Published in final edited form as:

Brain Res. 2008 May 1; 1207: 142–154.

Relationship between mRNA expression of splice forms of the $\zeta 1$ subunit of the N-methyl-D-aspartate receptor and spatial memory in aged mice

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Abstract

Age-related changes in the protein and mRNA expression of some of the splice forms of the $\zeta 1$ (NR1) subunit of the NMDA receptor have been seen in mice and rats. The present study was designed to determine whether individual splice forms of the $\zeta 1$ subunit of the NMDA receptor within prefrontal/frontal cortical regions contribute to memory deficits during aging and whether experience in learning tasks can influence the expression of the splice forms. mRNA expression of 4 splice forms ($\zeta 1$ -1, $\zeta 1$ -3, $\zeta 1$ -a and $\zeta 1$ -b) and mRNA for all known splice forms ($\zeta 1$ -pan) were examined by *in situ* hybridization. mRNA for C-terminal splice forms, $\zeta 1$ -1 (+C1 and +C2 cassettes) and $\zeta 1$ -3 (+C1 and +C2'), showed significant declines during aging in several brain regions even though overall $\zeta 1$ -pan mRNA expression was not significantly affected by aging. This suggests that these splice forms are more influenced by aging than the subunit as a whole. There was an increase in the expression of $\zeta 1$ -a (–N1 cassette) splice form in the behaviorally-experienced old mice relative to the younger groups. Old mice with high levels of mRNA expression for the $\zeta 1$ -a splice form in orbital cortex showed the best performances in the working memory task, but the poorest performances in the cued, associative learning task. These results suggest that there is a complex interaction between $\zeta 1$ splice form expression and performance of memory tasks during aging.

Keywords

NMDA receptor; memory; aging; learning; splice variants; NR1

1. Introduction

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

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memory that is affected by increasing age and can be studied in animals such as rodents and primates who experience age associated memory impairments (Barnes, 1988; Gage et al., 1984; Rapp et al., 1987).

There is evidence that brain regions such as the prefrontal cortex and hippocampal formation are associated with spatial memory functions (Gallagher et al., 2003; Greenwood, 2000; Tisserand and Jolles, 2003). All of these structures show greater decline in their volume in individuals with declining memory capabilities than the individuals with stable memory over time (Persson et al., 2006). This decline in prefrontal cortex volume has been speculated to alter the functional connectivity of cortical circuits contributing to cognitive aging (O'Donnell et al., 1999). A type of glutamate receptor known as the N-methyl-D-aspartate (NMDA) receptor is abundant in these areas of the brain (Bockers et al., 1994; Scherzer et al., 1998). These receptors are important for long term potentiation, one proposed mechanism for the formation of memory by strengthening of synapses (Cotman et al., 1989; Lynch, 1998). Inhibition of this receptor by antagonists results in impaired learning and memory abilities in rodents (Alessandri et al., 1989; Mondadori et al., 1989; Morris et al., 1986). Among the glutamate receptors, NMDA receptors are more susceptible to effects of aging (Magnusson, 1997a; Magnusson, 1997b). It has been observed that NMDA receptors experience changes in gating behavior, magnesium block and response to transmitter with increasing age in rodents and primates (see review Magnusson, 1998b). In autoradiographic experiments [³H] glutamate binding to the receptor decreases with increased age, suggesting a reduction in receptor activity with increased age (Kito et al., 1990).

The NMDA receptor is composed of four or five protein subunits from three different families of proteins, the $\zeta 1$ (NR1), ζ (NR2) and NR3 family in the rodents. Four members in the ζ family, one member in the $\zeta 1$, with eight splice variants, and two members in NR3 families have been identified and cloned (Eriksson et al., 2002; Ikeda et al., 1992; Ishii et al., 1993; Kutsuwada et al., 1992; Matsuda et al., 2002; Meguro et al., 1992; Yamazaki et al., 1992). Expression studies in *Xenopus* oocytes indicate that the $\zeta 1$ subunit is sufficient for a functional channel (Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Yamazaki et al., 1992).

The $\zeta 1$ subunit contains three splicing sites, one in the N-terminal (N1 cassette) and the other two in the C-terminal (C1 and C2 cassettes) region (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Yamazaki et al., 1992). Since the C2 cassette contains the stop codon, in its absence, additional sequences become a part of the protein and is known as the C2' cassette (Zukin and Bennett, 1995). Depending on whether the N1, C1 or C2 cassettes are present or absent, they make eight different splice variants. The splice variants of NR1 ($\zeta 1$) present in the receptor complex determine a number of properties of the ion channel, such as affinity to agonist and antagonists, zinc modulation and spatio-temporal expression in the brain (Dingledine et al., 1999; Laurie and Seeburg, 1994; Prybylowski and Wolfe, 2000; Zhong et al., 1995). Due to these properties, differential effects of aging on the different splice variants could lead to important changes in the physiology and pharmacology of the NMDA receptor in the aged brain.

