

BRIEF COMMUNICATION

Attenuated Lesion-Induced *N*-Methyl-D-Aspartate Receptor (NMDAR) Plasticity in the Dentate Gyrus of Aged Rats following Perforant Path Lesions

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Young animals demonstrate a significant upregulation of *N*-methyl-D-aspartate receptor 1 (NMDAR1) in the outer molecular layer (OML) of the dentate gyrus following a total unilateral ablation of the perforant path, and this response presumably facilitates a degree of functional recovery. Aged animals have attenuated responses to lesion-induced synaptic plasticity as compared with young subjects, and in fact display decreased synaptogenesis and sprouting following a unilateral perforant path lesion. To investigate the response of NMDAR1 in the dentate gyrus of aged animals to perforant path ablation, 24-month-old Sprague-Dawley male rats received a unilateral knife cut of the angular bundle. Our results demonstrated that aged animals displayed a blunted response to lesion-induced NMDA receptor-mediated plasticity, suggesting that aged animals have an impaired ability to respond to deafferentation through an increase in NMDA receptor levels in the deafferented zone.

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The perforant path provides the main cortical input to the hippocampus, originating primarily from layer II of the entorhinal cortex, and terminating primarily in the outer two-thirds of the molecular layer of the dentate gyrus (3). Following a unilateral transection of these fibers, an acute deafferentation of a segregated zone on the distal dendrites of hippocampal dentate gyrus granule cells occurs (i.e., outer molecular layer), followed by axonal sprouting of the remaining cortical afferents, reactive synaptogenesis, and a partial return of physiological and behavioral function (18, 19, 27).

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Recently, our laboratory studied the effects of a perforant path lesion on *N*-methyl-D-aspartate receptor (NMDAR) plasticity through analysis of the NMDAR1 subunit, which is required for a functional NMDA receptor. This receptor has been shown to play a key role in synaptic plasticity such as long-term potentiation (LTP), a cellular model of learning and memory (7). We showed that within 5 days postlesion in young adult male rats there is a 60% increase in the intensity of NMDAR1 subunit immunofluorescence restricted to the denervated outer molecular layer and the granule cell somata, in comparison to both the nonlesioned side and either side of control animals (10). These results were consistent with a previous study demonstrating an increase in NMDA binding in the hippocampus following a unilateral lesion of the entorhinal cortex (29).

Many experimental paradigms have demonstrated attenuated or altered plasticity in aged animals. In rats, LTP has been shown to have a faster rate of decay in aged animals (4). Using the unilateral perforant path lesion to examine plastic responses to denervation, studies have shown a decreased sprouting response and synaptogenesis, as well as decreased lesion-induced upregulation of growth-associated proteins in the hilus (9, 13, 14, 21, 23, 28, 30). Therefore, since functional alterations in NMDAR-mediated plasticity exist during aging, as do attenuated responses of aged animals to lesion-induced plasticity, the present study was designed to examine the response of the NMDAR1 subunit following a perforant path lesion in the dentate gyrus. To accomplish this, we quantitatively evaluated changes in NMDAR1 immunofluorescence intensity level within the outer molecular layer (OML) and the inner molecular layer (IML) of the dentate gyrus 5 days following transection of the perforant path in 24-month-old male rats.

Animals, surgery, and tissue processing. A total of ten 24-month-old male Sprague–Dawley rats were used in this study (Harlan Sprague–Dawley, Indianapolis, IN). Prior to arrival at the Mount Sinai School of Medicine Center for Laboratory Animal Sciences, animals were given an *ad libitum* diet at Harlan Sprague–Dawley (Techclad, Harlan Spargue–Dawley). Animals were individually housed in a temperature-controlled vivarium (25°C) with a 12-h light/dark cycle, and food and water were available *ad libitum* in home cages throughout the experiment. Five of the aged animals received a unilateral transection of the angular bundle and five aged animals were not operated on. Animals were anesthetized with 30% chloral hydrate and placed into a Kopf stereotaxic frame (Kopf Inc., Tujunga, CA). Stereotaxic transections of the perforant path (angular bundle) were made with an extendable Scouten wire knife (Kopf Inc., Tujunga, CA) that was inserted into the brain 1 mm anterior and 6.3 mm lateral to lambda, to a level 5 mm ventral from the surface. The knife was extended 2.5 mm medially, raised 4 mm, and then retracted. This procedure was repeated at a position 1 mm anterior and 5.3 mm lateral to lambda. All rats were anesthetized and perfused transcardially 5 days after surgery with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were immediately removed, blocked, and postfixed in 4% paraformaldehyde for 6 h. Sections from the rostral hippocampus were then cut in a coronal plane on a vibratome at a setting of 50 μm .

