression of the C1 cassette was associated with a more youthful search strategy, while higher expression may have contributed to a less accurate search. These results provide evidence for a change in the importance or role of GluN1 subunits of the NMDA receptors in memory across aging.

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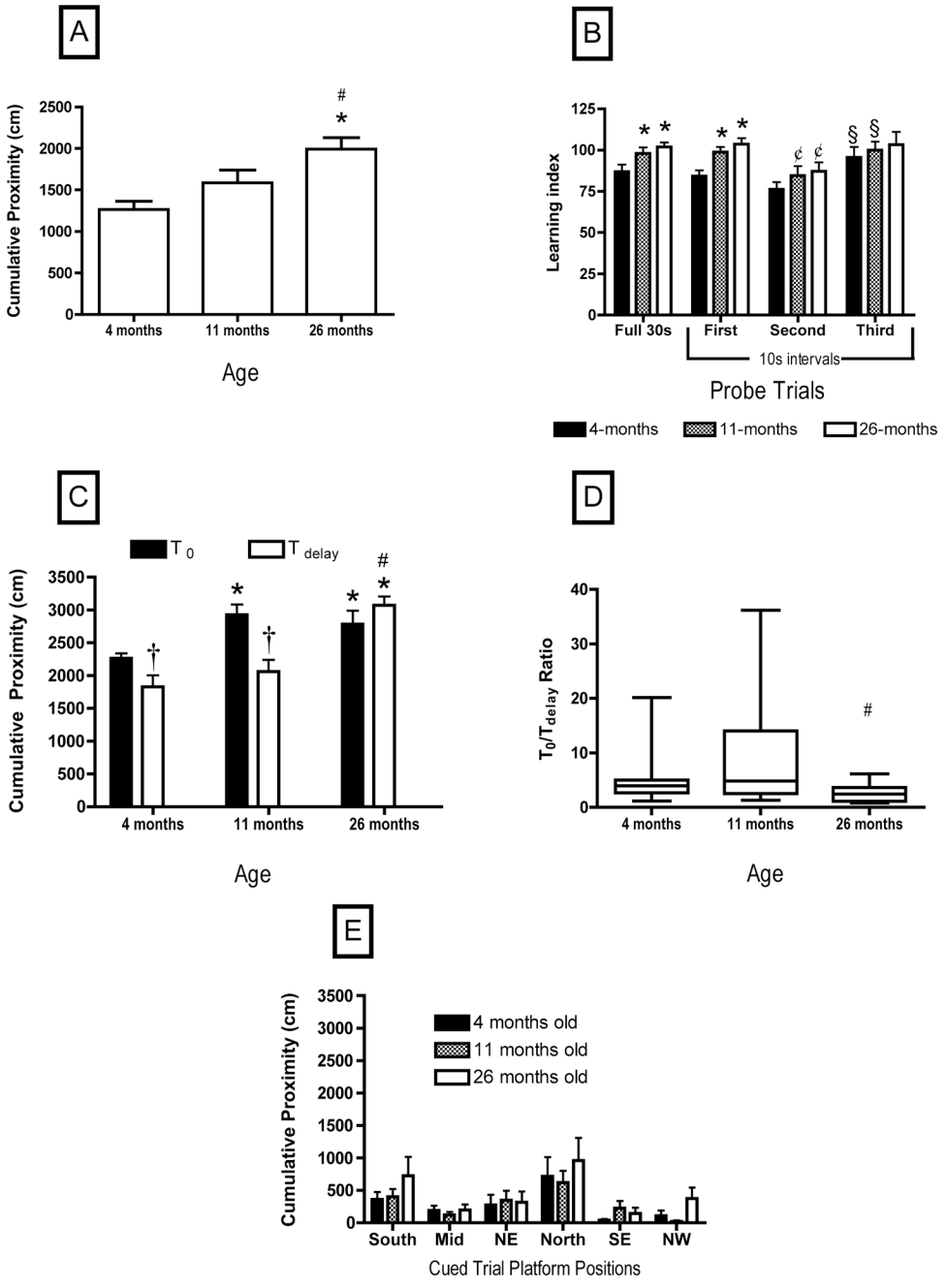

Fig. 1.

Memory performance of mice in different behavioral tasks. Graphs showing cumulative proximity scores of mice averaged across 12 days of place trials (A) and learning index scores calculated from the full 30s and three 10s intervals of probe trials (B) for the reference memory tasks; cumulative proximity scores for naïve (T_0) and delayed (T_{delay}) trials (C) and the ratio T_0/T_{delay} (D) averaged across all working memory sessions, and cued control trials at the 6 different platform positions (E) for the 4, 11 and 26 month old mice. A lower cumulative proximity score (A, C, E) or learning index value (B) indicated more time spent closer to the platform location. * $p < .05$ for differences from 4-month old animals for the same trials, # $p < .05$ for differences from 11-month old animals for the same trial(s), ¢ p

< .05 for difference from the first interval of probe trial in the same age group, § $p < .05$ for difference from the second interval of probe trial in the same age group, † $p < .05$ for differences from overall performance in naïve trials (T_0) within same age group. A-C, E) Graph bars = mean, error bars = SEM. Statistical analysis performed with ANOVA and Fisher's protected LSD post-hoc tests.