There is evidence that the $\zeta 1$ subunit of the NMDA receptor shows declines in mRNA and protein expression during the aging process in C57BL/6 mice (Magnusson, 2000; Magnusson et al., 2002), but some studies in the same strain of mice show no change in $\zeta 1$ during aging (Magnusson, 2001). One difference between these studies was the addition of a behavioral experience in the later study (Magnusson, 2001). The influence that behavioral testing may have on the $\zeta 1$ subunit needs to be further explored. Calorie restriction also leads to an upregulation of $\zeta 1$ subunits in middle aged and aged mice (Magnusson, 2001). This suggests that the expression of the $\zeta 1$ subunit during aging is variable and potentially susceptible to intervention.

It has been suggested that learning experience stabilizes synaptic modification and improves NMDA receptor expression (Quinlan et al., 2004; Sun et al., 2005). Because the $\zeta 1$ subunit mRNA shows variability during aging and could possibly be influenced by learning experience, we postulated that the overall expression of $\zeta 1$ subunits during aging is a function of differential changes in individual splice forms and its stabilization is due to learning experience influencing expression of the individual splice forms. In the present study, one aim was to determine if expression of individual splice forms were differentially affected by aging in prefrontal and/or frontal regions. The other aim was to determine roles of individual splice forms in learning ability and whether they were affected by experience in learning tasks. The focus was on the splice forms that were found previously to be most affected by aging, $\zeta 1$ -1 and $\zeta 1$ -3 (Magnusson et al., 2005), and on the $\zeta 1$ -a (-N1 cassette) and $\zeta 1$ -b (+N1 cassette), which had not been assessed yet in C57BL/6 mice across ages.

2. Results

2.1 Spatial reference memory

There was a significant main effect of age on performance in the reference memory place ($F_{(2, 30)} = 7.1$, $p = 0.003$; Fig. 1A) and probe trials ($F_{(2, 30)} = 4.5$, $p = 0.02$; Fig. 1B). Twenty-six month old mice had significantly higher cumulative proximity scores than both 11 and 4-month olds in place trials (Fig. 1A) and higher average proximity scores than the 4-month old mice in probe trials (Fig. 1B) when averaged over all the respective trials. There was a significantly lower cumulative proximity value for the 4-month old mice between place trials on day 1 and day 12 ($t_{(22)} = 6.74$, $p < 0.001$, Fig. 1A) and between probe trials 1 and 6 ($t_{(22)} = 2.57$, $p = 0.02$, Fig. 1B). Both the mid-aged and old mice also had significantly lower cumulative proximity values in place trial day 12 than place trial day 1 ($t_{(22)} = 6.66$, $p < 0.001$ and $t_{(22)} = 2.6$, $p = 0.02$ respectively, Fig. 1A) and in probe trial 6 than probe trial 1 ($t_{(22)} = 2.43$, $p = 0.02$ and $t_{(22)} = 4.07$, $p < 0.001$ respectively, Fig. 1B). A learning index was calculated from the overall performances in the probe trials as described by Gallagher and coworkers (Gallagher et al., 1993). The 26-month old mice had significantly higher learning index scores than the 4-month old mice ($F_{(1, 19)} = 9.4$, $p = 0.006$, Fig. 1C). The young (7.23 ± 0.28 cm/s) and mid-aged (6.71 ± 0.28 cm/s) mice were observed to have significantly faster swim speeds than the old mice (5.09 ± 0.14 cm/s) in the first day of training in the place trials ($p < 0.001$, not shown).

2.2 Spatial working memory

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

2.3 Cued control task

The animals analyzed above in the reference and working memory tasks showed no significant main effect of age, $F_{(2, 30)} = 1.5$, $p = 0.23$, on cumulative proximity scores in the cued control trials (Fig. 1F). Animals in all the age groups had lower cumulative proximity scores in their

cued trials than the place and working memory trials in all the platform positions except the north (Fig. 1). Swim speeds of the young (8.36 ± 0.42 cm/s) and mid-aged (7.92 ± 0.40 cm/s) animals across all the platform positions in cued tasks were significantly faster than the old (6.35 ± 0.36 cm/s) animals ($p < 0.001$, not shown).

2.4 mRNA expression of $\zeta 1$ splice forms

2.4.1 $\zeta 1$ -pan—There were no significant main effects of behavioral experience and no significant interactions between age and behavior on $\zeta 1$ -pan mRNA expression in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. A representative film image of hybridization to $\zeta 1$ -pan mRNA in a young mouse is shown in Fig. 2A. In the deep layers of lateral orbital cortex, there was a significant increase of mRNA expression from 4 to 11-months of age ($p = 0.02$; Fig. 3A). No other individual regions exhibited effects of age or experience (examples in Fig. 3A).