Immunocytochemistry and histochemistry. Adjacent tissue sections were incubated for 48 h with either a monoclonal antibody to NMDAR1 (54.1, 4.5 $\mu\text{g}/\text{ml}$) or synaptophysin (Boehringer Mannheim, Indianapolis, IN; 1:10) in PBS. After being washed in PBS for 30 min, the sections were incubated for 2 h in a solution of biotinylated anti-mouse IgG H&L in PBS (1:200). All sections were then washed for 30 min, and transferred to a solution containing a solution of FITC-conjugated avidin (1:200) in PBS for 1 h. Following a 30-min rinse, the sections were mounted on gelatin-coated slides and coverslipped with Vectashield. Histochemical staining for cytochrome oxidase activity was carried out on a third series of sections according to a protocol described previously (32). Synaptophysin immunoreactivity and cytochrome oxidase histochemistry were used to verify a complete perforant path lesion.

Confocal laser scanning microscopy (CLSM) and quantitative evaluation. Quantitative CLSM analysis of NMDAR1 was performed on images obtained from a Zeiss LSM 410 inverted confocal microscope (Zeiss Inc., Oberkochen, Germany) as described in several reports from our laboratory (2, 10–12, 25). All imaging parameters were set at the beginning of the study so as to yield a high-resolution image for both bright and dim sections without regions above the

maximal pixel intensity of 255 (Contrast/Brightness, Zeiss imaging software, Zeiss Inc.). For each animal analyzed, high-magnification images were obtained from six adjacent regions of identical area (2039 μm^2) within the IML and outer molecular layer OML of three randomly selected sections from both sides. The borders for the IML and OML were determined from the synaptophysin-immunoreacted sections, as well as those sections reacted for cytochrome oxidase. Each digitized image consisted of a $512 \times 512 \times 8$ -bit pixel array in which every pixel was assigned a gray level intensity value ranging from 0 to 255. Subtraction of background immunofluorescence was accomplished with a photometric offset (Zeiss imaging software, Zeiss Inc.) to establish a pixel intensity threshold below which a pixel would have no contribution to the average pixel intensity of the field. The average pixel intensity of the portion of the field above threshold was determined, thus representing the immunofluorescence intensity or area of the granule cell dendrites. The data from young animals to examine potential age-related differences in the response of NMDAR1 to a perforant path lesion were taken from (10) ($n = 5$).

Statistical Analysis. Statistical analyses were performed using StatView 5.0 (Abacus Concepts, Inc., Berkeley, CA) running on a Power Macintosh 8100/100 (Apple Computer, Cupertino, CA). Potential differences in the nondenervated (i.e., contralateral) versus denervated (i.e., ipsilateral) hemispheres were assessed using Student's paired *t* tests (one-tailed). Potential group differences in fluorescence intensity between young and aged lesioned and control animals were evaluated by a repeated-measures ANOVA and follow-up, pairwise contrasts (Bonferroni–Dunn tests). Significance was set at $P < 0.05$.

Qualitative observation of perforant path lesions and NMDAR1. All perforant path lesions were verified with adjacent sections that were processed for cytochrome oxidase histochemistry (Fig. 1A) and synaptophysin immunoreactivity (data not shown). Following the unilateral perforant path lesion, no qualitative differences in cytochrome oxidase histochemistry and synaptophysin were observed between the OML and IML in the contralateral the dentate gyrus. However, ipsilateral to the lesion, both cytochrome oxidase and synaptophysin were depleted in the OML as compared with the IML, demonstrating that a complete unilateral lesion was performed. All animals had complete unilateral lesions in the present study [i.e., complete depletion of cytochrome oxidase (Fig. 1A) and synaptophysin in the ipsilateral dentate gyrus (data not shown)].

