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Density and distribution of NMDA receptors in the human hippocampus in Alzheimer's disease

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We examined the distribution and density of N-methyl-D-aspartate (NMDA) displaceable L-[^{3}H]glutamate binding sites in human hippocampal samples obtained postmortem from Alzheimer's disease (AD) patients and from age-matched controls. Binding to NMDA receptors was stable for at least 72 h postmortem, and the pharmacological profile corresponded to that described using electrophysiology. NMDA receptors were concentrated in the terminal fields of major hippocampal pathways including the perforant path, Schaffer collaterals and the hippocampal output to the subiculum, all of which are proposed to use an excitatory amino acid transmitter. Little if any change in hippocampal receptor density was observed in AD patients compared to age-matched controls except in one case where major hippocampal cell loss occurred. The distribution of NMDA receptors did, however, correspond to the predilection for neuritic plaques and neurofibrillary tangles in hippocampal subfields.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized pathologically by the presence of neurofibrillary tangles, neuritic plaques and neuronal cell loss in specific cortical and subcortical areas[1,2,12–16]. Several investigators have reported severe and consistent neuronal degeneration in the entorhinal cortex, subiculum and hippocampus[12–16], which is suggested to functionally disconnect the cortex from the hippocampus[13,14].

In previous studies we demonstrated that axon sprouting occurs in the dentate gyrus of the hippocampal formation in the brain of AD patients[8]. The loss of entorhinal afferents triggers compensatory growth by the remaining inputs to the dentate gyrus. We have postulated that this mechanism rebuilds the circuitry and slows the functional decline in this critical relay[3,4]. In order for this process to be operative, however, it is necessary that the appropriate postsynaptic receptors be present to serve these afferents.

The excitatory amino acids glutamate, aspartate and possibly related compounds are thought to be the transmitters of the entorhinal input to the hippocampus, of major intrahippocampal pathways, and of the hippocampal output to the subiculum[5,7,22]. Of particular relevance is the excitatory amino acid receptor which recognizes N-methyl-D-aspartate (NMDA), which is concentrated in the terminal fields of these major hippocampal pathways[10,19–22]. NMDA receptors are thought to mediate memory formation since NMDA receptor antagonists block long-term potentiation[11] (a presumed synaptic analogue of memory) as well as learning in a spatial task[23]. Thus, the presence of NMDA receptors is critical for normal memory functions.

We examined the distribution and density of NMDA receptors in the human hippocampus using a newly developed autoradiographic assay[20,21]. NMDA receptor distribution can be visualized using L-[^{3}H]glutamate and when binding conditions are optimized (low temperature, no CaCl, short incubation times, etc.), the NMDA binding site is preferentially labeled and the pharmacological profile of NMDA-displaceable L-[^{3}H]glutamate binding corresponds with that of NMDA receptors obtained in electrophysiological measures. Previously, Greenamyre and coworkers reported that L-[^{3}H]glutamate bind-

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ing sites, which had a low affinity for quisqualate and which were equated with NMDA receptors, decrease by about 50% in the cerebral cortex of AD patients[9] and are also markedly reduced in the hippocampus[34].

Human hippocampal samples were obtained postmortem from neurologically normal (5 males, one female, ages 55–74 years, postmortem delay 5–18 h) and from Alzheimer's disease (5 males, two females, ages 62–85 years, postmortem delay 4–29 h) patients. Alzheimer's disease diagnosis was confirmed by the presence of numerous neurofibrillary tangles and neuritic plaques using Bodian's or Bielshowsky's silver stain. At autopsy, the brain was divided midsagittally and one half was placed in Formalin for neuropathologic diagnosis and the other hemisphere dissected and frozen in either isopentane or powdered dry ice and stored at −70 °C. For autoradiograms, hippocampal tissue was cut in transverse sections (6 μm) in a cryostat and thaw-mounted onto gelatin and chrom-alum subbed slides. Representative 30 μm sections were also stained with Cresyl violet and for acetylcholinesterase (AChE) activity[24]. Autoradiography was performed according to the previously published methods[20,21] and the slides were placed against LKB ^{3}H-sensitive film along with brain-paste

