Aging is known to alter many physiological processes within the brain including synaptic responses, long-term potentiation, learning, and memory. Aging has also been shown to alter the expression and distribution of N-methyl-D-aspartate (NMDA) receptors in many different brain regions, including the hippocampus. Additionally, we have recently reported that young adult rats show an activity-dependent increase in the surface expression of NMDA receptors. We have extended these observations in the present study in aged animals and have found that aged Fischer 344 rats fail to show activity-dependent changes in the surface distribution of NMDA receptors. In conjunction with this observation we have also noted that aged rats show an expression deficit in the C2 splice variant of the NR1 subunit. This subunit is thought to play an important role in neuronal signaling, these observations suggest possible new areas of dysfunction in this receptor that might underlie age-related deficits in neuronal physiology.

Aging has long been known to have detrimental effects upon the function of the nervous system, but the precise molecular mechanisms involved have not been elucidated. Multiple signaling pathways involved in inflammation, antioxidant defenses, developmental gene regulation, and cellular signaling have been implicated as playing a role in the age-related deterioration (1–9). The N-methyl-D-aspartate (NMDA) receptor is thought to play an important role in neuronal signaling because of its ability to flux calcium. This occurs in response to glutamate and concomitant depolarization, thus allowing the NMDA receptor to function as a “coincidence detector.” This capability has led some to suggest that the NMDA receptor could be involved in “Hebbian” synaptic plasticity. We and others (5, 9–14) have noted that as part of the aging process, a specific decrease in the expression and function of the NMDA receptor occurs in multiple areas of the brain. Moreover, we have recently demonstrated a correlation between the amount of NR2B subunit of the NMDA receptor in the hippocampus and spatial learning task performance in aged rats (15).

Long-term potentiation (LTP) is a long lasting increase in synaptic efficacy, which can be produced by repeated stimulation of neurons in many regions of the brain including the hippocampus. We have recently reported that LTP stimulation produces a redistribution of NMDA receptors from an intracellular pool to the surface of hippocampal neurons from young adult rats (16). To extend these studies, we have examined the effect of LTP stimulation upon the surface distribution of NMDA receptors in Fischer 344 rats at 4, 16, and 24 months to examine the effect of aging upon this activity-dependent redistribution of receptor. Additionally, in young adult rats, there is a preferential redistribution of the C2 cassette-containing NR1 splice variant subunits to the surface. We therefore also examined the expression of NR1 splice variants in aged animals.

EXPERIMENTAL PROCEDURES

Animals—Fisher 344 rats were obtained from the National Institute of Aging breeding colony at Harlan Sprague-Dawley at 6, 16, and 24 months of age. After sacrifice the brains were removed and the hippocampi dissected out. 400-μm slices were prepared on a McIlwain tissue slicer and allowed to recover at 32 °C in a perfusion chamber containing ACSF (124 mM NaCl, 4 mM KCl, 1 mM MgSO4, 2.5 mM CaCl2, 10 mM dextrose, 1 mM KH2PO4, 25.7 mM NaHCO3) at a rate of 4 ml/min for 90 min. For surface expression experiments, CA1 mini-slices were prepared directly from isolated CA1 as described previously (16).

Semiquantitative Western Blotting—Tissue samples for protein expression were sonicated into Tris-buffered SDS (10 mM Tris, pH 8.0, 2 mM EDTA, 1% SDS). Protein concentrations were determined using a modified BCA assay (Pierce). Samples were loaded onto 7.5% SDS-PAGE along with a five-point standard curve for comparative analysis. Whole rat brain homogenate was used as the standard with aliquots from the same batch freshly prepared before each set of gels was run. Gels were transferred to polyvinylidene difluoride membrane using a subharine transfer apparatus (Owl Scientific). Following transfer, blots were dried and blocked with 5% milk or 3% BSA in Tris/HCL, pH 7.4, 100 mM NaCl, 0.1% Tween 20). Primary antibody was applied for 16 h at 4 °C in 1% milk or 1% BSA in TTBS. Secondary antibody was applied for 2 h at 25° in 1% milk or 1% BSA in TTBS. Gels were developed using SuperSignal (Pierce) and were imaged and analyzed using an Alpha Imager (Alpha Innotech). Quantitation was performed using Excel (Microsoft), and statistics were performed using StatView (SAS Institute). Antibodies to NR2A, NR2B, and NR1 splice variants (N-terminal cassette, C1 cassette, C2 cassette, C2’ alternate reading frame), and synapsin were produced in our laboratory and have been described previously (16). Antibodies were obtained commercially, NR1 from PharMingen and GluR2 from Chemicon.