NMDAR1 immunostaining was observed in the cytoplasm of the granule cells and throughout the extent of the dendritic arbor; however, the OML of the dentate gyrus ipsilateral to the lesion appeared to be more

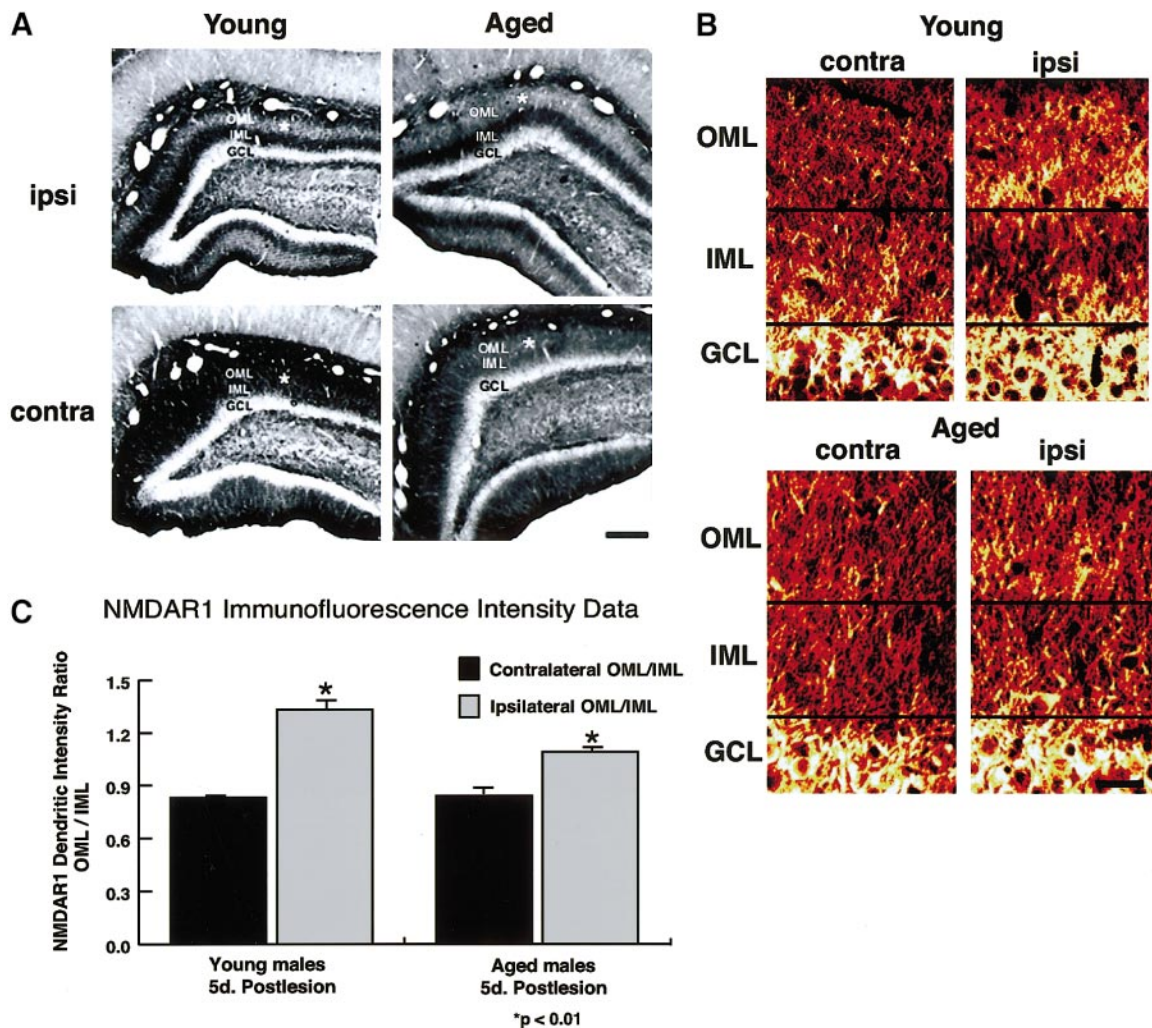


FIG. 1. (A) Coronal cytochrome oxidase-stained sections through the ipsilateral and contralateral sides of both young (10) and aged animals. Note the decreased intensity of staining in the OML (asterisks) on the side ipsilateral to the lesion in comparison with that of the OML of the contralateral side. Bar = 250 μm . (B) Digitized confocal microscopic images of NMDAR1 immunofluorescence in the dentate gyrus of a young adult (~5 months) and aged (24 months) rat examined 5 days postlesion. The images on the left of each pair represent fields from the nonlesioned, contralateral side, while the images on the right represent fields from the lesioned, ipsilateral side. The images from the young adult animal have been previously published (10). Note that in comparison to the relative homogeneity of immunofluorescence throughout the entire molecular layer on the contralateral side, an overt increase in the immunofluorescence intensity in the OML compared with the IML is apparent on the ipsilateral side of animals in both groups. It is also apparent that the increase in intensity in the OML of the young adult rat is greater than the increase observed in the aged rat. Bar = 30 μm . (C) Bar graphs comparing OML/IML ratios of NMDAR1 immunofluorescence intensity values in young adult (5 months) and aged (24 months) male rats 5 days postlesion. Statistical analyses revealed that the ipsilateral OML/IML ratios of both the young adult and aged groups were significantly increased (asterisks) compared with the corresponding contralateral OML/IML ratios (P values < 0.01, Student's paired t test). Comparisons of absolute values revealed that in the young adult rats OML intensity on the ipsilateral side was increased compared with the contralateral side, while IML intensity did not differ between the two sides (10). In the aged animals, as in the young animals, there was a significant increase in OML intensity on the ipsilateral side compared with the contralateral side, while IML intensity did not differ between the two sides ($P < 0.05$; Student's paired t test). Between-group comparisons revealed that the OML/IML ratios of the lesioned sides were significantly lower in the aged animals than in the young adults (Bonferroni-Dunn: $P < 0.0001$), while there was no difference in the OML/IML ratios of the nonlesioned sides (Bonferroni-Dunn: $P > 0.50$). Values represent means \pm SEM of five rats per group. OML, outer molecular layer; IML, inner molecular layer; GCL, granule cell layer.

