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No nerve growth factor response to treatment with memantine in adult rats

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Running Headline:

Nerve growth factor and memantine treatment

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Summary

Nerve growth factor (NGF) is the most widely examined neurotrophin in the experimental models of Alzheimer´s disease (AD) and has been shown to prevent the retrograde degeneration of cholinergic neurons. In this study we examined NGF and cholineacetyltransferase (ChAT) changes in several rat brain regions after excitotoxic lesion of the entorhinal cortex with quinolinic acid and tested the effect of memantine on spatial learning in the radial maze after lesion. We observed a significant increase (+26%, $p=0.02$) of NGF concentrations in the hippocampus of the lesioned rats when compared to sham-lesioned rats. Chronic treatment with memantine showed no significant effect on the NGF increase in the hippocampus ($p=0.72$). The ChAT activity was significantly increased in the lesioned rats when compared to controls (+16%,$p<0.05$) and did not depend on treatment with memantine. In spite of this, memantine improved performance of the radial maze. This indicates that memory improving effects of memantine observed in experimental animals and in clinical studies are probably not related to changes in brain NGF content, whereas the observed NGF increase in the denervated hippocampus is probably trauma-related reflecting impaired retrograde transport of hippocampal NGF.

Key words:

Nerve growth factor (NGF), memantine, choline acetyltransferase (ChAT), memory, excitotoxic lesion

Introduction

Glutamate is involved in learning and memory processes and developmental plasticity (Riedel et al. 2003). The neuroprotective potential of both competitive and uncompetitive NMDA receptor antagonists have been confirmed in numerous studies (Meldrum and Garthwaite 1990). Therefore memantine, a partial NMDA receptor channel blocker has been used for several years for the treatment of dementia and positive effects on cognitive as well as behavioural symptoms have been reported (Parsons et al. 1999, Reisberg et al. 2003).

The major glutamatergic input to the hippocampus originates in the entorhinal cortex which is one of the early regions affected by Alzheimer´s disease (AD), that could play a predominant role in the initial pathological cascade of the disease (Hyman et al. 1986). In rats, lesioning of this area with quinolinic acid, a selective agonist of the NMDA-subtype of glutamate receptors which has excitotoxic properties, leads to spatial memory deficit such as working memory (WM) impairment and reduced acquisition of reference memory (RM) in the radial maze (Zajaczkowski and Danysz 1997, Zajaczkowski et al. 1996). Memantine has been shown to improve performance in radial maze of animals with such cortical lesion (Zajaczkowski et al. 1996). Since in the study by Zajaczkowski and colleagues the effect of memantine was not immediate but required 3-4 days to develop, it was hypothesized in this article that neuronal growth factors might have contributed to this effect.

Nerve growth factor (NGF) is the most widely examined neurotrophin in experimental models of AD. Studies performed in aged rats demonstrated that NGF infusions could reverse age-associated declines in basal forebrain cholinergic neurons and correct spatial memory deficits (Fischer et al. 1987). Thus, NGF has been discussed to play a critical role in neuroregeneration and plasticity of the basal forebrain (Hörtnagl and Hellweg 1997, Hellweg et al. 1998).

In this study we examined NGF changes in several brain regions after excitotoxic lesion with quinolinic acid, which is a glutamatergic denervation model resembling some aspects of AD (Myhrer 1993) with an attempt to correlate these changes to improved performance seen during memantine treatment. Since NGF stimulates the expression and activity of cholineacetyltransferase (ChAT) (Hellweg et al. 1998, Siegel and Chauhan 2000), the ChAT activity in the hippocampus, septum and frontal cortex was also studied.

Material and Methods

Animals

37 male Sprague-Dawley-Rats (250-280g) were used for the experiments. The animals were housed under standard conditions (22°C) on a 12 h light, 12 h dark cycle and had free access to tap water, laboratory chow for the time of learn training was reduced to 15g/day. Ethical approval of the experimental protocol was obtained from the *Regierungspräsidium Darmstadt* (II 17a-19c 20/15 project: F-77-42)

Surgical procedures

The lesion method has been described in detail before. After decapitation of the animals, CNS tissue samples of anterior cortex, posterior cortex, hippocampus and striatum were stored at -70°C until homogenization.