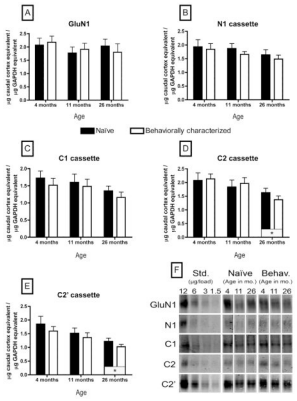

Fig. 2.

Protein expression of all NMDA receptor GluN1 subunits and the GluN1 subunits containing the N or C-terminal cassettes. A-E) Graphs showing protein expression of all GluN1 splice variants together (A) and GluN1 subunits containing N-terminal cassette N1 (B) or C-terminal cassettes C1 (C), C2 (D) or C2′ (E) in prefrontal/frontal cortical regions of mouse brain. (F) Representative images of the blots used for quantification of the above-mentioned cassettes and all GluN1 subunits. GAPDH was used as a loading control and data were normalized with the amount of GAPDH expressed in each lane. * $p < .05$ for difference in expression of protein from 4-months old animals when data were collapsed across behavioral experience groups. Graph bars = mean, error bars = SEM. Statistical analysis performed with ANOVA and Fisher's protected LSD post-hoc tests.

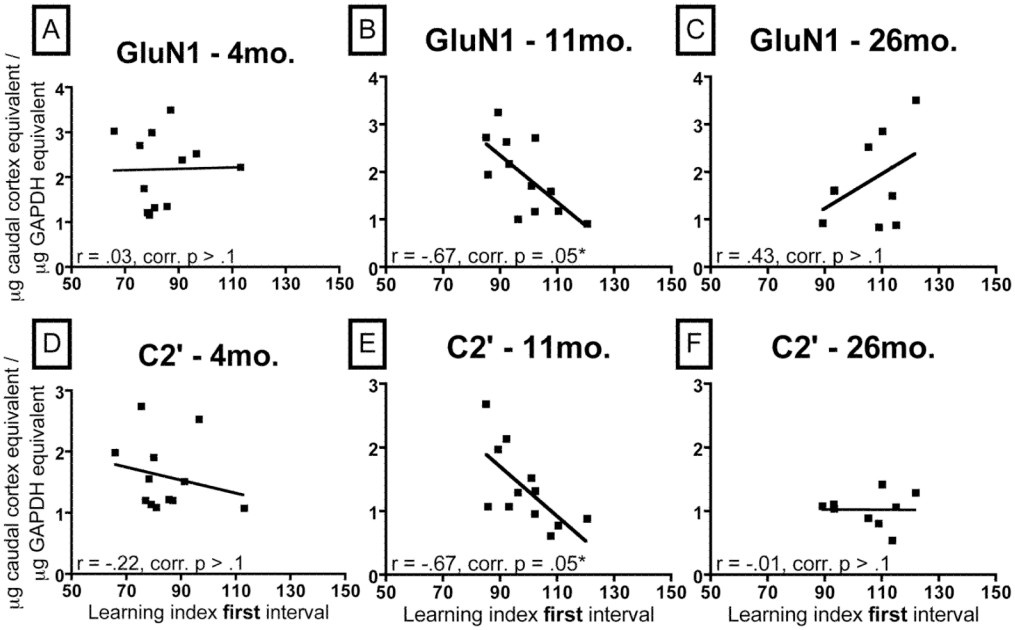

Fig. 3.