2.4.2 $\zeta 1$ -1 splice form—Expression patterns of $\zeta 1$ -1 splice form mRNA showed no significant main effects of behavioral experience and no significant interactions between age and behavior in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. A representative film image of hybridization to $\zeta 1$ -1 mRNA in a young mouse is shown in Fig. 2B. Analysis of individual brain regions showed some effects of aging on the mRNA expression of $\zeta 1$ -1 splice form. There was a significant reduction of mRNA expression from 4 to 11-months of age ($p = 0.04$) and from 4 to 26-months of age ($p = 0.004$) in superficial layers of insular cortex (Fig. 3B). Deep layers of insular cortex ($p = 0.04$) and lateral orbital cortex ($p = 0.03$) showed a significant decrease in mRNA expression between 4 and 26-month old animals (Fig. 3B). There was a significant reduction of mRNA expression between 4 (262 ± 12 pmol labeled $^{33}\text{P}/\text{mm}^2$ tissue) and 26-months (221 ± 15 pmol labeled $^{33}\text{P}/\text{mm}^2$ tissue) of age in superficial somatosensory cortex ($p = 0.03$). No other individual regions exhibited effects of age or experience (not shown).

2.4.3 $\zeta 1$ -3 splice form—Analysis of individual brain regions indicated some significant effects of behavioral experience on the expression of $\zeta 1$ -3 splice forms, so the behavioral groups were analyzed separately (Table 1). It was observed that there was a significantly higher expression of $\zeta 1$ -3 mRNA ($p = 0.04$) in 11-month old behaviorally-characterized animals than the 11-month old naïve animals in superficial layers of ventral orbital cortex (Table 1). Effects of age on $\zeta 1$ -3 mRNA expression were not observed in the naïve animals, but were observed in the behaviorally-characterized animals in some of the brain regions (Table 1, Fig. 2C & D). A significant reduction in mRNA expression of $\zeta 1$ -3 was observed in 11 and 26-month old animals as compared to the 4-month old behaviorally-characterized animals ($p = 0.01$ for both) in the deep layers of insular cortex (Table 1). In the superficial layers of insular cortex, a significantly lower mRNA expression in 11-month old animals than the 4-month old behaviorally characterized animals was observed ($p = 0.03$). A difference in mRNA expression between 4 and 26-month old behaviorally-characterized animals was observed in deep layers of medial prefrontal ($p = 0.04$), primary motor ($p = 0.01$) and somatosensory cortices ($p = 0.02$) and between 11 and 26-month old animals in superficial layers of medial prefrontal cortex ($p = 0.02$).

2.4.4 $\zeta 1$ -a splice form—Analysis of individual brain regions indicated significant or near-significant main effects of experience, so the two behavioral groups were analyzed separately (Table 2). A significant increase in mRNA expression was observed in 26-month old behaviorally characterized as compared to naïve animals in deep ($p = 0.02$) and superficial ($p = 0.03$) layers of insular cortex (Table 2). No significant differences in mRNA expression between the three age groups were observed in the naïve animals but differences were found in the behaviorally characterized animals across age groups (Table 2, Fig. 2E, F). Significantly

higher expressions of mRNA in 26-month old than the 4-month old animals were observed in deep layers of medial prefrontal ($p < 0.001$), insular ($p = 0.003$), secondary motor ($p = 0.003$), primary motor ($p = 0.01$) and somatosensory cortices ($p = 0.03$) and superficial layers of ventral orbital ($p = 0.03$), medial prefrontal ($p = 0.003$) and somatosensory cortices ($p = 0.04$). Significant increases in expression of mRNA between 11 and 26-month old animals were observed in deep layers of insular ($p < 0.001$), lateral orbital ($p = 0.03$), medial prefrontal ($p = 0.008$), secondary motor ($p = 0.001$), primary motor ($p = 0.005$) and somatosensory cortices ($p = 0.005$) and superficial layers of insular ($p = 0.02$), ventral orbital ($p = 0.02$), lateral orbital ($p = 0.04$) and somatosensory cortices ($p = 0.001$).

2.4.5 $\zeta 1$ -b splice form—There were no significant main effects of behavioral experience and no interactions between age and behavior on $\zeta 1$ -b mRNA expression in any of the brain regions analyzed, so data within age groups was collapsed across naïve and behavioral treatment groups. In the individual brain regions, there was a significant reduction of mRNA expression from 4 (246 ± 12 pmol labeled $^{33}\text{P}/\text{mm}^2$ tissue) to 11-months (204 ± 11 pmol labeled $^{33}\text{P}/\text{mm}^2$ tissue) of age ($p = 0.02$) in superficial layers of insular cortex when collapsed across experience groups. There were no other regions that showed significant difference between age groups (not shown).

2.5 Correlations

We performed correlations between mRNA densities in the insular and orbital regions and learning index for reference memory and T_0/T_{delay} ratio for working memory using individual old animals alone in order to determine if the reference and working memory performance correlated with the mRNA densities for $\zeta 1$ splice forms in different brain regions.