Homogenization procedure

Each frozen tissue sample of the various brain regions was homogenized by ultrasonication in 10-20 vol of deionized water at +4°C after addition of 2 ml homogenate buffer (0.1 M Tris/HCl; 0.4 NaCl; pH 7.0; 0.1% NaN_3 (acid) and a variety of protease inhibitors and restored immediately at –70°C; for details see.

Pharmacological treatment with memantine

Alzet osmotic minipumps for delivering drugs s.c. were implanted on the back of the animals under Hypnorm anaesthesia (0.04 ml/100g i.m.) 7-13 days after lesioning (4 days before the training). Animals that did not receive drugs underwent the complete surgical procedure but pumps were not actually implanted. Memantine (20 mg/kg/per day) was dissolved in 0.9% sodium chloride solution and delivered with Alzet minipumps (with an infusion rate of 5.0 and 0.5 µl/h, respectively). The serum concentrations of memantine used in this study were in the therapeutical range (1,2 µM) (Zajaczkowski et al. 1996).

Memory training

One week before surgery the animals were handled (2 days) and allowed to adapt to the apparatus (3 days). In the habituation phase on the third day the rats were placed once in the maze in groups of four to five for 5 min. and food pellets were scattered randomly throughout the maze (four to five pellets per rat). In the training phase on the fourth day the food cups were placed at the end of each arm. The four randomly selected arms were baited with one pellet of food each. Rats were placed individually into the center of the maze and remained inside the maze until they had collected all food pellets but not for longer than 5 min. Rats

were given three to five trials, depending on their performance (collection of all pellets within 5 min). On the fifth day the same procedure was repeated except that the rats were placed in the center of the maze in the plastic tube, which was then removed after 15s. The exclusion criterion of 10 min. was applied at every trial.

Surgery was performed 4-6 days after adaptation as described above. Training began 9-13 days after surgery. Rats were placed into the tube that was removed after 15 s. In each trial four of the arms were baited with one pellet of food. The position of the baited arms was left unchanged during the entire experiment. Each rat was given four trials daily for 12 days. The rats were observed by a video camera and each entry with all four paws into each arm was scored. The rats remained inside the maze until they had collected all food pellets until 10 min had elapsed, whichever came first. Rats requiring more than 10 min to finish the trial were excluded from further experiments. Two types of errors were scored: working memory errors, i.e. entry into an arm already visited during the same trial, and reference memory errors, i.e., each entry into an arm that was never baited. Additionally the time spent by the rat in the maze during each trial was measured for evaluation of general activity including anxiety and motivation.

Determination of NGF

NGF levels in the re-thawed homogenates were determined by a fluorometric, enzyme-linked immunoabsorbent assay (ELISA) with a detection limit of 50 fg NGF/assay as described formerly. The previous protocol was simplified as described elsewhere.

Determination of ChAT activity

The activity of ChAT was determined radiochemically, with several modifications which have been described previously.

Statistical analysis

Frequency of working memory errors and frequency of reference memory errors were evaluated. The data were analysed by two way analysis of variance (ANOVA; treatment and blocks as factors) followed, if significant, by the Newman Keuls test for pairwise comparisons (SigmaStat software, Jandel Scientific). The number of rats in each group was seven to ten.

Statistical analysis of NGF values was performed by a two way analysis of variance (ANOVA; lesion and memantine as factors); if significant Newman Keuls test was used for post hoc analysis. Significance was accepted at $p<0.05$. All data are shown as means ± SEM.