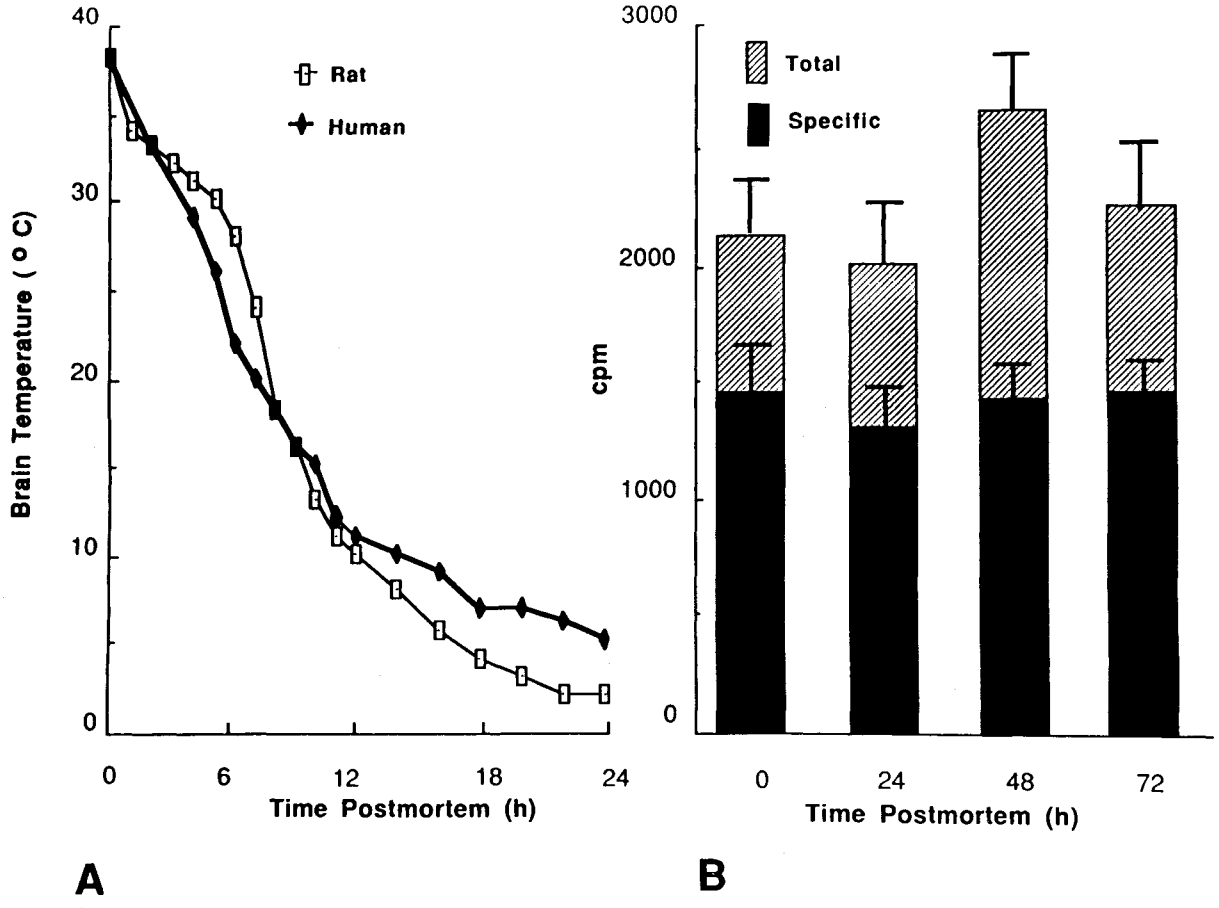

Fig. 1. A: approximation of the postmortem cooling rate of the human brain using a rat model. Data for the human brain cooling rate under normal mortuary conditions were obtained from ref. 31. Conditions for the rat model are described in the text. B: L-[^{3}H]glutamate binding in rat brain at various times postmortem. Conditions were such that the cooling rate of the rat brain approximated that observed in the human brain postmortem, as shown in A. Total binding represents the counts obtained from two coronal sections of rat brain containing the hippocampal region incubated under conditions described in the text, with no quisqualate or SITS added. Specific binding refers to total binding minus that observed in the presence of 100 μM NMDA. Values are the mean ± S.E.M. of 5 samples determined in triplicate. The non-specific binding largely represents binding to quisqualate receptors and to transport sites. Binding to these sites was minimized in autoradiographic studies by using ion-free buffers at 0–4 °C and by the inclusion of quisqualate (5 μM) and SITS (100 μM)[21].