2.5.1 Reference memory—Learning index scores as a measure of reference memory performance, did not correlate significantly with the mRNA expression of the whole $\zeta 1$ -pan or the individual splice forms in the old animals at a significance level of .05 (not shown). We observed a trend for a negative correlation ($r = -.76$, corrected $p = .08$) between $\zeta 1$ -a mRNA densities in superficial layers of lateral orbital cortex and learning index scores (Fig. 4A). Higher densities were associated with better performances in the reference memory task.

2.5.2 Working memory—The T_0/T_{delay} ratios, which represents working memory performance, were significantly positively correlated with mRNA expression of $\zeta 1$ -a splice variant in the deep layers of ventral orbital cortex ($r = .82$, corrected $p = .04$; Fig. 4B). Higher T_0/T_{delay} values indicate better working memory performance; so positive correlations indicated high $\zeta 1$ -a mRNA densities associated with better performance. No other significant correlations were found (not shown).

2.5.3 Cued control task—We performed correlations of cued trial performance with mRNA expression of different splice forms. The results indicated positive correlations for only $\zeta 1$ -a mRNA expression with cued trial performance in deep and superficial layers of ventral orbital cortex and lateral orbital cortex (Fig. 4C, r range = .93 to .86, corrected p range = .003–.01). A correlation of T_0/T_{delay} ratio with cued trials indicated a positive relationship between measurements (Fig. 4D). However, poor performance (a higher score) in cued trial performance in this case was associated with better performance (higher score) in working memory tasks and vice versa.

3. Discussion

This study provides new evidence for a differential effect of behavioral experience on the expression of different N- and C-terminal splice forms of the $\zeta 1$ subunit during aging and

evidence for a role of the ζ 1-a splice form within orbitofrontal brain regions in working memory and associative memory performances in aged mice. The oldest mice showed significant deficits in both spatial reference and spatial working memory ability as compared to the young and middle-aged mice. There was little effect of aging on expression of mRNA that is found in all known splice variants, but mRNA for two of the C-terminal splice forms, ζ 1-1 and ζ 1-3, showed significant declines during aging in several brain regions. Behavioral experience was associated with an up-regulation of the ζ 1-a mRNA in prefrontal and frontal cortex regions in old mice. Within the old mice high ζ 1-a was associated with good working memory performance, but poor associative memory performance.

3.1 Reference and Working Memory Performance

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

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

Swim speed differences were observed between different ages of mice in each of the water maze tasks used. Therefore, the traditional measure of latency was not used. Path length was also not considered a good option with these mice, because older ones tend to float when initially placed in the tank (unpublished observation). Cumulative and average proximity measures used in this study were corrected for ideal path cumulative proximity by using individual swim speed for that trial. This measure thus is less affected by swim speed than latency and more reflective of the bias for the platform than path length (Gallagher et al., 1993). With these measures, we observed similar age related changes in both place and probe trials. The probe trial measurements were resistant to the differences in swim speed because the trial time was same for all probe trials. The use of the ratio for working memory also reduced swim speed influences by being a within individual measurement. In addition, despite differences in swim speed, the cued control task showed no aging differences.

In the working memory tasks, we detected a decline in performance in older animals as compared to the young in both the naïve and delayed working memory trials. Better performance of the young, compared to older mice in the naïve trials suggests that they learned something about the platform location or had a better search strategy than the older mice. Lack of improvement in the delayed working memory trials was observed only in the case of 26-month old mice. Similar results were obtained in our previous experiment with C57BL/6 mice where middle-aged mice performed well in the delayed working memory trials but the old mice were impaired (Magnusson et al., 2003). To study working memory in rats using the water maze, several investigators have considered using multiple trials within one session to represent working memory (Galea et al., 2000; Kikusui et al., 1999; Lehmann et al., 2000), whereas several others have used only the first trial after a naïve swim (Frick et al., 1995; Morris et al.,

1986; Steele and Morris, 1999). Frick and coworkers have shown that out of the four trials in working memory tasks, trials 1 and 2 were associated with working memory and trials 4 and 5 were more associated with reference memory (Frick et al., 1995). In our previous experiment we used trial 4 as the delayed trial, but it was not clear if the mice were using working memory for that trial (Magnusson et al., 2003). In the present study we used the second trial (next trial after naïve swim) as the delayed trial (10 minutes delay after naïve swim) and found similar results in both middle-aged and old mice.

