Hippocampal 3H-CPP binding and spatial learning deficits in aged rats

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Recent evidence has shown that either intraventricular or intrahippocampal injection of the NMDA antagonist D-2-amino-5-phosphonovalerate (AP5) produces a spatial learning deficit. This deficit is similar to that observed in aged animals. The spatial learning capacity of aged rats could, then, be related to changes in the status of hippocampal NMDA receptors. This study assessed hippocampal NMDA receptor binding in young and aged rats using the potent NMDA antagonist 3H [(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP). Initial characterization experiments indicated that 3H-CPP bound selectively to a class of receptors in hippocampal tissue with high affinity (39.5 nM). The 3H-CPP receptor binding capacity was significantly reduced in experimentally naive aged rats (24-25 months) as compared with young animals (5 months). A comparable reduction in 3H-CPP binding was found in a comparison of aged and young rats that had been trained on a spatial task in the Morris water maze. Furthermore, the reduction in 3H-CPP binding was correlated with the severity of the learning deficit present within the aged group. It was concluded that a reduction in hippocampal NMDA sites, as measured by 3H-CPP binding, may contribute to the emergence of cognitive deficits in aged animals.

Glutamate receptors that are activated by N-methyl-D-aspartate (NMDA) are highly concentrated in the hippocampal formation (Monaghan & Cotman, 1985; Monaghan, Holets, Toy, & Cotman, 1983; Monaghan, Yao, Olverman, Watkins, & Cotman, 1984), a neural system long considered to be crucial for certain memory processes (Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe & Nelde, 1978). Recent evidence has also demonstrated the involvement of a hippocampal NMDA mechanism in synaptic plasticity (Cotman & Monaghan, 1988; Ernston, Lynch, & Bliss, 1987; Harris, Ganong, & Cotman, 1984; Morris, Anderson, Lynch, & Baudry, 1986), thus linking this type of receptor to long-term changes in hippocampal function. Activation of NMDA receptors mediates the induction of long-term potentiation (LTP) following high-frequency stimulation, as evidenced by the blockade of hippocampal LTP (but not normal synaptic transmission) by the selective NMDA antagonist amnrophosphonovaleric acid (AP5) (Cotman et al., 1988; Ernston et al., 1987; Harris et al., 1984; Morris et al., 1986). LTP is widely considered to be a form of activity-dependent strengthening of synaptic transmission that may provide a mechanism for information storage in the mammalian brain (Bliss & Lomo, 1973; Tyler, 1986).

A case for the involvement of hippocampal NMDA-dependent LTP in information storage is further supported by studies that have demonstrated a selective learning impairment produced by AP5 at doses that also interfere with LTP. An initial study reported that intraventricular AP5 administration, at doses sufficient to block hippocampal LTP in rats, impaired learning of a spatial task in the Morris water maze; this task is highly sensitive to a variety of manipulations that disrupt normal hippocampal function. The deficit produced by AP5 was selective because it spared learning of another task, visual discrimination, which is also relatively insensitive to hippocampal dysfunction (Morris et al., 1986). More recently, a spatial learning deficit was observed in animals that received microinjections of AP5 directly into the hippocampus (Morris, Halliwell, & Bowery, 1989). Spatial learning deficits have also been reported following administration of noncompetitive NMDA antagonists (Shapiro & Caramanos, in press; Robinson, Crooks, Shinkman, & Gallagher, 1989).

Spatial learning deficits that are similar to those produced by NMDA antagonists are often found in aged rats. For example, many studies have reported an age-related deficit in spatial learning in the Morris water maze (Gage, Dunnett, & Bjorklund, 1984; Pelleymounter, Smith, & Gallagher, 1987; Rapp, Rosenberg, & Gallagher, 1987). Furthermore, aged rats exhibit deficits in a variety of other spatial learning tasks, including the Barnes circular hole board and the radial-arm maze (Barnes, & McNaughton, 1980; Barnes & McNaughton, 1985; De Toledo-Morell, Morell, & Fleming, 1984; Wallace, Krauter, & Campbell, 1980). The dynamics of LTP are also affected by age: LTP appears to reach its maxi-

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mum more slowly (and decay more rapidly) in old rats than in young ones (Barnes et al., 1980; Barnes et al., 1985).