Results

Effects of chronic treatment with memantine on working and reference memory in rats with lesioned entorhinal cortex

No significant difference occurred between errors of untreated and memantine treated control (without lesion) animals for the working and reference memory (Figs 1 and 2). Also no significant difference could be detected between sham-lesioned and non-lesioned animals. All lesioned rats had significantly more errors when compared to controls and sham-lesioned animals. Also, in lesioned animals, there was a positive effect of a treatment with memantine on reference memory but not working memory errors. At the end of the training (after 12 days) this difference between the groups (except lesion alone) could not be detected any more.

NGF concentrations in several brain regions after lesion of the entorhinal cortex and a 14 day treatment with memantine

A two way analysis of variance showed that the lesion method had a significant effect ($p=0.0205$) on the NGF concentration in the hippocampus ($p<0.05$) while the treatment with memantine had no effect ($p=0.725$). The a posteriori performed student Newman keuls test revealed a significant difference in the hippocampal NGF concentration between lesioned and sham-lesioned rats ($p<0.05$, +26%), while the other pairwise differences were statistically not significant (see Figure 3).

ChAT-activity in the hippocampus, frontal cortex and septum after a entorhinal lesion and a 14 day treatment with memantine

ChAT-activity (μmol/min/mg) has been detected in homogenates of hippocampus, frontal cortex and septum after lesion of the entorhinal cortex and a 14 day treatment with memantine. No significant difference was detected between sham-lesioned and lesioned animals both in terms of memory training and NGF concentrations. Thus, for the statistical analysis of the ChAT activity these animals were considered as one control group. For the concentrations in the hippocampus a two way analysis of variance revealed the lesion method has a significant effect ($p=0.0314$, $df=1$), while the factor treatment had no significant effect ($p=0.1692$, $df=1$). The ChAT activity in the hippocampus was significantly increased (+16%) in the lesioned rats ($p<0.05$) when compared to controls (sham lesioned and untreated). For the concentration of ChAT in the frontal cortex a two way analysis of variance showed that the lesion method ($p=0.0133$, $df=1$) and the treatment ($p=0.0288$, $df=1$) showed a significant effect. A significant ($p<0.05$) increase of ChAT activity in the frontal cortex of lesioned rats was found by up to 11%, when compared to control animals (see Figure 4).

Discussion

Convincing evidence indicates that the function of the cholinergic septohippocampal system is intimately linked to the presence of endogenous NGF (Van der Zee et al. 1995) and that after entorhinal cortex lesion septal cholinergic plasticity seems to be related to changes of NGF expression (Van der Zee et al. 1992, Conner et al. 1994). In particular, NGF is known to increase ChAT activity and expression in cholinergic neurons of the basal forebrain (Thoenen et al. 1987, Scott und Crutcher 1994). The basal synthesis of NGF was shown to be activity-dependently regulated by NMDA-receptors (Zafra et al. 1991). However, in this study we found no influence of 14 day memantine treatment on NGF protein concentrations in comparison to untreated animals in lesioned and non-lesioned rats.

ChAT activity measured in the hippocampus, frontal cortex and septum was unchanged except for a significant increase in the hippocampus of lesioned animals. This finding is in line with observations of an increase of ChAT activity in the hippocampus following lesion of the entorhinal afferents (Storm-Mathisen 1974). We hypothesize that NGF, which exerts neurotrophic actions on cholinergic neurons of the basal forebrain and regulates ChAT activity, could be responsible for the observed ChAT increase. The entorhinal cortex lesion might possibly induce only minor changes of the NGF protein concentration, which probably failed to be detected, as the projections from the entorhinal cortex to the frontal cortex are widely ramified.

However, not only the entorhinal cortex lesion increased the ChAT activity in the present study, but also chronic treatment with memantine brought about a significant increase of ChAT activity in the frontal cortex (+11%). As memantine showed no direct influence on NGF concentrations investigated so far, it is noteworthy that memantine and NGF increased the cholinergic neurotransmission probably in an independent manner. Memantine led to a significant increase of ChAT activity in the present study independently of an entorhinal cortex lesion. These results are in line with the hypothesis that the glutamatergic system influences the function of cholinergic neurons in the central nervous system via NMDA-receptors (Wenk et al. 1997, Giovannini et al. 1994, Hasegawa et al. 1993). In summary, an increase of the cholinergic neurotransmission can be regulated by NGF and memantine in a synergistic manner probably via different mechanisms.