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

3.2 mRNA Expression of $\zeta 1$ splice forms

There have been different results seen for the expression pattern of $\zeta 1$ -pan in the frontal cortex region. Our lab has observed significant effects of aging on $\zeta 1$ -pan protein expression (Magnusson et al., 2002) and mRNA in frontal cortex (Magnusson, 2000; Magnusson et al., 2005), yet in another study, there was no effect of aging on the $\zeta 1$ -pan mRNA expression (Magnusson, 2001). This suggests that some factor(s) produce variability in the effects of aging on $\zeta 1$ -pan. $\zeta 1$ -pan mRNA expression in the present study only showed declines between young and middle-aged mice in one region, lateral orbital cortex. There were significant reductions of $\zeta 1$ -1 mRNA with increased age in insular, lateral orbital and superficial somatosensory regions. In the present study, $\zeta 1$ -3 mRNA was affected by aging in insular, medial prefrontal and primary motor areas, but only in the behaviorally-experienced animals. In our previous study, $\zeta 1$ -1 and $\zeta 1$ -3 were affected by aging when $\zeta 1$ -pan showed significant declines with aging (Magnusson et al., 2005). The present results suggest that $\zeta 1$ -1 and $\zeta 1$ -3 mRNA expression were affected before there were significant signs of aging overall in $\zeta 1$ -pan mRNA expression. In addition, they showed that behavioral experience was associated with a significant age-related decline in $\zeta 1$ -3 mRNA expression that wasn't seen in the naïve mice.

The $\zeta 1$ -1 and $\zeta 1$ -3 splice forms both contain the C1 cassette, which is abundant during development, present at about 50% of the total $\zeta 1$ subunit, and present in slightly higher amounts in adult stages, at about 58% in the rat cortex (Prybylowski and Wolfe, 2000). This cassette is important for NMDA receptor functioning as it contains phosphorylation sites, two for PKC and one for PKA activity (Tingley et al., 1997). In culture, C1 cassettes target NR1 subunits to the receptor rich domains (Ehlers et al., 1995; Ehlers et al., 1998). The cassette appears to interact with two proteins, γ -tubulin and neurofilament L, which contribute to clustering of receptors (Ehlers et al., 1998; Lin et al., 1998). These studies (Ehlers et al., 1995; Ehlers et al., 1998; Lin et al., 1998; Tingley et al., 1997) suggest that decreases in C1, as observed in aged mice, could lead to decreased localization of NMDA receptors in receptor clusters and less potentiation of receptor function due to decreased phosphorylation. Our preliminary results on protein expression of different cassettes from these same animals indicated that decline in both C1 and C2 cassette expressions occurred during aging (unpublished observation).

Expression of $\zeta 1$ -a mRNA in the naïve animals was not affected by the process of aging. In the behaviorally experienced animals however, $\zeta 1$ -a mRNA expression was significantly increased in aged animals in most of the brain regions analyzed. The $\zeta 1$ -a splice form doesn't contain the insertion cassette N1. Splicing out of the N1 insertion cassette has been shown to reduce affinity for agonist by almost five fold (Durand et al., 1993) and decrease the current amplitude in oocytes expressing NR1 subunits (Hollmann et al., 1993; Zheng et al., 1994).

Presence of N1 insert has also been found to attenuate stimulation by polyamines, proton inhibition and zinc modulation (Durand et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Zheng et al., 1994). In rat brain, ζ 1-a splice forms have been found to be the most abundant form of splice variants and are expressed all throughout the brain (Laurie and Seeburg, 1994; Laurie et al., 1995). A higher expression of ζ 1-a mRNA in the 26-month old animals indicated that there was a higher amount of receptor protein without the N1 insert. This might lead to a significantly lower response of the NMDA receptor population to the transmitter glutamate but a higher response to other modulators (Durand et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Zheng et al., 1994). ζ 1-b mRNA expression showed no significant effects of aging. It is not clear why there wasn't a corresponding decrease in the ζ 1-b splice form containing the N1 cassette.

3.3 Correlations

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

No significant correlations between mRNA expression of any of the splice forms and learning index (measure of reference memory performance) were observed in any of the brain regions analyzed. A near-significant relationship however, was observed between high ζ 1-a mRNA density in lateral orbital cortex and good performance in the spatial reference memory task. Reference memory, involving recall of stored information, has been found to be associated with orbital frontal cortex (Nyberg et al., 1995; Paradiso et al., 1997).

Higher expression of the ζ 1-a splice form in ventral orbital cortex was associated with better working memory performance. This finding fits with human studies in which higher activity in the orbital region was observed in older individuals performing working memory tasks in a functional MRI study (Cook et al., 2007). Since there was an association of poor performance in cued trials with good performance in working memory tasks, the correlation of cued trials and mRNA expression of ζ 1-a in prefrontal orbital cortex was not unexpected. Dependency of associative learning on orbital frontal cortex has been shown by an inability of rats to access cues with orbitofrontal cortex damage (Gallagher et al., 1999). It is, however, not clear why working memory and associative learning in the cued task should be negatively correlated.