In light of these similarities, this study examined the possibility that spatial learning deficits exhibited by aged rats are associated with altered NMDA binding in the hippocampal formation. The present study, then, compares binding of the highly potent NMDA agonist 3H-3-{[(±)-2-carboxyphperazin-4-yl]propyl-1-phosphonic acid (CPP; Lehman et al., 1987; Murphy, Schneider, Boehm, Lehman, & Williams, 1987) in experimentally naive young and aged rats. In addition, groups of young and aged rats were trained on a spatial task in the Morris water maze, and hippocampal 3H-CPP binding was assessed for these subjects in order to compare the binding characteristics of the aged animals with their performance in the behavioral task.

Method

Subjects

Fourteen young (5 months) and 22 aged (24-25 months) male Long-Evans rats obtained from Charles River Laboratories were subjects in the study. The animals in the aged groups were obtained as pathogen-free retired breeders at 8-9 months of age. The young pathogen-free animals were resident in the Department of Psychology vivarium at least 1 month prior to the experiment. The vivarium is climate controlled at 25°C. All animals were housed in single cages on a 12:12-h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided ad lib.

Radioligand Binding Assay

3H-CPP (specific activity = 30.2 Ci/mM) was obtained from New England Nuclear. D and L-AP5 were obtained from Cambridge Biochemical Corporation. L-glutamate, kainic acid, and quisqualic acid were obtained from Sigma Chemical Company. Unlabeled CPP was obtained from Research Biochemicals, Natuck, MA.

Following decapitation of the animals, hippocampi were rapidly dissected on an ice-cold block and frozen on dry ice. Hippocampi were stored at -70°C for at least 1 month prior to the experiment. The animals were obtained from the vivarium at least 1 month prior to the experiment. The vivarium is climate controlled at 25°C. All animals were housed in single cages on a 12:12-h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided ad lib.

Behavior

The water maze apparatus used in the behavioral training was a circular steel tank (diam = 1.83 m, depth = 0.58 m) with a removable white escape platform (diam = 10.2 cm, height = 34.5 cm) centered in the SE quadrant of the tank. The tank was filled to a depth of 35.5 cm with water (27°C) opacified by the addition of 0.9 kg powdered milk so that the escape platform was camouflaged and submerged 1 cm below the water surface. The maze was surrounded by white muslin curtains intersecting at each quadrant for ease of access. Dark extramaze patterns were affixed to the curtains at all times to provide an arrangement of spatial cues. During training, the subjects were videotaped with a Panasonic WV-241 P closed circuit TV camera with a 4-mm wide-angle lens suspended above the center of the maze. Training trials were observed by the experimenter on an RCA 12-in. monitor screen.

All subjects were handled (approximately 2 min/day) for 7 days prior to training. On Day 1 of training, the animals were habituated to the water maze by being placed, for 90 sec, in the water tank, with the escape platform removed. The animals were then returned to their home cages.

Starting on Day 2, each animal completed three trials per day for a maximum of 12 consecutive days of testing. During a trial, each animal was placed in the maze facing the wall at one of four equally spaced entry points (entry points corresponded with the North, South, East, and West poles of the maze). These entry points varied from trial to trial in such a way that an animal was placed in the maze at each entry point once across every four trials. During a training trial, the animal was allowed to swim for a total of 120 sec or until it reached the escape platform, where it was allowed to remain for 20 sec. Escape latencies were measured as the time elapsed from the animal's release into the water until its escape onto the platform. If an animal failed to reach the platform within 120 sec, the experimenter placed it onto the platform for 20 sec. Following the 20-sec interval on the platform, the animal was placed into a dry holding cage for 60 sec, at which point the second trial began. After the third trial, the animal was returned to its home cage. During training, the escape platform remained in the same location in the maze across trials.

