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Nerve growth factor response to excitotoxic lesion of the cholinergic basal forebrain is slightly impaired in aged rats

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Running Headline:
Nerve growth factor content after basal forebrain lesion

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Summary

Nerve growth factor (NGF) promotes survival and function of basal forebrain cholinergic neurons. We studied NGF and choline acetyltransferase (ChAT) activity after partial quisqualic acid induced lesions of the basal forebrain in 3 and 27 months-old rats, in order to investigate whether NGF-related regeneration is disturbed in old age. 2 weeks post lesion, ChAT activity decreased by 25 to 32% in adult and old rats. 3 months post lesion, the ChAT deficit receded in adult rats, but remained unchanged in old rats. 2 weeks post lesion, NGF levels were reduced by 36 to 44% , but there was no significant difference between adult and old rats. 3 months post lesion, we found increased NGF levels by 44% in the posterior cortex of adult rats. These results indicate that the compensatory NGF increase in the posterior cortex after partial cholinergic lesion of the basal forebrain is slightly impaired in old age.

Keywords

Nerve growth factor (NGF), choline acetyltransferase (ChAT), aging, quisqualic acid, cholinergic basal forebrain, regeneration

1. Introduction

The cholinergic system of the basal forebrain plays a prominent role in learning and memory [Durkin (1989)]. Cholinergic neurotransmission seems to be essential for attention and short term memory [Hörtnagl and Hellweg (1997), Sarter and Bruno (1994)].

There are several mechanisms discussed to underlie the cholinergic cell loss within the basal forebrain during aging and in Alzheimer´s disease [as reviewed by Wenk and Willard 1998]. There is compelling evidence that nerve growth factor (NGF) acts throughout life on the cholinergic basal forebrain system and the striatum [Thoenen et al. (1987)]. In these regions of the central nervous system (CNS) NGF plays an important role for the support of the neuronal population and the maintenance of a compensatory and regenerative function in neurodegeneration [Knüsel et al. (1994), Thoenen et al. (1987)]. In the cholinergic basal forebrain, NGF stimulates the activity and expression of choline acetyltransferase (ChAT) and protects endangered cholinergic neurons against atrophy and neurodegeneration [Hörtnagl and Hellweg (1997), Scott and Crutcher (1994), Thoenen et al. 1987), Hellweg et al. (1998)]. In the present study, ChAT activity as a marker of the cholinergic system and NGF levels have been determined after a quisqualic acid-induced lesion of the basal forebrain (NBM) in old and adult rats, and at two time points. Possible differences between old and adult animals have been compared, mechanisms of regeneration the two groups have been discussed and the time course of the two markers after a possible compensatory response three months after the lesion have been investigated. The main goal of the study was to investigate whether there are differences between old and adult rats in regenerative abilities after an acute lesion of the NBM[1].

2. Materials and Methods

2.1. Animals

50 old (27 months-old) and 32 adult (3 months-old) female Wistar rats were used for the lesion experiments. As detailed below (2.3), control groups consisted of 5 old and 5 adult rats, which received no treatment to exclude nonspecific effects of the stereotaxic operations. Ethical approval of the experimental protocol was obtained from the Senator for Health in

[1] Part of this has been published in abstract form previously [Hellweg et al. (1996)].

Berlin (n° 92/91). The animals were housed under standard conditions (22°C) on a 12 h light, 12 h dark cycle and had free access to laboratory chow and tap water.

2.2. Materials

Chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Other reagents were obtained from Sigma Chemical Company (Deisenhofen, Germany). Anti-mouse-NGF-antibodies (clone 27/21), and anti-mouse-β-NGF (clone 27/21)-β-galactosidase-conjugate were purchased from Boehringer Mannheim. Mouse-NGF was provided by Prof Rohrer (Max-Planck-Institute for Brain Research, Frankfurt/Main, Germany).

2.3. Surgical procedures

The lesioning method has been described in detail before [Steckler et al. (1993)]. Briefly rats were lesioned under ketamine/Xylosin anesthesia . Quisqualic acid (0.12 mol/l dissolved in 0.1 M phosphate buffer, pH 7.4) was injected in 25 adult and 16 old rats A: 0.2 mm anterior to bregma, L: 3.4 mm lateral to the midline, V: 7.0 mm ventral to the dura, 0.5 µl, and A: 1.0 mm, L: 2.6 mm, V: 7.2 mm, 0.5 µl. Each infusion was delivered over 2.5 min and the needle was left in place for another 2 min after injection. 25 adult and 16 old rats received bilateral sham lesions.