In summary, there were heterogeneous temporal and regional effects of aging on the different splice forms of the NMDA receptor complex. Aging of the ζ 1-pan subunit of the NMDA receptor seemed to be contributed by the combinatorial effects of aging on ζ 1-a, ζ 1-1 and ζ 1-3 splice forms. Up-regulation of ζ 1-a splice form message, influenced by behavioral experience, seemed to have benefited working memory ability in aged animals. Associative learning was negatively affected by the same increases. Overall these results indicate that ζ 1 pan mRNA expression is regulated by a combination of complex changes in the different splice forms.

4. Experimental Procedure

4.1 Animals

A total of 72 C57BL/6 mice (National Institute on Aging, NIH) from three different age groups (four, ten and twenty-six months of age) were used for the study. They were fed *ad libitum* and housed in cages under 12hr light and 12hr dark cycle. The animals were divided into two behavioral groups; naïve and behaviorally-characterized, containing twelve animals from each age group. Animals in the behaviorally-characterized group were subjected to learning

experience with the use of the Morris water maze as discussed below. The animals in the naïve group were housed for the same amount of time as the behaviorally-characterized animals. After the behavioral testing, all animals were euthanized with exposure to CO₂ and decapitated. The brains were then harvested, frozen rapidly with dry ice and stored at -80 °C until further processing.

4.2 Behavioral Testing

Spatial reference and working memory and cued control task ability were tested using the Morris Water Maze. A 4-foot diameter metal tank was covered with white contact paper and filled with water that was made opaque white with non-toxic paint. A platform was placed 1 cm below water level. Spatial cues consisted of figures of geometric shape and other items such as toys and pieces of cloth. The cues were placed high on walls of both the room and the tank. There were seven different platform positions located at five different distances from the tank wall. Trials were video taped using a camera placed above the center of the tank on the ceiling of the room. Paths of the trials were analyzed by using the “SMART” video tracking system (San Diego Instruments, San Diego, CA). There were different entry points for each trial and the mice were placed in the tank facing the wall.

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

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

4.2.3 Spatial working memory—On Days 15 through 22, mice were tested in a spatial working memory task (Magnusson et al., 2003). The task consisted of two sessions per day for 8 days. There was a two-day break between sessions eight and nine. The platform positions were changed between each session. Each session consisted of 4 trials. The naïve trial started by placing a mouse into an entry point facing the tank and allowing it to search the platform for a maximum of 60 s (T_0) after which the mouse was allowed to remain on the platform for 30 s (first test trial) followed by cage rest for 10 minutes (delay period). In the second trial it was placed in the water at a different entry point from the naïve trial and allowed to search the platform for a maximum of 60 s (T_{delay}). The mouse was again allowed to stay on the platform for 30 s and allowed to rest in the cage for 60 s. The mouse was placed into the water 2 more times at 2 different entry points and allowed to find the platform for 60 s (T_2 and T_3). They spent 30 s on the platform and rested in the cage for 60 s between trials. They were then placed into their cages until the next session. If the mouse failed to find the platform within the designated 60 s for any of the trials, it was led to the platform by the experimenter. The entry points within one session were randomly assigned for each trial, with no entry points repeated within a session. Working memory was assessed between T_0 and T_{delay} . The extra sessions were performed based on previous findings that mice need additional trials to show improvement between trials (Magnusson et al., 2003).

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

4.3 Brain Sectioning

The frozen brain from each animal was cut mid-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 before hand. Coronal brain sections of 12 μ m thickness, representing animals from each age and behavioral group were placed on each slide and kept frozen at -80°C until used. Slides were divided into 12 cutting groups, each group containing animals from each age and behavioral group. Position of animal brain sections on slides were changed randomly between each cutting groups to control for the variability while washing during *in situ* hybridization

4.4 In situ hybridization

Oligonucleotides used for the *in situ* hybridization were commercially prepared (Macromolecular Resources, Colorado State University, Fort Collins, CO). The sequences used were: ζ 1-a, AACTGCAGCACCTTCTCTGCCTTGGACTCCCGTTCCTCCA; ζ 1-b, GCGCTTGTTGTCATAGGACAGTTGGTCGAGGTTTTCATAG; ζ 1-1, TCCACCCCGGTGCTCGTGTCTTTGGAGGACCTACGTCTC; ζ 1-3, GATATCAGTGGGATGGTACTGCGTGTCTTTGGAGGACCTA; and ζ 1-pan, GCACAGCGGGCCTGGTTCTGGGTTGCGCGAGCGCGACCACCTCGC (Laurie and Seeburg, 1994).

Oligonucleotides were labeled with ^{33}P -dATP (Perkin Elmer, Waltham, MA) of specific activity: 3103 to 3238 Ci/mM using terminal deoxyribonucleotidyl transferase (Invitrogen Corp., Carlsbad, CA) and purified in Microspin G-25 columns (Amersham Bioscience, Piscataway, NJ). The specific activities for the labeled oligonucleotides were calculated to be 40 to 140 dpm/fmol of ζ 1-a probe, 85 to 139 dpm/fmol of ζ 1-b probe, 87 to 155 dpm/fmol of ζ 1-1 probe; 81 to 140 dpm/fmol of ζ 1-3 probe, and 105 to 130 dpm/fmol of ζ 1-pan probe, depending on the labeling experiment.