Numerous studies have shown that the cholinergic neurons in the basal forebrain are predominantly involved in short term and spatial memory formation and attention (Hörtnagl and Hellweg 1997). Thus, an increased cholinergic neurotransmission, as observed in the present study after treatment with memantine could well contribute to its positive effect on learning in the lesioned rats.
In the present study we found an increase of NGF protein concentration in the hippocampus after lesion of the entorhinal cortex, which is in line with several other studies showing an increase of NGF after different cerebral lesions. The NGF increase seems to be part of a cascade of events after damage of the adult brain, possibly reflecting neuronal plasticity (Lindvall et al. 1994, Hellweg et al. 1998).

In conclusion a memantine treatment-related increase of the cerebral NGF content could not be shown in the present study, thus NGF alterations are unlikely to contribute to the cognitive effects of memantine. However, the present data suggest that increase in cholinergic activity after memantine treatment might play such a role in lesioned rat brain.

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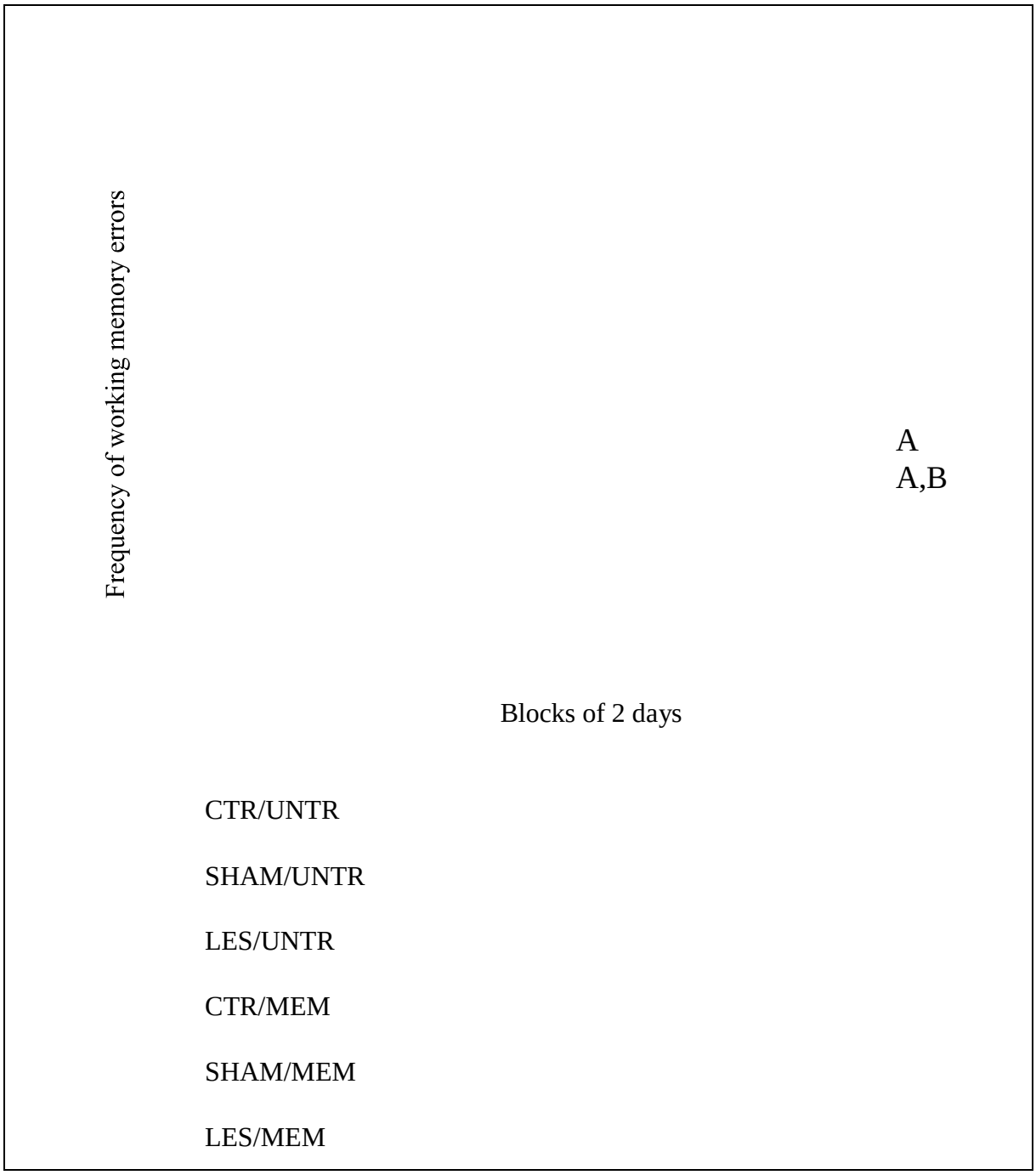