The partial lesioned and sham-lesioned animals were divided into two groups and decapitated after 2 weeks and 3 months post lesion. The control animals were decapitated at the age of 3 and 27 months, respectively. In the first experimental group, i.e. 3 months post stereotaxic lesion, the main control group consisted of sham-treated animals. Unlesioned animals were used as a second control group to estimate the size of a non-specific effect of the stereotaxic intervention. With respect to the second experimental group, i.e. 27 months post streotaxic lesion, we assumed that NGF and ChAT levels would not change significantly due to aging alone over the 2.5 months period between the two time-points when animals were sacrificed [cf. Hellweg et al. (1990)]. This is the reason why we only sacrificed untreated animals at one point in time. Dissected CNS tissue samples of anterior cortex, posterior cortex, hippocampus and striatum were stored at -70°C until homogenization, as described elsewhere [Hellweg et al. (1990)].

2.4. 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 Hellweg et al. (1989). Repetitive freezing and thawing of homogenates is known not to interfere with the quantification of NGF [Korsching and Thoenen (1987)].

2.5. 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 elsewhere in detail [Hellweg et al. (1989)]. The previous protocol for this two-site ELISA was simplified in part and these modifications proved to not impair accuracy and sensitivity of the assay [Hellweg et al. (1992)]. The measured levels of NGF were corrected for mean recoveries of added mouse 2.5 S NGF (125 pg/ml), which were determined in each assay. Determinations of recovery, specific and unspecific NGF binding (the latter against mouse IgG_1 obtained from MOPC 21) involved quadruplicate fluorescence determinations for each tissue sample.

2.6. Determination of ChAT activity

The activity of ChAT was determined according to the method of Fonnum (1975), with several modifications which have been described previously [Auburger et al. (1987)]. Each sample was measured twofold with a specific inhibitor of ChAT (N-hydroxyethyl-4-(1-naphtylvinyl)-pyridiumbromide in a concentration of 0.3-0.5 mg/ml) in order to determine unspecific activity. In the substrate solution (pH=7.4) which contained 16 mM choline chloride, 0.2 mM Eserin, 0.5 mM Acetyl-Coenzyme A, 0.3 M NaCl and 100 mM NaH_2PO_4 x H_2O, 200 µl [^{3}H]acetyl-CoA has been reconstituted with a specific activity of 3.4 Ci/mmol. Measurements without ChAT-inhibitor were carried out in triplicates. Each assay did not contain more than 28 samples in order to limit time differences between individual measurements. To exclude inter-assay-variance, samples of one brain region of all animal groups were measured in the same assay.

2.7. Statistical analysis

All data are presented as mean ± one standard deviation (SD). Since NGF concentrations do not follow a normal distribution a non-parametric approach was used to test the differences in

the NGF serum concentrations between the two groups. In order to determine significance of observed differences between NGF contents and ChAT-activities between animal groups, Kruskall-Wallis test was employed to test homogeneity of the distributions of NGF concentration between the three groups simultaneously. As an *a posteriori* test Wilcoxon's two-sample test was applied to test for pairwise differences. Differences were considered significant at $p<0.05$. NGF concentrations have been percent-corrected for each control group to avoid the influence of inter-assay-variance [Hellweg et al. (1989), (1990), (1992)].

3. Results

3.1. ChAT activity

In adult animals the stereotaxic lesion of the NBM induced a significant reduction of the choline marker ChAT after 2 weeks in the anterior (-31%, p=0.007), posterior cortex (-38%, p=0.001) and in the hippocampal brain samples (-23%, p=0.002) compared to adult untreated and sham treated controls. The reduction of ChAT activity in two of the three brain regions was also significant in comparison to sham-lesioned animals (anterior cortex –25%, p=0.002, posterior cortex –32%, hippocampus –20%, p=0.016). Old animals also showed a reduction of ChAT activity in the anterior cortex (p=0.019) when compared with sham lesioned and control animals (Figure 1). A slight damage of the cholinergic system was detected in sham-treated animals at all ages, but this was only significant in the hippocampus of old animals in comparison to untreated controls (ChAT-reduction –11%, p=0.032).

Three months post lesion, the reduction of ChAT activity in adult and old animals was significant compared to sham-treated controls in the anterior (-14%, p=0.002; -19%, p=0.001, respectively) and posterior cortex (-15%, p<0.001; -17%, p<0.003, respectively). In the hippocampus old animals showed a slight reduction, which did not reach significance (p=0.093) (figure 2).

In old control treated animals a significant reduction of ChAT activity was observed in the striatum (-24%, p=0.032) compared to adult controls, and in old untreated control animals in the anterior cortex (-12%, p=0.032) compared to adult controls (Figures 1 and 2).

