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NMDA RECEPTORS IN FRONTAL CORTEX AND HIPPOCAMPUS OF ALCOHOL CONSUMERS

Eulalia Villegas^{1*}, Ramon Estruch^{1,2}, Guadalupe Mengod³ & Roser Cortés³

1) Department of Internal Medicine, Hospital Clínic, Barcelona, Spain

2) CIBEROBN Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Spain

3) Department of Neurochemistry and Neuropharmacology, IIBB, CSIC-IDIBAPS, CIBERNED, Barcelona, Spain.

Correspondence to:

*Eulalia Villegas, M.D. Current address:

Hospital Dos de Maig

Dos de Maig 301

08025 Barcelona, Spain

E-mail: eulalia.villegas@sanitatintegral.org

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Key words: Alcoholism, glutamate receptors, human brain, MK801, radioligand binding, receptor autoradiography.

ABSTRACT

Specific binding of [³H]MK801 to N-methyl-D-aspartate (NMDA) receptors in the frontal cortex and hippocampus (CA1 and gyrus dentatus) was measured by receptor autoradiography in 16 Caucasian chronic alcohol consumers free of clinical manifestations of alcoholism, and compared to 16 Caucasian control subjects. Binding densities were not significantly different between heavy and moderate drinkers, neither between alcohol consumers that were abstinent or non-abstinent before death, nor between ethanol drinkers and controls. Continued alcohol consumption, in the absence of hepatic, neurologic or psychiatric disorders related to alcoholism, does not alter the binding properties of NMDA receptors in the brain areas studied.

Running head short title: "NMDA receptors in alcohol consumers"

A number of studies have suggested that, at least partially, alcohol-induced brain damage associated to chronic alcohol consumption is mediated through changes in glutamatergic NMDA receptors. This is supported, by the effects of drugs such as acamprosate, a partial NMDA antagonist used in relapse prevention in alcohol-dependent patients (Hammarberg et al., 2009). Only few studies have addressed the analysis of glutamate receptors in relation to alcohol abuse in humans and have provided controversial results (Cummings *et al.* 1990; Dodd *et al.* 1992; Freund & Anderson 1996, 1999; Ridge *et al.* 2008). Our hypothesis is that permanent neuronal damage produced by chronic alcohol consumption is in part due to progressive changes in NMDA receptors, and that these changes might develop before the beginning of clinical manifestations of chronic alcoholism, including dependence, craving and abstinence. Thus, we assessed here the density of NMDA receptors in frontal cortex and hippocampus –brain areas known to be notably affected by chronic alcohol consumption– in a group of 16 alcohol consumers (AC) (16 male, mean age 58 ± 12 years, post-mortem delay 19 ± 8 hours) in comparison to a control group of 16 non-drinkers (14 male and 2 female, mean age 60 ± 12 years, post-mortem delay 17 ± 5 hours) using autoradiographic techniques with the specific NMDA-antagonist [^3H]MK801. AC subjects included had not suffered any hepatic, neurologic or psychiatric disorders related to alcoholism. They were classified as moderate consumers (40 to 80 g of absolute alcohol daily for at least 10 years) or heavy consumers (more than 80 g of absolute alcohol daily for at least 10 years, enough to develop chronic alcoholic pathology). We also discriminated between AC cases that were abstinent (alcohol withdrawal minimum one week before death) or non-abstinent (last ethanol ingestion less than 12 hours before death). In consequence, it is unlikely that these subjects would undergo a withdrawal situation that could eventually involve acute changes in NMDA receptors (See **Supplementary Material** for additional information).

Densities of [^3H]MK-801 binding were measured by microdensitometry on film autoradiograms in frontal cortex (layers II-VI), and CA1 (oriens and pyramidal layers) and gyrus dentatus (GD, molecular layer) of the hippocampus (Fig. 1), and are summarized in Table 1. Statistical analysis (unpaired t-test, GraphPad Prism) revealed the absence of significant differences between the different

groups analyzed. Covariance analysis (SPSS for Windows) indicated no influence of liver fibrosis in our sample (See **Supplementary Material** for detailed data).