Figure 1:
The effect of chronic infusion of memantine (20mg/kg per day) on working memory by rats in the radial maze after bilateral lesion of the entorrhinal cortex. **A** shows a significant effect ($p<0.05$) when compared to untreated controls. **B** shows a significant effect ($p<0.05$) when compared with lesioned rats without memantine treatment.
CTR/UNTR: control animals without memantine treatment. **SHAM/UNTR**: shamlesioned animals without memantine treatment; **LES/UNTR**: lesioned animals without memantine treatment. **CTR/MEM**: Control animals with memantine treatment. **SHAM/MEM**: sham-lesioned animals with memantine treatment. **LES/MEM**: lesioned animals with memantine treatment.

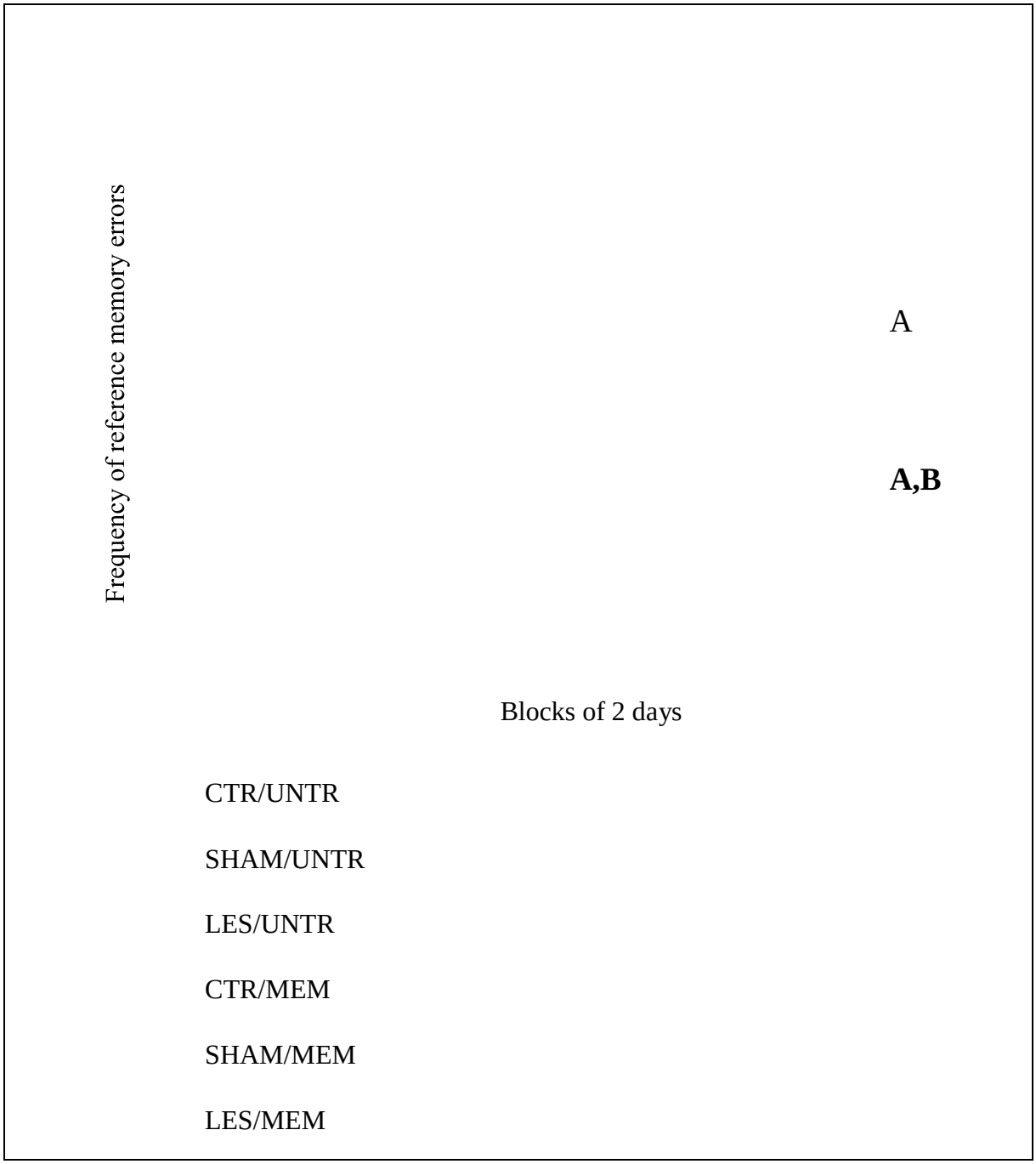

Figure 2:
The effect of chronic infusion of memantine (20mg/kg per day) on working memory by rats in the radial maze after bilateral lesion of the entorrhinal cortex. **A** shows a significant effect ($p<0.05$) when compared to untreated controls. **B** shows a significant effect ($p<0.05$) when compared with lesioned rats without memantine treatment.
CTR/UNTR: control animals without memantine treatment. **SHAM/UNTR**: shamlesioned animals without memantine treatment; **LES/UNTR**: lesioned animals without memantine treatment. **CTR/MEM**: Control animals with memantine treatment. **SHAM/MEM**: sham-lesioned animals with memantine treatment. **LES/MEM**: lesioned animals with memantine treatment.