Every sixth trial consisted of a probe trial during which the escape platform was removed and the animal was allowed to swim for 30 sec. These trials were used to assess the development of a spatial bias for the location of the escape platform. Two measures were used in evaluating spatial bias: quadrant time and annulus crossings. Both measures were obtained from videotapes of the probe trials. Quadrant time refers to the number of seconds an animal spent in the quadrant of the maze that had formerly contained the escape platform. Similarly, annulus crossings are the number of
times the animal traversed the exact former location of the escape platform. A criterion performance was achieved when an animal spent at least 10 sec in the training quadrant and crossed the training annulus at least twice during a probe trial. This criterion represents significant spatial learning; animals trained with a varying location for the escape platform do not exhibit a comparable spatial bias on probe trials (unpublished observations). After each rat achieved criterion performance in this study, behavioral testing of that rat was terminated.

Approximately 10 days after the completion of behavioral testing, the subjects were sacrificed. Naive animals were sacrificed immediately after removal from their home cages. Necropsy data were obtained by grossly inspected the pituitary gland of each aged animal for evidence of tumors. Blood samples from a subset of the aged subjects (n = 5) were screened for a panel of murine viral antibodies.

RESULTS

All aged animals included in the data analysis were healthy, as determined by routine assessment during the experiment and necropsies performed at sacrifice. Tests for murine viral antibodies performed on blood samples from a subset of the aged subjects were negative, confirming the absence of pathogens in this colony as indicated by routine screening.

Under the conditions used in this assay, 3H-CPP bound to washed hippocampal tissue from young, naive rats in a saturable and specific manner, with an average $B_{\text{max}}$ of 2.47 (± 0.36) pmol/mg protein and an average apparent $K_d$ of 39.5 nM (± 7.5). A saturation curve representing four preliminary experiments where 3H-CPP was incubated with hippocampal tissue from young rats is shown in Figure 1. Drug inhibition studies indicated that 3H-CPP binding was specific to NMDA-type glutamate receptors, in that the IC$_{50}$ values for unlabeled CPP, L-glutamate, and D-AP5 were 73.0, 75.8, and 171.8 nM, respectively. IC$_{50}$ values for non-NMDA glutamatergic agonists, such as quisqualate and kainic acid, were much higher, ranging from 12.4–15.2 μM, respectively. 3H-CPP appeared to bind to NMDA-type receptors in a stereospecific manner, since the L-isomer of AP5 competed for 3H-CPP sites with an IC$_{50}$ of 50.1 μM, in contrast to the D-isomer, which exhibited an IC$_{50}$ of 171.8 nM. IC$_{50}$ values were derived from Hill plots for each of the drugs tested. Competition curves for each of the drugs tested were monophasic, suggesting that these compounds were competing for a single class of noncooperative sites labeled by 3H-CPP. None of the Hill coefficients generated from these characterization studies were significantly different from 1.0. These characterization data are summarized in Table 1. 3H-CPP was also dependent upon pH, in that binding was maximal at pH of 7.6, and was decreased by more than half in buffers as acidic as pH 6.0 or as basic as pH 9.0. 3H-CPP binding was also decreased by half

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**Figure 1.** Specific binding of 3H-CPP to hippocampal tissue pooled from young animals in four separate experiments. Nonspecific binding was defined in the presence of 10 μM L-glutamate and represented 30%–40% of total binding at 3H-CPP concentrations near the $K_d$ and 60%–70% of total binding at saturating concentrations of 3H-CPP. The inset illustrates a Scatchard plot of 3H-CPP binding using a concentration range of 3.1–300 nM 3H-CPP. The average $B_{\text{max}}$ in this plot was 2.35 pmol/mg protein and the $K_d$ was 27.0 nM.
in the presence of 2.0 mM Ca** and by two-thirds with the addition of 120 mM Na* to the incubation buffer (data not shown).