Our results suggest that binding to frontal cortex and hippocampal NMDA receptors remains unaltered after chronic consumption of high doses of alcohol in the absence of clinical manifestations of alcoholism. Previous studies on hippocampal homogenates reported higher densities of [³H]glutamate binding in 5 alcoholics compared to 3 controls, with concomitant decreases in specific NMDA binding (Michaelis *et al.* 1990, 1993). Other authors (Dodd *et al.* 1992) found no differences in [³H]MK801 binding in cortical membrane preparations between 10 alcoholics and 6 controls. Finally, Ridge *et al.* (2008) detected decreases in the expression of NR1, NR2A and NR2B NMDA receptor subunits in cerebral cortex only in cirrhotic alcoholics, but not in alcoholics without hepatic disease. Using receptor autoradiography, Freund & Anderson (1996, 1999) found significant increments in binding in frontal cortex (13 abstinent alcoholics and 13 controls) when using [³H]glutamate and the selective NMDA antagonist [³H]CGP39653. On the contrary, Cummins *et al.* (1990) showed reduced [³H]glutamate binding in hippocampus from 10 abstinent alcoholics.

Different reasons could explain the discrepancies between those data and our study. Clinical variations related to comorbidity such sudden death, cirrhosis, acute hypoxia or pneumonia could eventually alter glutamate binding (Piggott *et al.* 1992; Freund & Anderson 1996; Ridge *et al.* 2008). In our sample, we did not find an influence of liver fibrosis (a necropsy finding without previous clinical manifestations), supporting the study of Ridge *et al.* (2008) that suggests that hepatopathy, but not alcohol *per se*, could be the cause of changes in NMDA receptors.

Another possible explanation is related to the ligand used. MK-801 binds inside the previously opened ion channel of the NMDA receptor and it has been demonstrated that ethanol diminishes [³H]MK801 binding only in the absence of glycine, a modulator of channel aperture (Freund & Anderson, 1996). Since we used intact, fresh-frozen brain tissue, it is possible that glycine present in our sections might interfere with the binding of [³H]MK801 and therefore mask putative alterations present in samples from chronic alcohol consumers.

It might be considered that ethanol could interact with NMDA receptors in a manner not measurable by autoradiographic techniques. For instance, a decrease in specific NMDA receptor subunits could eventually be compensated by an accelerated synthesis of mRNA encoding the proteins involved. Finally, it could be speculated that ethanol modifies other cellular events, such as the migration of receptor subunits from extrasynaptic sites in the plasmatic membrane to synaptic functional loci, or receptor assembly, among others.

Thus, a number of fundamental questions remain open, and further studies on a greater number of human brain samples are necessary. Combined studies of histological assessment with autoradiography with different ligands and in situ hybridization can yield information on the effects of chronic ethanol consumption on functional mature NMDA receptors and some of their subunits.

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Table 1. Densities of [³H]MK 801 binding in controls and alcohol consumers.

Data are mean densities of specific [³H]MK 801 binding expressed in fmol/mg tissue (mean±SD). No statistically significant differences were found among the different groups.

Group	Frontal cortex	Dentate gyrus	CA1
Controls	71.62 ± 17.89 (n=16)	147.83 ± 34.94 (n=15)	173.18 ± 49.65 (n=15)
Alcohol consumers (AC) all	82.94 ± 20.57 (n=15)	139.26 ± 38.97 (n=13)	191.66 ± 70.19 (n=13)
>80 g/day consumers	81.16 ± 16.25 (n=11)	131.28 ± 36.91 (n=11)	187.18 ± 75.94 (n=11)
40-80 g/day consumers	87.86 ± 32.39 (n=4)	183.14 ± 6.92 (n=2)	216.29 ± 4.61 (n=2)
Abstinent	79.71 ± 4.35 (n=3)	124.36 ± 18.93 (n=4)	165.75 ± 42.56 (n=4)
Non-abstinent	83.75 ± 23.05 (n=12)	145.88 ± 44.53 (n=9)	203.17 ± 78.90 (n=9)

Figure I. Autoradiographic images showing the total (A) and non-specific (B) binding of $[^3\text{H}]\text{MK801}$ in the hippocampus of a control subject. CA1: CA1 field; DG: dentate gyrus; PH: parahippocampal gyrus; wm: white matter. Bar= 4mm.