standards or ^{3}H-standards (Amersham) for a period of 4–8 weeks. Autoradiograms were analyzed by computer-assisted image analysis (Spatial Data Systems). The distribution of specific binding was determined by subtracting the levels of binding found in various brain regions in the presence of 200 μM NMDA from the levels of total ligand binding in the respective regions. Inclusion of 5 μM quisqualate and 100 μM 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid (SITS, a chloride channel blocker) markedly reduced non-NMDA-specific binding but did not alter levels of NMDA-specific binding, thus allowing NMDA receptor binding to be visualized directly using L-[^{3}H]glutamate[21]. Chemicals were obtained from either Sigma (St. Louis, MO) or Tocris Chemicals (Essex, U.K.).

To permit quantitative comparisons of binding levels determined in human tissue samples with various postmortem times, we evaluated the postmortem stability of NMDA binding sites using a rat model in which the postmortem brain temperature cooling curve approximated that observed in human tissue[31] (Fig. 1A). Rats were sacrificed by cervical dislocation and placed into a styrofoam box containing a 500 ml bottle of water at 37 °C and covered with styrofoam chips. This was left at room temperature for 6 h and then placed in a refrigerator at 4 °C. Brain temperature was monitored using a thermistor inserted approximately 0.5 cm into the brain and connected to a chart recorder for continuous monitoring of temperature. Whole tissue sections were prepared and treated as described above, but instead of fully air-drying the tissue, the sections were wiped off the slide with Whatman GF/B glass fiber filters. The radioactivity in these sections was then determined by liquid scintillation spectrophotometry. No change was observed in NMDA-displaceable L-[^{3}H]glutamate binding up to 72 h postmortem (Fig. 1B). Thus, the distribution and density of NMDA receptors can be readily measured in the human brain obtained postmortem.

Electrophysiologically defined NMDA antagonists[11,21] were effective in blocking NMDA-displaceable L-[^{3}H]glutamate binding whereas similar compounds without agonist or antagonist activity were without effect (Table II). Moreover, displacers of L-[^{3}H]glutamate binding at other electrophysiologically defined glutamate receptor subclasses, including kainate (1 μM), amino-3-hydroxy-5-methyl-isoxazole (AMPA, 5 μM), quisqualate (5 μM), and the chloride channel blocker, SITS (100 μM), did not alter specific levels of binding. These results support the NMDA receptor identification of the NMDA-sensitive binding site and indicate that rodent and human NMDA receptors are pharmacologically similar with respect to these measures.

The distribution of NMDA sites in human hippocampus closely resembled that found in rat brain[19–22]. In brains of non-AD patients, highest binding levels were found in the stratum radiatum and stratum pyramidale of CA_1 and subiculum. Moderate binding densities were found in the dentate molecular layer, stratum oriens and stratum lacunosum-moleculare. Low levels of binding were observed in hilus and CA_3 of stratum radiatum and stratum pyramidale (Table I; Fig. 2A). In hippocampal samples obtained postmortem from Alzheimer's disease patients a similar binding profile was observed, with no significant differences in binding densities (Table I, Fig. 2B). Furthermore, no correlation was observed between

TABLE I

Density of NMDA-sensitive L-[^{3}H]glutamate binding sites in hippocampal subfields

Values represent the amount of L-[^{3}H]glutamate binding (fmol/mg protein) inhibited by the presence of 200 μM NMDA and are the mean ± S.E.M. for the average of 2–4 sections of 6 control and 7 Alzheimer's disease hippocampi. The dentate/CA_1 ratio is the average density of NMDA receptors in the dentate gyrus molecular layer compared to the average receptor density in CA_1 stratum radiatum and stratum pyramidale.