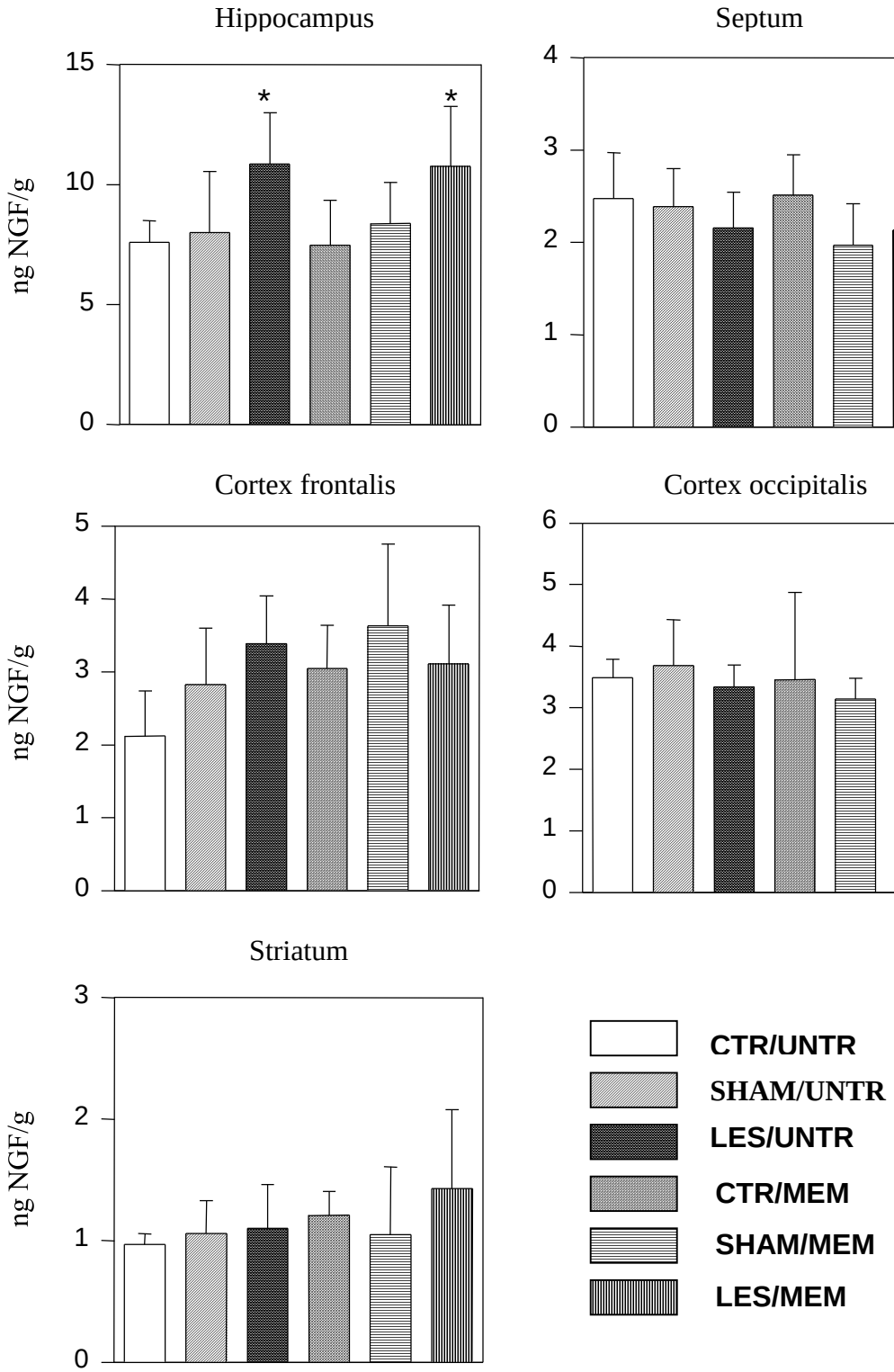

Figure 3:
Nerve growth factor concentrations (ng/g) in the hippocampus, septum, frontal cortex, occipital cortex and striatum after bilateral lesion of the entorhinal cortex and a 14 day treatment with memantine. (MEM; 20mg/kg/24h); * significant difference when compared to sham lesioned animals. **CTR/UNTR**: control animals without memantine treatment. **SHAM/UNTR**: shamlesioned animals without memantine treatment; **LES/UNTR**: lesioned animals without memantine treatment. **CTR/MEM**: Control animals with memantine treatment. **SHAM/MEM**: sham-lesioned animals with memantine treatment. **LES/MEM**: lesioned animals with memantine treatment.