Supplementary Material

Cases. Studies were performed on 32 Caucasian subjects, 16 chronic alcohol consumers (AC) and 16 psychiatrically normal controls. Data related to alcohol consumption were obtained from medical records and confirmed by reports from close family members. History of alcohol consumption including length of abstinence before death was registered. All AC selected had not been not previously diagnosed of any alcohol associated disorder. Consequently, none fulfilled any criteria of alcohol dependence (DSM-IV) or diseases associated to alcoholism (ICD-10). Patients with a known history of neurological or psychiatric disorders or intravenous drug abuse were excluded. Detailed information on the individual characteristics of subjects, including alcohol consumption data, is summarized in Supplementary Table 1. Storage time of tissues was in all cases lower than three years. One case died after a gastrointestinal haemorrhage but did not present alterations in mesenteric and portal circulation that could suggest that the bleeding was from hepatic origin, as observed at autopsy.

Collection of brain samples and preparation for autoradiography. Brains were collected at autopsy at the Department of Pathology of the Hospital Clinic and the Forensic Anatomical Institute of Barcelona (Spain) within the first 28 hours after death. A standardized neuropathological evaluation was done on formalin-fixed blocks of each brain. In the AC group, no anatomical evidences of Wernicke-Korsakoff syndrome were found.

Once the brains were removed from the cranium, the right hemisphere was cut into approximately 2 cm-thick blocks, which were immediately frozen on dry ice and kept at -80°C. Coronal sections (14 µm thick) were obtained from frozen blocks of frontal cortex and hippocampus using a microtome-cryostat (Leitz 1720, Germany), thaw-mounted onto glass slides previously coated with APTS and kept at -20°C until use. Experiments were run following a matched-pair design so that each pair included sections from one alcoholic brain and its matched control.

Supplementary material Table 1. Summary of cases

Case	Age (years)	Sex	PMI (hours)	Alcohol (g/day)	Smoker	Ethanol abstinence before death	Co-morbidity	Pathological changes in necropsy	Cause of death
C1	45	M	23.5	None	Yes	–	–	–	Accident
C2	43	M	23.5	None	Yes	–	–	–	CHD
C3	69	M	13.0	None	Yes	–	–	–	CRA
C4	74	M	13.5	None	Yes	–	CHD	–	PE
C5	67	M	9.5	None	Yes	–	–	–	CHD
C6	47	M	16.0	None	Yes	–	Hepatitis C + HT	–	CHD
C7	59	M	6.0	None	None	–	Pulmonar fibrosis	–	CRA
C8	43	W	20.0	None	None	–	–	-	Cancer
C9	65	M	15.0	None	None	–	–	Aortic aneurism	CHD
C10	74	M	16.0	None	Yes	–	CHD	–	CHD
C11	47	M	16.0	None	None	–	–	–	CHD
C12	72	M	20.0	None	Yes	–	–	–	CHD
C13	66	W	22.0	None	Yes	–	HT	–	CHD
C14	59	M	12.0	None	Yes	–	CHD	CHD	CHD
C15	62	M	22.0	None	Yes	–	CHD	CHD	CHD
C16	70	M	19.0	None	Yes	–	–	–	CHD
A1	49	M	5.5	40-80	No	NO	–	–	Traumatism
A2	57	M	27.0	>80	No	NO	Tuberculosis	–	CRA
A3	44	M	21.0	>80	Yes	YES	COPD	–	CRA
A4	67	M	16.0	>80	Yes	YES	–	Liver fibrosis	CRA
A5	50	M	28.0	>80	No	NO	–	–	Sudden death
A6	66	M	23.0	40-80	Yes	NO	Diabetes mellitus	–	Sudden death
A7	34	M	28.0	>80	None	NO	–	–	CRA
A8	43	M	24.0	>80	Yes	NO	–	–	CRA
A9	66	M	11.0	>80	None	NO	Diabetes mellitus	–	Traumatism
A10	72	M	20.0	40-80	Yes	NO	HT	–	CHD
A11	74	M	24.5	40-80	None	NO	–	Liver fibrosis	Sudden death
A12	51	M	21.5	>80	Yes	NO	–	–	Sudden death
A13	63	M	23.5	>80	None	NO	CHD	–	Sudden death
A14	56	M	10.0	>80	Yes	YES	–	Liver fibrosis	CHD
A15	73	M	14.0	>80	Yes	NO	COPD	–	GIH
A16	64	M	6.0	>80	None	YES	–	–	CHD

(Supplementary table 1): A: Alcohol consumers; C: Controls; CHD: Coronary heart disease; COPD: Chronic obstructive pulmonary disease; CRA: Cardio-respiratory arrest; GIH: Gastrointestinal hemorrhage; HC: Hepatic cirrhosis; HT: Hypertension; M: Man; PE: Pulmonary embolism.; PMI: Postmortem interval; W: Woman

Quantitative receptor autoradiography for NMDA receptor binding.