The behavioral assessment in this experiment was included in order to evaluate the relationship between impaired learning capacity and the status of NMDA binding in aged rats. With the exception of 1 aged rat, all animals reached criterion by the sixth probe trial. For the purpose of data analysis, this animal was assigned a score of 7. As shown in Figure 2, many aged animals required more training to reach criterion than did the young subjects. The average number of training trial blocks required to reach criterion for the young and aged groups was 2.1±.3 and 4.0±.5, respectively. Statistical comparison of these data revealed a significant age difference (t = 2.67, p < .02), indicating that the aged group learned at a slower rate than did the young group. A proportion of the aged rats, however, learned at a rate that was similar to that of the young rats, whereas others were distinctly impaired, falling outside the entire range of performance for the young group.

Analysis of 3H-CPP binding for these trained groups revealed Bmax values that were lower for the aged animals than for their younger counterparts (t = 2.99, p < .01; see Table 2). Kd values did not differ significantly between the two age groups. Similarly, 3H-CPP binding experiments in a separate group of naive animals indicated that maximal binding was significantly decreased in the aged hippocampus (t = 2.41, p < .05), with no significant age difference in affinity for 3H-CPP (this is also illustrated in Table 2). Furthermore, Bmax values for the trained aged animals correlated with the number of trials required to attain criterion (r = −0.58, p < .01). This modest, but significant, correlation indicates that larger decreases in 3H-CPP binding were associated with more pronounced learning deficits among the aged subjects. As Figure 3 illustrates, 3H-CPP binding for aged animals depended on how rapidly they acquired the task. Although 3H-CPP binding was similar in young and aged animals that required no more than 15 training trials to reach criterion, it was significantly reduced in the aged animals that required more extended training (p < .01).

**DISCUSSION**

Our initial characterization studies indicated that 3H-CPP binds to a single class of sites in a manner that is consistent with that of an NMDA receptor. In general,
the induction of LTP at perforant/dentate synapses in vivo and impairs spatial learning in the water maze (Morris et al., 1986). Thus, altered NMDA binding may contribute to a decline in the plastic properties and information storage capacity of hippocampal circuitry with age.

The present results do not indicate whether the loss of 3H-CPP binding is particularly pronounced in any subregion(s) of the hippocampal formation. There is normally a high concentration of NMDA binding sites in the dentate gyrus (Monaghan et al., 1985), sites that are presumably localized on neurons that are targets of perforant path innervation. This innervation becomes sparser in the aged rats' brain (Barnes, 1979), and a marked loss of perforant path innervation is observed in Alzheimer's Disease (Hyman, Van Hoesen, Damasio, & Barnes, 1984; Hyman, Van Hoesen, & Damasio, 1987). Thus, decreased 3H-CPP binding might occur in conjunction with perforant path denervation. There is also the possibility that a decrease in 3H-CPP binding in our homogenate assay reflects neuron loss in the hippocampus proper. Decreased numbers of neurons in the CA1 and CA3 regions of the hippocampus were previously observed in aged Long-Evans rats (Meaney, Aitken, van Berkel, Bhatnagar, & Sapolsky, 1988). In a more recent study, it was found that aged Long-Evans rats with spatial learning impairments had substantially greater cell loss in these regions.

These characterization data are in agreement with earlier reports (Murphy et al., 1987; Wong & Threlkeld, 1987). The $K_d$ values for 3H-CPP binding in our assay, however, were lower (almost 10-fold) than those reported by Murphy et al. (1987), although they were similar to those reported by Wong et al. (1987). This seems reasonable, since the majority of our assay conditions were similar to those of Wong and Threlkeld (1987). A recent study reported that unlabeled CPP was capable of displacing 3H-flunitrazepam binding in a filtration assay at lower concentrations than it displaced 3H-CPP in a centrifugation assay (White, Bender, & Swinyard, 1988). We found that unlabeled flunitrazepam competed for sites labeled by 3H-CPP with an average IC$_{50}$ of 889 nM (a 10-fold lower affinity than unlabeled CPP), using a centrifugation assay. This indicates that, under our assay conditions, it is unlikely that 3H-CPP binding represents labeling of benzodiazepine sites.