intense by immunofluorescence than the IML (Fig. 1B). This pattern of NMDAR1 immunostaining following a 5-day unilateral perforant path lesion is consistent with observations seen in the dentate gyrus of young animals with a 5-day perforant path lesion (10). Unop-

erated control rats had a similar pattern of NMDAR1 immunostaining as the contralateral hemisphere of the lesioned rats (data not shown). This pattern of staining in the unoperated controls is similar to that described in young animals. Recent work from our

laboratory demonstrated that no age-related changes occur in NMDAR1 in intact aged male rats (2).

Quantitative assessment of NMDAR1 following a unilateral perforant path lesion. To assess the potential effect of age on NMDAR1 plasticity following a 5-day unilateral perforant path lesion, CLSM was used to quantify the distribution of NMDAR1 in the OML versus the IML. While the aged animals did upregulate NMDAR1, an ANOVA revealed significant differences between the young and aged lesion and control animals (main effect of age $F_{(3,16)} = 30.00$, $P < 0.0001$; main effect of the lesion $F_{(1,16)} = 97.30$, $P < 0.0001$; age \times lesion interaction, $F_{(3,16)} = 30.84$, $P < 0.0001$). Within the lesioned aged animals, the ratios of the denervated (i.e., ipsilateral) OML versus IML as compared with the nondenervated (i.e., contralateral) hemisphere were significantly different (Fig. 1C; Student's paired t test; $t_{(4)} = -5.263$, $P < 0.01$). These data demonstrate that aged animals, similar to young rats, upregulate NMDAR1 following a perforant path lesion. Consistent with a previous study of young animals from our laboratory, quantitative assessment of the ratio of the nondenervated (i.e., contralateral) OML versus IML of aged animals revealed a ratio of 0.85 (10). Comparisons of the ratio of the contralateral OML/IML from the present study with that from (10) revealed no significant differences between the young and aged animals (Fig. 1C; Bonferroni-Dunn: $P > 0.50$). However, the ratio of the OML versus the IML of the dentate gyrus ipsilateral to the lesioned hemisphere in the aged animals revealed a ratio of 1.09, far less than the ratio described from a previous study of young animals of 1.33 (10). Statistical analyses revealed a significant difference in the ratios between the young and aged animal groups (Fig. 1C; Bonferroni-Dunn: $P < 0.0001$).