Incubation with [^3H]MK-801 was performed as described previously (Gulya *et al.*, 1991). For each brain block, three consecutive sections were used, two for the determination of total binding and one for the nonspecific binding. Slide-mounted tissue sections were preincubated for 30 min in 50 mM Tris-HCl buffer (pH 7.4 at 25 °C). Afterwards the slides were incubated for 120 min at 25 °C in Tris-HCl buffer containing 20 nM [^3H]MK801 (250.63 Ci/mmol; NEN DuPont, Boston, MA, USA). Nonspecific binding was determined in the presence of 2 μM unlabeled MK801. After incubation, the slides were given three 1-min rinses in Tris-HCl buffer. The sections were then rapidly dried under a stream of cold air.

Autoradiograms were obtained by apposing the sections against ^3H -sensitive film (Amersham Hyperfilm-[^3H], Amersham, UK) for 5 weeks at 4 °C. The film was co-exposed with sets of tritiated microscale standards (calibrated in nCi/mg tissue) (^3H -Microscales, Amersham). The autoradiograms were developed and fixed with Kodak developer and fixer at 18 °C. Sections were also stained with cresyl violet for anatomical reference and identification of the structures.

The densities of [^3H]MK801 binding were measured by microdensitometry in layers II-VI of the frontal cortex, the molecular layer of the gyrus dentatus, and oriens and pyramidal layers of the CA1 hippocampal field using a computerized image analysis system (MCID-M4, Imaging Research Inc., St. Catharines, Ontario, Canada). Standardization curves derived from the tritiated standards were used to convert optical density readings of the autoradiograms into nCi/mg of tissue equivalents and transformed to fmol of bound [^3H]MK801/mg protein. Non-specific binding was homogeneous and low in all regions. Images were enlarged ca. x20 with a zoom to obtain a resolution that could allow discriminating among hippocampal layers. Five measures were taken from each structure in every sample and the corresponding means were considered as

single density data points. The non-specific binding was subtracted from the mean of total binding densities obtained in duplicate sections.

Statistical analysis. We used descriptive statistics with means and standard deviations for the baseline characteristics of the patients. Comparisons of binding densities between groups were done using the unpaired t-test, since the variables showed a normal distribution according to the results of the Kolmogorov-Smirnov test (GraphPad, Prism). All statistical tests were 2-tailed, and the significance level was 0.05.

In order to rule out significant changes on binding data secondary to variables such as age, gender, PMI, storage time, alcohol consumption, hepatic fibrosis, benzodiazepine consumption, smoking history, sudden death, we performed a covariance analysis for the three regions studied (SPSS 14.0, SPSS Inc., Chicago, Illinois).

Subject clinical data

Covariance analysis of our data indicated that consumption low doses of benzodiazepines at night for sleeping disturbances reported for two cases, had no influence on binding data. According to this analysis there was an effect of sudden death on the statistical results which would need to be further confirmed.

Alcohol abstinence:

Several studies have shown that chronic ethanol ingestion produces increases in MK-801 binding to NMDA receptors in rat brain, and that the persistence of changes in MK-801 binding runs in parallel with the time-course for ethanol withdrawal seizure susceptibility, usually 24 hours (Gulya et al. 1991). In contrast, the human timing for the development of clinical withdrawal is more prolonged. The clinical manifestations can begin in a period between few hours and two days after alcohol cessation and then increment during the next days, when the patient can suffer severe symptomatology such as delirium tremens (MacKeon et al. 2008). Due to these chronologic differences between human and rodents, we considered alcohol consumers as non-abstinents when they had ingested alcohol less than 12 h before death, since it is unlikely that they

would undergo a withdrawal situation that could eventually involve acute changes in NMDA receptors.

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