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

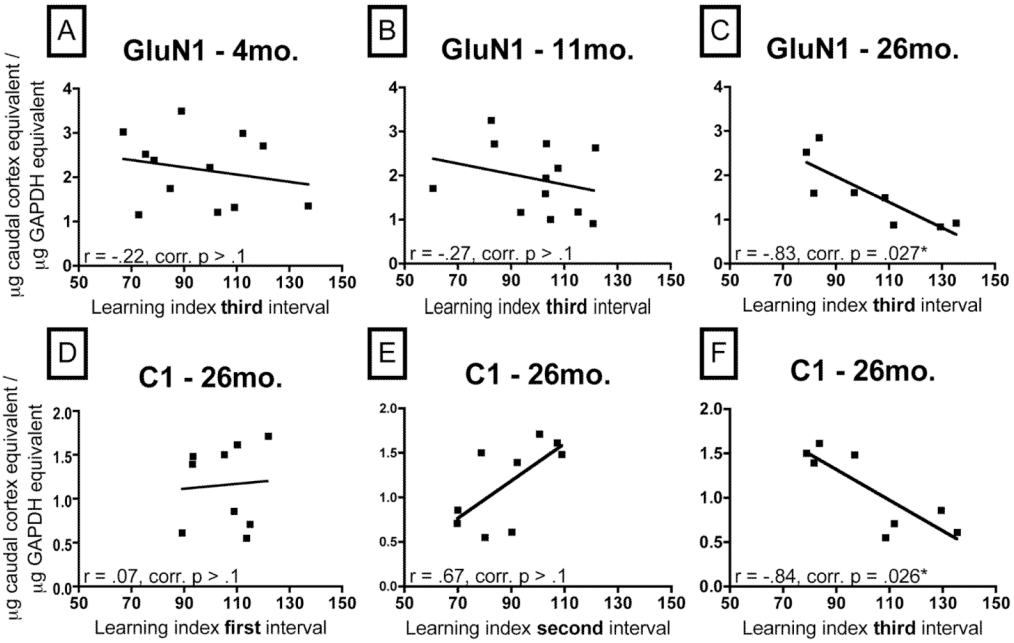

Fig. 4.

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

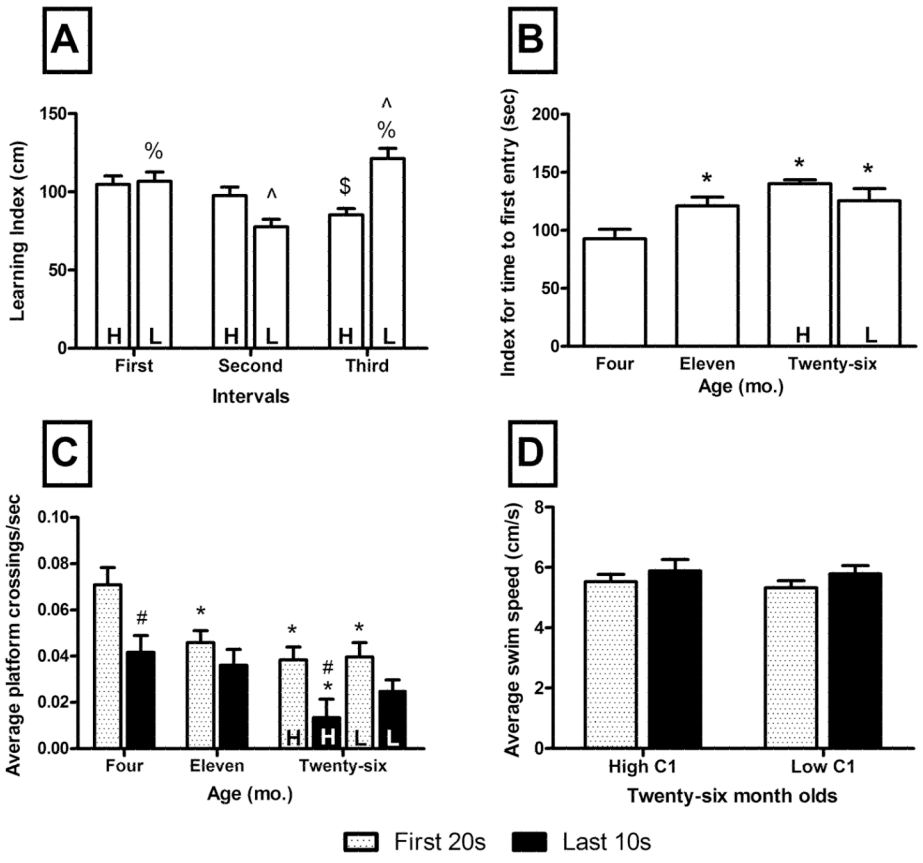

Fig. 5.

Relationship between high and low C1 cassette expression in aged mice and performance during the probe trials for reference memory. A) Graph showing learning index scores for aged animals with high (H) and low (L) C1 cassette/GAPDH expression during the three different 10-second intervals for probe trials of reference memory. B) Graph showing time taken to reach the former platform location for the first time, indexed across the probe trials, for the three age groups of animals. The 26 month olds were separated into High C1 (H) and Low C1 (L) groups. C) Graph showing the number of times/second that animals from the three age groups reached the platform location during the first 20 and last 10 seconds, averaged across the probe trials for reference memory. The 26 month olds were separated into High C1 (H) and Low C1 (L) groups. D) Swim speed of twenty-six month old animals divided into High C1 and Low C1 groups during the first 20 second and the last 10 second of the probe trials. H = twenty-six month old animals with high C1 cassette protein expression (C1 cassette/GAPDH), L = twenty-six month old animals with low C1 cassette protein expression. % = difference from animals in the second interval of the same C1 group. $ = difference from animals in the first interval of the same C1 group. ^ = difference from animals with low C1 expression in the same interval. * = difference from the four month olds in the same interval or full probe trials. # = difference from the average platform crossings/sec in the first 20 seconds in the same age or C1 group. mo. = months. Graph bars = mean, error bars = SEM.

Table 1

Swim speeds of mice of different age groups in different behavioral tasks.

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

mo., months of age,

*p < .05 for difference from the 4 month old animals.

†p < .05 for differences from the 11 month old animals.

Data indicate mean SEM.

Table 2

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

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

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