To determine the source of the age-related difference in NMDAR1, comparisons of the absolute values of NMDAR1 immunofluorescence were made in the aged lesioned animals. NMDAR1 in the ipsilateral OML of the aged animals appeared to be upregulated compared with the contralateral OML, and quantitative evaluation of NMDAR1 immunofluorescence intensity in the OML from the nondenervated dentate gyrus, compared with the denervated hemisphere, revealed a significant difference in the aged animals (Student's paired t test: $t_{(4)} = -2.533$, $P < 0.05$). There was no detectable difference in immunofluorescence intensity in the IML between the two sides (Student's paired t test: $t_{(4)} = 1.158$, $P > 0.84$). A previous study from our laboratory demonstrated that there was a significant difference between the ipsilateral and contralateral OML in young lesioned animals (Student's paired t test: $t_{(4)} = -3.380$, $P < 0.02$), but no detectable difference in the IML of those same subjects (Student's paired t test: $t_{(4)} = 1.831$, $P > 0.14$) (10). Moreover,

ratios of NMDAR1 in the OML versus the IML in the unoperated control aged animals revealed ratios of 0.94 and 0.96. These ratios are consistent with a previous study from our laboratory demonstrating that in young unoperated control animals the ratio of NMDAR1 in the OML versus the IML was 0.85 and 0.89 (10). Statistical analyses comparing the ratios of these different groups revealed no significant differences between the young and old unoperated controls (Bonferroni-Dunn: both P values > 0.05 ; data not shown). These data suggest that the difference in the ratios of the denervated hemisphere of the young and aged rats is due to an attenuated upregulation in the OML of aged animals.

Our results demonstrate an attenuated response of NMDAR1 plasticity following a perforant path lesion in aged male rats. This attenuated response of lesion-induced NMDAR1 plasticity in aged animals is consistent with previous literature suggesting that there are blunted responses to lesion-induced plasticity in aged animals. Previous studies have described a decreased sprouting response and synaptogenesis, as well as a reduced upregulation of growth-associated proteins in the hilus in aged animals following a perforant path lesion (9, 13, 14, 21–23, 28, 30). One mechanism that might underlie the blunted sprouting response and synaptogenesis is a loss of plasticity of NMDA receptors, since NMDA receptor antagonists have been shown to regulate dendritic spine density in the hippocampus (17, 33).

Attenuated responses have been described in aged animals in other experimental paradigms. For example, blunted hypothalamic responses have also been described in aged female animals in response to estrogen deprivation. Multiple studies have demonstrated that the luteinizing and gonadotropin-releasing hormone response that follow a short-term ovariectomy is blunted in aged animals compared with young subjects (6, 8, 15, 20, 26, 31). Recent findings from our own laboratory have demonstrated that the estrogen-induced increase in spine density observed in young animals following a short-term ovariectomy and estrogen replacement is attenuated in aged animals (1). In addition, it may be that the attenuated response of NMDAR1 to a perforant path lesion in aged animals may result from a delayed compensatory response. As stated above, the sprouting response and synaptogenesis following a perforant path lesion are attenuated in aged animals in a short-term experimental interval but the aged brain achieves a similar response to the young one over a longer period (13, 14, 22). Therefore, it will be necessary to perform experiments in aged subjects that examine a full time course of different postlesion intervals to determine if the blunted NMDAR1 response is maintained or eventually reaches the same level as in a young animal. In addition, it will be necessary to determine whether the other NMDA re-

ceptor subunits, NMDAR2A and NMDAR2B, that constitute the channel in the adult brain also display a blunted response to a perforant path lesion. A recent study demonstrated that there are reductions in the levels of these two subunits in the aged hippocampus (16). Alterations in the response of the levels of these subunits may underlie some of the functional changes observed in NMDA receptors in the aged brain (5, 24).

CONCLUSION

These data and other cited findings suggest that a possible age-related decrement in the ability to mount a plasticity response may occur at several levels. This blunted capacity to modify both structural and molecular elements of synaptic circuitry may be a critical adjunct to chronic age-related decrements that have been described in that it compromises the aged animal's ability to counter potentially negative perturbation in neural circuits as effectively as a young animal.

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