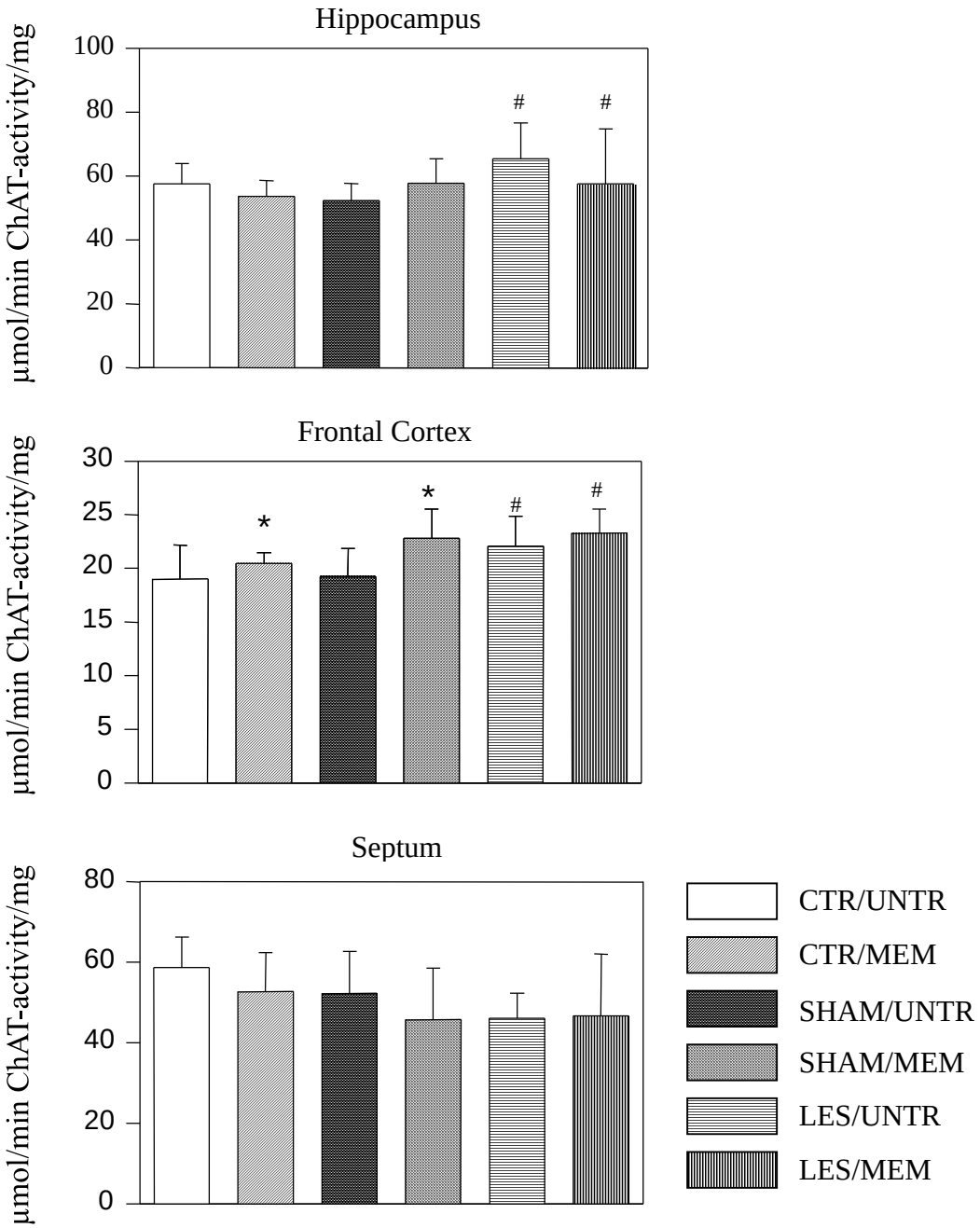

Figure 4

ChAT activity (µmol/min/mg) in the hippocampus, septum, frontal cortex, occipital cortex and striatum after bilateral lesion of the entorhinal cortex and a 14 day treatment with memantine. (MEM; 20mg/kg/24h); # significant difference when compared to sham lesioned animals; * significant difference of memantine treatment. **CTR/UNTR**: control animals without memantine treatment. **SHAM/UNTR**: shamlesioned animals without memantine treatment; **LES/UNTR**: lesioned animals without memantine treatment. **CTR/MEM**: Control animals with memantine treatment. **SHAM/MEM**: sham-lesioned animals with memantine treatment. **LES/MEM**: lesioned animals with memantine treatment.