Region	*Control*	*Alzheimer's*
Dentate gyrus molecular layer		
Inner	337 ± 38	345 ± 39
Middle	288 ± 35	258 ± 37
Outer	179 ± 31	184 ± 33
Hilus		
CA_{4-2}	73 ± 9	83 ± 11
CA_{4-1}	117 ± 15	134 ± 13
Stratum radiatum		
CA_3	68 ± 12	98 ± 17
CA_1	406 ± 38	403 ± 72
Stratum pyramidale		
CA_3	103 ± 17	149 ± 26
CA_1	441 ± 49	391 ± 60
Stratum oriens		
CA_1	228 ± 47	188 ± 37
Stratum lacunosum-moleculare		
CA_1	161 ± 22	180 ± 29
Dentate/CA_1	0.66 ± 0.24	0.82 ± 0.24

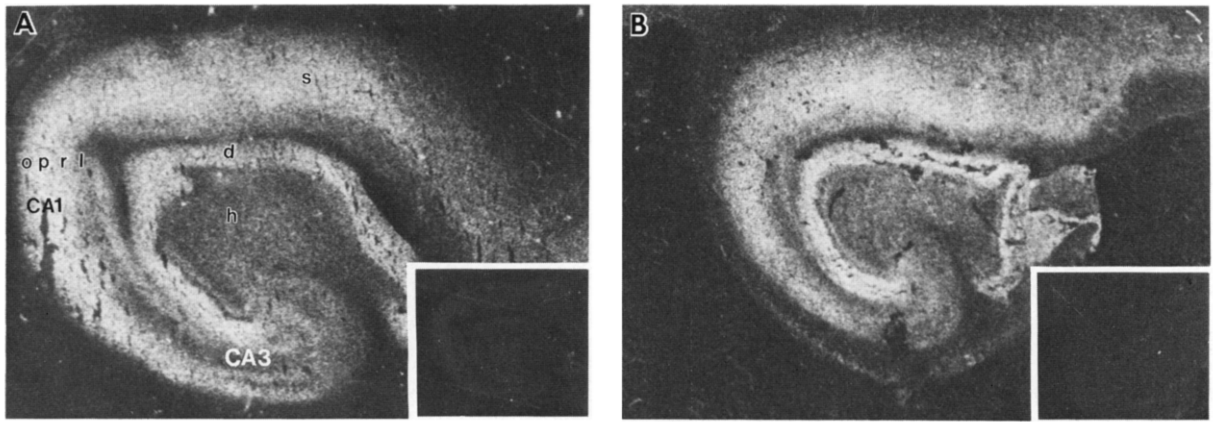

Fig. 2. Autoradiograms of NMDA-sensitive L-[^{3}H]glutamate binding sites in transverse hippocampal sections obtained postmortem from a control patient (A) and from an individual with Alzheimer's disease (B). Insets represent non-NMDA-specific binding in adjacent sections, determined in the presence of 200 μM NMDA. The autoradiograms were printed directly from Ultrofilm (LKB), with bright areas representing regions of high binding site density. d, dentate gyrus molecular layer; h, hilus; l, stratum lacunosum-moleculare; o, stratum oriens; p, stratum pyramidale; r, stratum radiatum; s, subiculum.

NMDA receptor density and age or postmortem delay ($r^2 = 0.166$ and 0.271, respectively, in CA_1 str. radiatum) and attempts to subdivide the AD group based on age of onset did not reveal any differences in the density or distribution of NMDA receptors. In one case, however, a 60% decrease in binding density in CA_1 was observed along with slightly increased binding levels in the dentate gyrus molecular layer. The ratio of binding levels in the dentate molecular layer vs CA_1 str. radiatum and str. pyramidale in the 6 other AD patients examined (0.60 ± 0.09) was very similar to that observed in 6 control cases (0.66 ± 0.24) whereas this ratio was 2.1 in the AD case noted above. Neuronal counts in CA_1 in this AD patient were the lowest of those examined, being decreased by over 50% compared to the other AD cases.

TABLE II

Comparison between the inhibitory potencies of a series of ω-phosphonic acid glutamate analogs on NMDA-sensitive L-[^{3}H]glutamate binding determined by quantitative autoradiography in CA_1 stratum radiatum of control human hippocampus using 100 μM of the phosphonate

AP-4, 2-amino-4-phosphono-butyrate; AP-5, 2-amino-5-phosphonopentanoate; AP-6, 2-amino-6-phosphonohexanoate; AP-7, 2-amino-7-phosphonoheptanoate; AP-8, 2-amino-8-phosphonooctanoate.