The two major findings of this study are that (1) maximal 3H-CPP binding in the hippocampal formation was markedly reduced in aged rats and (2) the magnitude of this reduction was related to the severity of the spatial learning deficit in the aged animals. Hippocampal 3H-CPP binding was reduced approximately 45% in aged animals whether they were naive or trained in the Morris water maze; in fact, at each age, 3H-CPP binding capacity was very similar for trained and naive rats. This indicates that training itself did not affect 3H-CPP binding. 3H-CPP binding, however, did correlate significantly with the number of trials to criterion for the aged subjects and was significantly lower in "impaired" aged rats than in both their "unimpaired" aged cohorts and young rats. Taken together, these data suggest that the reduction in hippocampal 3H-CPP binding may contribute to the spatial learning deficit observed in aged animals.

The decrease in hippocampal 3H-CPP binding in aged rats that were impaired in the water-maze task agrees with other work demonstrating that the function of NMDA receptors is critical in spatial learning tasks (Morris et al., 1986; Shapiro et al., in press; Robinson et al., 1989). It has further been suggested that hippocampal NMDA receptors may be important in spatial learning because they are necessary for the induction of long-term potentiation (LTP). This suggestion is based on the observation that a chronic regimen of AP5 administration blocks the induction of LTP at perforant/dentate synapses in vivo and impairs spatial learning in the water maze (Morris et al., 1986). Thus, altered NMDA binding may contribute to a decline in the plastic properties and information storage capacity of hippocampal circuitry with age.

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### Table 2

|          | Naive          | Trained         |
|----------|----------------|-----------------|
| Bmax     | Kd (nM)        | Bmax            |
| Young    | 2.47 (+/- .36) | 39.5 (+/- 7.5)  |
|          | 2.39 (+/- .26) | 45.8 (+/- 3.0)  |
| N=6      |                | N=8             |
| Aged     | 1.37 (+/- .29) | 24.2 (+/- 6.7)  |
|          | 1.33 (+/- .17) | 34.2 (+/- 5.1)  |

*Differs significantly from young, p < .05. **Differs significantly from young, p < .01.

| Group          | 3H-CPP BOUND (pmol/mg protein +/- s.e.m.) |
|----------------|------------------------------------------|
| Young          | ![Graph](image)                          |
| Aged Unimpaired| ![Graph](image)                          |
| Aged Impaired  | ![Graph](image)                          |

![Graph](image) Figure 3. Average B$_{max}$ values of 3H-CPP binding in young and aged animals that were trained in the water maze. All animals in the young group reached criterion in three probe trials. The aged "unimpaired" animals also reached criterion by the third probe trial, whereas the aged "impaired" group required an excess of three probe trials to reach criterion. Analysis of these data indicated a significant difference between groups [F(2,19) = 12.2, p < .001]. Post hoc comparisons revealed that B$_{max}$ values for the aged impaired group differed significantly from B$_{max}$ values for the young and aged unimpaired groups (ps < .01). The young and aged unimpaired groups, however, did not differ from one another.
than did either young rats or aged animals with a relatively intact spatial learning capacity (Issa, Gauthier, Rowe, & Meaney, 1989; M. Meaney, personal communication). The topography of NMDA binding in the aged brain could be resolved in further studies using in vitro autoradiography techniques. The present data suggest that defining the behavioral capacities of aged animals used in such an analysis would be highly desirable.

A growing number of studies indicate that age-related changes in neurobiological parameters within the hippocampal formation are associated with learning deficits on spatial tasks. These include the rate of decay for LTP (Barnes, 1979; Barnes et al., 1985), the rate of kindling (DeToledo-Morell et al., 1984), the complement of perforated synapses in the dentate gyrus (Geinesman, DeToledo-Morell, & Morell, 1986a, 1986b), neurochemical content (Jiang, Owyang, Hong, & Gallagher, 1989), and neural activity as reflected in altered hippocampal 2-deoxyglucose metabolism (Gage, Kelly, & Bjorklund, 1984). For each of these measures of hippocampal integrity and/or function, the severity of change in the aged brain bears a predictable relationship with the degree of spatial learning impairment. The present results provide added detail to an emerging role for hippocampal dysfunction underlying the mild/moderate cognitive impairments that often accompany normal aging.

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