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

addition of 50-fold excess non-radiolabelled oligonucleotide to the hybridization solution on some slides. Slides were exposed to Kodak Biomax films for 3–8 days depending on the splice form along with slide containing ^{14}C standards. Brain and standard images were captured using a Macintosh G4 computer with a Powerlook 2100 XL scanner (UMAX, Taiwan) and NIH Image software. Quantitative densitometry was performed on the images from four sections for total hybridization and two sections for nonspecific hybridization from each animal with the use of NIH Image software. The different prefrontal and frontal cortex brain regions analyzed for mRNA expression were deep (cortical layers IV–VI) and superficial (cortical layers II–III) layers of ventral orbital cortex, lateral orbital cortex, medial prefrontal cortex (areas containing cingulate cortex, infralimbic cortex and prelimbic cortex), insular cortex (areas containing both granular and agranular insular cortex), secondary motor cortex, primary motor cortex and the somatosensory cortex (areas containing both primary and secondary somatosensory cortex). The sections ranged from 0.38 to 1.94 mm rostral to bregma (Paxinos and Franklin, 2001). Specific signal was determined by subtracting nonspecific hybridization from total hybridization. Nonspecific hybridization of ^{33}P labeled with mRNAs of different splice forms ranged from 16 to 53% of total hybridization. The ^{14}C standards were used to convert optical density to fmol of labeled ^{33}P -dATP/mm² tissue (Eakin et al., 1994).

4.5 Data analysis

Data for behavioral testing were analyzed as described earlier (Magnusson et al., 2003). Cumulative proximity was used to measure performance in the place, working memory and cued trials. Cumulative proximity was obtained from the Smart system according to the method of Gallagher and coworkers (Gallagher et al., 1993), and was manually corrected for start position. Briefly, the animal's distance from the platform, or proximity measure, was measured by the computer every 0.2 seconds for the duration of the animal's swim. These proximity measures were then added together to give a cumulative proximity. The proximity measures were corrected for start position by calculating the cumulative proximity for the ideal path, based on swim speed and starting point and subtracting this from the cumulative proximity measurement from the tracking system. Average proximity to the platform was used to assess performance in the probe trials (Gallagher et al., 1993). The data was collected similar to the cumulative proximity measure, but after correcting for starting point, the proximity measures were averaged over the 30s trial (Gallagher et al., 1993). Learning index scores were calculated from the probe trial data according to Gallagher and coworkers (Gallagher et al., 1993). The mean average proximity measurements for the young mice in the first probe trial (probe trial 1) were divided by the mean measurements for the young mice in each separate probe trial in order to obtain a multiplier for each probe trial. The multipliers obtained were as follows: 1.00, 1.32, 1.25, 1.66, 1.70, 1.35 for probe trials 1–6, respectively. For each mouse, the average proximity scores for each trial were multiplied by the respective multipliers for each trial and the products were summed to obtain a learning index score for that mouse. For both cumulative and average proximity and learning index scores, higher values represented poorer learning ability and lower values indicated better learning performance. Proximity measures were used to assess performance in these studies because they are less influenced by swim speed differences than more traditional measures such as latency to reach the platform (Gallagher et al., 1993; Magnusson, 1998a). The proximity measures are also more sensitive to some of the alternative strategies that animals can use to find the platform that may not involve place learning (Gallagher et al., 1993). The learning index score provides similar information to traditional measurements of time spent in the correct quadrant, but has the added advantage of providing a single value that can represent the spatial bias in multiple probe trials and also reflect the learning curve by being weighted to reward those animals who acquire the task faster (Gallagher et al., 1993).

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

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

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

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

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

Acknowledgements

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

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Fig. 1.

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

Fig. 2.

Representative film images of mRNA hybridization of different splice forms of behaviorally characterized animals. In situ hybridization images from a young mouse for $\zeta 1$ -pan (A) and $\zeta 1$ -1 (B). $\zeta 1$ -3 mRNA showed higher density of hybridization in some areas in young behaviorally-characterized animal brain (C) than in the old animals from the same behavioral group (D). $\zeta 1$ -a mRNA showed lower densities of hybridization in many regions of young animal brains (E) than in the old animals (F). Representative in situ image from a young mouse for $\zeta 1$ -b (G). Diagrams adapted from Paxinos and Franklin (2001) showing analyzed brain regions of the prefrontal and frontal cortex of mice used in this study (H & I). Even numbers indicate superficial layers (II and III) and odd numbers indicate deep layers (IV-VI) of prefrontal and frontal cortical regions of mouse brain. 1, 2 ventral orbital cortex; 3, 4 lateral orbital cortex; 5, 6 medial prefrontal cortex; 7, 8 secondary motor cortex; 9, 10 primary motor cortex.

cortex; 11, 12 somatosensory cortex and 13, 14 insular cortex. Standard images and the equivalent pmol of labeled $^{33}\text{P}/\text{mm}^2$ tissue are shown to the right of each brain image.