AP (X)	*% Inhibition*
AP-4	24.9
AP-5	78.7
AP-6	0
AP-7	96.2
AP-8	3.8

The results on the presence of NMDA sites in the majority of AD cases indicate that the receptors do not inevitably decline in the course of the disease. Receptor density is preserved except in cases where there is extremely severe cell loss. The similar density and distribution of NMDA receptors in hippocampal samples obtained postmortem from AD patients, as compared to age-matched controls, was surprising in view of the previously reported general loss of these receptors in the cortex[9] and hippocampus[34]. These contrasting results may be the result of differences of the severity of the disease in the two studies, or they may reflect different methods. Greenamyre et al. equated NMDA receptors with low-affinity quisqualate sites, which were defined as L-[^{3}H]glutamate binding sites not displaced by 2.5 μM quisqualate, measured in the presence of added Cl^- and Ca^{2+} ions[9,10,34]. Under similar conditions we have shown that over 50% of the Cl^--dependent, quisqualate-insensitive L-[^{3}H]glutamate binding is also insensitive to NMDA[19,22]. Evidence is accumulating that this Cl-enhanced, NMDA insensitive L-[^{3}H]glutamate binding represents a chloride-dependent high-affinity uptake or transport site rather than

a neuronal postsynaptic receptor[26,33]. It is therefore uncertain whether the previously reported loss of L-[^{3}H]glutamate binding in cortical regions of the Alzheimer's disease brain represented a loss of postsynaptic NMDA receptors, of Cl-dependent high-affinity uptake sites, or of both uptake sites and receptors. A loss of chloride-dependent glutamate transport sites would not be unexpected in view of the loss of sodium-dependent [^{3}H]D-aspartate uptake sites in AD[25].

Previously we reported that axon sprouting occurs in the hippocampus of AD patients[8]. As neuronal cells die, remaining healthy neurons sprout new connections which are postulated to augment the actions of residual fibers, or in some cases assume the functions of lost inputs[3,4]. For this compensatory response to be operative, the postsynaptic receptors which mediate the normal synaptic functions of the original circuitry must be present. The presence of NMDA receptors in AD in the appropriate hippocampal subfields suggests that the corresponding circuits have the capacity for long-term potentiation and memory formation and thus can participate in hippocampal plasticity.

NMDA receptors, however, are also involved in the progression of excitotoxic pathologies[27,32] and may play a similar role in AD. The pattern of hippocampal neuronal vulnerability in AD has previously been recognized to be similar to that in status epilepticus, hypoxia/ischemia and hypoglycemia[2]. In animal models of these latter disorders, high levels of the excitatory amino acids glutamate and aspartate have been shown to replicate the pattern of damage[30], as do excitotoxins acting at NMDA receptors[28]. Moreover, NMDA receptor antagonists effectively prevent the secondary neuronal damage resulting from epilepsy, ischemia and hypoglycemia[17,18,29]. The recent report that glutamate and aspartate induce paired helical filament formation in cultured human neurons implies that excitatory amino acids may be involved in the pathophysiology of AD[6].

In animal models, the vulnerability of cortical and subcortical regions to excitotoxic insults appears to correlate with NMDA receptor density[21,32]. The correspondence between NMDA receptor density and the predilection for AD-neuropathology in the human hippocampus is consistent with a possible involvement of excitotoxic mechanisms in the pathogenesis of this disease. NMDA receptors are concentrated in CA_1, subiculum, and in the outer layers of the entorhinal cortex, all of which consistently have a high number of tangles, granulovacular degeneration, and Hirano bodies in AD[2]. Furthermore, the dentate gyrus molecular layer, also rich in NMDA receptors, has a predilection for neuritic plaques[15]. If excitotoxic mechanisms do contribute to the pathophysiology of Alzheimer's disease, NMDA receptor antagonists represent potential therapeutic agents.

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Note added in proof

The loss of NMDA receptors associated with severe cell loss in CA, has been confirmed in 3 additional AD cases.