Electrophysiology—Field EPSPs were obtained by the usual protocols. In brief, the Schaeffer collateral pathway was stimulated using a bipolar nichrome electrode, and recordings were made from the dendritic layer of CA1 using a silver electrode contained in a finely drawn glass capillary tube containing ACSF. These field EPSPs were considered as representing mainly AMPA receptor responses. To obtain NMDA receptor responses, AMPA EPSPs were blocked by the administration of 2-μM NBQX. For electrophysiological studies, 1 train of 1-s 100-Hz stimulation was delivered at 50% of the maximal NMDA receptor response. For biochemical studies, LTP was induced in the slice using a multi-rake electrode, which we have described previously. In short, 16 rake electrodes were fixed to a rigid support in a 4 × 4 matrix arrangement allowing for the stimulation of 4 slices at 4 rake positions.
each (16 stimulation points/mini-slice). Columns of 4 rake electrodes on the same slice were oriented parallel to each other and separated by 500 μm. Adjacent columns were separated by 2.5 mm. LTP was induced by the sequential delivery of four 1-s trains of 100-Hz stimulation per rake electrode separated by 30 s. Rake positions were stimulated in turn across the slice so that only one rake/slice was stimulating at any given time.

**Cross-linking for Surface Expression Analysis**—To assay basal and stimulation-dependent surface expression, we employed the membrane impermeable cross-linker, bis(sulfosuccinimidyl)suberate (BS3; Pierce) as described previously (16). For determination of basal surface expression values, slices were allowed to recover for a minimum of 90 min and transferred to either ACSF or ACSF containing 1 mg/ml BS3 for 45 min at 4 °C. To assay stimulation-dependent changes in surface expression, slices were harvested at 30 min post-LTP, and both stimulated and matched control slices were treated with cross-linker. A minimum of three slices per control or treatment groups was included for each animal. To terminate the reaction and quench remaining BS3, slices were washed three times in cold ACSF containing 20 mM Tris, pH 7.6. Surface expression was determined following semiquantitative Western blot analysis and a comparison of cross-linked samples to non-treated controls.

**RESULTS**

We have previously reported that aging is associated with a selective decrease in the expression of the NR1 and NR2B subunits of the NMDA receptor with no change in the expression of the NR2A subunit of the NMDA receptor or in the expression of the GluR2 subunit of the AMPA receptor (10). Other groups have found similar results, although age-related decreases in the expression of the NR2A subunit have also been reported (9, 11, 12, 14). In this report we have extended our previous studies by analyzing the surface expression of NMDA receptors in the CA1 region of the hippocampus across the life span of the Fischer 344 rat. We saw no significant differences in basal surface expression in any of the examined targets in rats at 6, 16, and 24 months of age (data not shown). Thus, in marked contrast to the expression of NMDA receptors in aged animals, which is down-regulated, the percentage of the receptor pool that is basally expressed at the surface remains relatively constant with age.

We next sought to examine the effect of aging upon LTP-related changes in surface expression of glutamate receptors in Fischer 344 rats at 6, 16, and 24 months using a multiple “rake” stimulation device (Fig. 1). Surface expression changes following HFS were measured by cross-linking of both stimulated and nonstimulated matched slices 30 min post-LTP. Samples were then used for semiquantitative Western blotting; as the cross-linker (BS3) is membrane-impermeable, only surface expressed molecules are cross-linked, rendering them unable to enter polyacrylamide gels. The internal pool of receptor, which is not cross-linked, is measured directly. Equal amounts of total protein are loaded, so that changes in surface expression with treatment are reflected in alterations in the levels of intracellular proteins. As previously described, LTP stimulation led to an increase in surface expression of NR2A, 2B, and NR1 in young adult animals (16 months). However, no LTP-related changes were seen in the surface expression of NR1, 2A, or 2B subunits in the 24-month group (Fig. 2). An intermediate effect was seen in the 16-month group. In this group, NR2A shows a trend toward a significant change indicating an increase in surface expression with LTP (mean % change = 26.58 ± 6.86%, n = 7, p = 0.11). NR2B was not significantly effected (mean % change = 14.58 ± 16.64%, n = 7, p = 0.39) nor was NR1 (mean % change = 9.61 ± 6.02%, n = 7, p = 0.52) or GluR2 (p = 1). Six-month-old animals showed a significant increase in the surface expression of NR1 (mean % change = 24.37 ± 6.72%, n = 6, p < 0.05) and NR2A (mean % change = 21.23 ± 1.68%, n = 6, p < 0.05) and no change in the surface expression of NR2B (mean % change = 14.76 ± 9.31, n = 6, p = 0.34) or GluR2 (mean % change = 3.69 ± 7.28%, n = 6, p = 0.88). Statistical analyses were performed by ANOVA with Fisher’s PLSD post-hoc test. B, representative immunoblots.
FIG. 3. NMDA receptor response with LTP stimulation. Young adult animals show a potentiation of the NMDA receptor response with the delivery of 1 s of 100-Hz stimulation via a bipolar electrode in the presence of NBQX to block AMPA receptor responses (mean % change = 28.4% ± 9.3%, n = 6, p = 0.005). Measurement of the NMDA receptor response in slices from 16-month-old Fisher 344 rats before and after a single train of HFS shows a trend toward an increase after HFS, but the change is not significant (mean % change = 24.2 ± 11.5%, n = 4, p = 0.14 paired t test). Measurement of the NMDA receptor response in slices from 24-month-old Fisher 344 rats before and after a single train of HFS (delivered at arrow) fails to show any changes (mean % change = 3.2 ± 9.2%, n = 4, p = 0.99 by paired t test).