Fig. 3. mRNA densities of $\zeta 1\text{-pan}$ and $\zeta 1\text{-1}$. Graphs showing densities of mRNA expression of $\zeta 1\text{-pan}$ (A) and $\zeta 1\text{-1}$ splice form (B) in prefrontal cortical regions of mice brain. * $p < 0.05$ for difference in expression of mRNA from 4-months old animals. Data indicate mean $\pm$ SE. Lat orb, lateral orbital cortex; vent orb, ventral orbital cortex; sup, superficial cortical layers II–III; deep, cortical layers IV–VI.

Fig. 4.

Graphs showing correlation analysis of mRNA for the $\zeta 1$ -a splice form in orbital cortices of old mice and performance in a reference memory task, as shown by learning index scores (A); in a working memory task, as shown by the ratio of T_0/T_{delay} (B); and in the cued control task, as shown by averaged cumulative proximity (cum prox; C). D) Graph showing correlation analysis between performance in the working memory task and cued trials. Please note that high learning index scores and cumulative proximities are associated with poor performance in reference and cued trials, respectively. High T_0/T_{delay} ratios indicate good performance. Lat orb, lateral orbital cortex; vent orb, ventral orbital cortex; T_0 , initial naïve trial; T_{delay} , first test trial with 10 minute delay from naïve trial; corr p, corrected p-value (p_{ACT} statistical method).

mRNA expression of ζ 1-3 in different brain regions of the prefrontal and frontal cortex in naïve and behaviorally-characterized groups in different age groups of mice. Data indicates mean $\pm$ SE.

Cortical regions		Naïve		Behaviorally-characterized	
		4-months	11-months	4-months	26-months
Insular cortex	-deep	261 $\pm$ 26	228 $\pm$ 7	274 $\pm$ 16	220 $\pm$ 15 [*]
	-superficial	255 $\pm$ 22	225 $\pm$ 14	260 $\pm$ 22	228 $\pm$ 13
Ventral orbital cortex	-deep	227 $\pm$ 26	218 $\pm$ 23	282 $\pm$ 40	198 $\pm$ 29
	-superficial	234 $\pm$ 28	224 $\pm$ 24	283 $\pm$ 38	220 $\pm$ 25
Lateral orbital cortex	-deep	222 $\pm$ 35	217 $\pm$ 15	273 $\pm$ 35	213 $\pm$ 20
	-superficial	218 $\pm$ 23	206 $\pm$ 17	248 $\pm$ 38	206 $\pm$ 16
Medial prefrontal cortex	-deep	250 $\pm$ 23	235 $\pm$ 8	253 $\pm$ 21	205 $\pm$ 15 [*]
	-superficial	242 $\pm$ 20	229 $\pm$ 15	237 $\pm$ 20	236 $\pm$ 22 [#]
Secondary motor cortex	-deep	238 $\pm$ 24	234 $\pm$ 11	252 $\pm$ 20	219 $\pm$ 10
	-superficial	276 $\pm$ 16	255 $\pm$ 16	259 $\pm$ 7	243 $\pm$ 8 [*]
Primary motor cortex	-deep	236 $\pm$ 24	220 $\pm$ 8	241 $\pm$ 12	201 $\pm$ 10
	-superficial	260 $\pm$ 25	253 $\pm$ 17	254 $\pm$ 24	212 $\pm$ 12 [*]
Somatosensory cortex	-deep	248 $\pm$ 24	219 $\pm$ 7	238 $\pm$ 23	212 $\pm$ 11 [*]
	-superficial	267 $\pm$ 25	241 $\pm$ 11	267 $\pm$ 20	235 $\pm$ 11

mRNA expression is measured in pmoles of labeled $^{33}\text{P}/\text{mm}^2$ of tissue.

^{*} p<0.05 for differences from 4-month old behaviorally-characterized animals

[#] p<0.05 for differences from 11-month old behaviorally-characterized animals

[†] p<0.05 for differences from the naïve animals in same age group

deep= cortical layers IV–VI

superficial= cortical layers II–III

Table 2

mRNA expression of $\zeta 1$ -a in different brain regions of the prefrontal and frontal cortex in both naïve and behaviorally-characterized groups in different age groups of mice. Data indicates mean $\pm$ SE.

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

mRNA expression is measured in pmoles of labeled $^{33}\text{P}/\text{mm}^2$ of tissue.

* p<0.05 for differences from 4-month old behaviorally-characterized animals

p<0.05 for differences from 11-month old behaviorally-characterized animals

† p<0.05 for differences from the naïve animals in same age group

deep= cortical layers IV–VI

superficial= cortical layers II–III