FIG. 4. NR1 splice variant total expression in young and aged animals. Total expression of NR1 splice variants was measured using semiquantitative Western blotting. N1 cassette containing splice variants: there is no significant difference between 2-month (mean = 2.92 ± 0.52, n = 8) and 22-month animals (mean = 3.06 ± 0.22, n = 8, p = 0.87). C1 cassette containing splice variants: no difference is seen between 2-month (mean = 3.19 ± 0.67, n = 8) and 22-month animals (mean = 3.10 ± 0.39, n = 24, p = 0.99). C2 cassette containing splice variants: a significant difference is seen between 2-month (mean = 3.45 ± 0.54, n = 8) and 22-month (mean 2.93 ± 0.21, n = 24) animals (p = 0.02). C2‘ cassette containing splice variants: no significant difference is seen between 2-month (mean = 4.84 ± 0.75, n = 8) and 22-month (mean = 4.32 ± 0.24, n = 24, p = 0.52) animals. All units are standardized to aliquots of the same batch of rat brain homogenate. All statistics performed across splice variants and across age by ANOVA with Fisher’s PLSD post-hoc test. All 2-month sets contain 8 animals and all 22 month sets contain 24 animals.

**DISCUSSION**

NMDA receptor dysfunction in the hippocampus of aged animals has been demonstrated by multiple groups using a variety of techniques (5, 13). We and others have also demonstrated age-related reductions in the expression of the NMDA receptor. In this report we extend those findings by examining the surface expression of the NMDA receptor subunits in the CA1 region of the hippocampus in 6-, 16-, and 24-month-old Fischer 344 rats. We have found that aging does not affect the basal surface expression of NMDA receptors. Although aged animals express less of the receptors, the percentage of total cellular receptor on the surface does not change.

We have recently documented activity-dependent changes in the surface expression of NMDA receptors (16). Extending those findings, we find that aged animals are deficient in the ability to increase the quantity of NMDA receptor at the surface of the neuron in response to activity. Young animals show a significant increase in the amount of NR1 and NR2A subunits at the surface of the neuron (NR2B increases are seen but are not significant), which is not seen in aged animals. Similarly, aged animals fail to show an increase in NMDA receptor-mediated current post-LTP stimulation as is seen in young animals. These findings, in combination, are suggestive of a plasticity deficit in the function of aged neurons, whereby aged animals are unable to increase the amount of NMDA receptor at the surface in response to stimulation. If NMDA receptors are important for forming a “synaptic tag” for synapse formation or enlargement, as has been suggested previously, aged animals should be compromised in their ability to increase synaptic contacts in this fashion. Thus, this described defect in activity-dependent regulation may indicate a mechanism for decreased plasticity in aging mediated by a decrease in the formation or enlargement of synapses with activity. Cycling of receptors and other proteins into membranes occurs both constitutively and with activation of various signaling cascades. Our data suggest that constitutive cycling of NMDA and AMPA receptors is not altered with age. In contrast, activity-dependent cycling of NMDA receptors appears to be altered profoundly as a function of age. The mechanism of this selective deficit in receptor trafficking is unknown; however, one clue may be found in our previous observation that the increase in surface expression of the NR1 subunit is preferential for NR1 subunit splice variants containing the C2 cassette. In examining the expression of NR1 splice variant proteins in aged animals, we find that there is a specific deficit in the expression of the C2 cassette containing subunits. It has been suggested that the cellular quantity of the NR1 subunit protein is determined in part by assembly with other subunits and that the differential assembly of subunits may determine the intracellular distribution of receptor (17–19). Thus, the age-related reduction in the amount of C2 splice variant NR1 subunit may impair the activity-dependent cycling of NMDA receptors. Age-related decreases in the expression of NR2 subunits could also contribute to this scenario, reducing the amount of total functional receptor that could be assembled.

These findings extend our understanding of the cellular distribution of NMDA receptors in aged animals and describe a new deficit in the activity-dependent regulation of the cellular distribution of these receptors in aged animals. Further studies are needed to elucidate the importance of activity-dependent alterations in the surface expression of NMDA in synaptic plasticity in aged animals.

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