3.2. NGF levels

NGF concentrations decreased significantly two weeks post lesion in the anterior cortex (-44%, p=0.014) and hippocampus (-36%, p=0.05) of adult animals compared to adult untreated controls (figure 3). In the hippocampus of old sham-lesioned animals NGF was significantly reduced

(-17%, p=0.049) as compared with untreated old controls. Three months post lesion, no differences in NGF concentrations between lesioned and sham-lesioned animals were found, neither in anterior cortex nor in hippocampus. In the posterior cortex of adult lesioned animals a significant increase of NGF as compared to sham-lesioned animals (+44%, p=0.008) was measured (figure 4). It could be argued that sample size was insufficient to compare NGF levels 3 months post lesion. As a matter of fact, the increase of NGF in the posterior cortex in young animals when compared to their control group (sham-treated animals of the same age) was highly significant in a Kruskal-Wallis and an a posteriori Wilcoxon rank sum test (P-value 0.008), which means that the probability of a type I error was below 1%.

In the striatum of adult sham-lesioned animals NGF concentrations were significantly increased compared to untreated controls (+40%, p<0.04).

4. Discussion

In the present study, an initial decrease of NGF levels after acute lesion was observed in excitotoxin-lesioned as well as in sham-lesioned animals, which was more pronounced in the anterior cortex of adult rats treated with quisqualic acid (-44%, p<0.014) compared to sham-treated controls. The principal reason for the observed NGF reduction could therefore be the traumatic lesion of the neurons, which project from the NBM to the cortex or an unspecific destruction of NGF-synthesizing cells in the region of injection. This corresponds to a temporary NGF reduction previously reported in the hippocampus and dentate gyrus after a transient ischemia of the basal forebrain in rats [Shozuhara et al. (1992)] and the observed time course of NGF and ChAT activity is highly consistent with the results of a detailed morphologic study of Unger and Schmidt (1992).

As has been shown in recent lesion studies by our group, the intraventricular infusion of the cholinergic toxin ethylcholine aziridinium (AF64A) caused a dose-dependent decrease of hippocampal ChAT activity with a maximum in the first week and a partial recovery between weeks one and four after the infusion [Hellweg et al. (1997), (2001)]. In response to the treatment with AF64A, NGF-mRNA increased steadily during the observation period of 7 weeks, whereas NGF-protein levels only decreased transiently in the ventral part of the

hippocampus after 3 weeks [Hellweg et al. (1997), (2001)]. These observations suggest that an increased synthesis of NGF is part of an endogenous compensatory regeneration, but that the utilization of NGF as well as the transiently elevated levels of NGF observed within the denervated hippocampus may occur at a faster rate than NGF-mRNA levels [Hellweg et al. (1997)]. By contrast, the nearly complete damage of the cholinergic basal forebrain results in an increase of NGF-protein content in the denervated target regions without changes in NGF expression [Hörtnagl and Hellweg (1997)], leading to the hypothesis that the loss of retrograde transport is the reason for the incapability of neurons to rescue cholinergic functions [Korsching et al. (1986), Yu et al. (1996)].

The increase of NGF in the posterior cortex of adult animals 3 months post lesion could be interpreted as an improved NGF-dependent compensation mechanism in this brain region, accompanied by an increased ChAT activity (from –32% to –15%). An NGF-dependent increase of ChAT activity has been reported previously in a lesion study with intracerebroventricularly injected 192 IgG-saporin, which is a conjugate of the monoclonal antibody for the p75 neurotrophin receptor (p75NTR) and the ribosome-inactivating protein saporin, which produces dose-dependent selective lesions of cholinergic basal forebrain neurons [Waite et al. (1994), Winkler et al. (1998)]. Winkler et al. (1998) showed that NGF restored ChAT activity in 50% p75NTR-mediated deafferentiation (24% increase in the parietal cortex, and 57% increase in the frontal cortex), but not in 80% deafferentiated neurons. Previous findings indicate that the cholinergic basal forebrain is not saturated by endogenous NGF levels, and cholinergic markers can be elevated by additional trophic factor treatment [Winkler et al. (1998), Winkler and Thal (1995)]. In adult (i.e., 2-5 months) compared to old (i.e., 24 months) Fischer-344-rats, increased NGF levels after lesion of the septal neurons have been observed in the hippocampus [Scott and Crutcher (1994)], which could be interpreted as an impairment of the compensatory increase of neurotrophins after denervation in old age [Scott and Crutcher (1994)]. The increase of the observed NGF-protein levels could be due to an increased cerebral de-novo-synthesis as an intrinsic compensation mechanism, as well as a secondary accumulation of NGF-protein in the target region resulting from a decreased retrograde axonal transport of NGF in lesioned cholinergic neurons [Hellweg et al. (1998)].

In the present study, reduced ChAT activity was observed in old rats compared to adult untreated animals in the anterior cortex (-12%) and the striatum (-24%). This corresponds to previous reports of significant losses of ChAT activity with aging [Gallagher et al. (1990), Williams (1991)]. Our data in lesioned animals are also in line with assessments of ChAT

activity and ChAT immunoreactive neuronal profiles after infusion of colchicine in the NBM showing the largest decrease 7 days post lesion with a recovery of both of these markers in the parietal cortex after 84 days [Shaughnessy et al. (1998)]. This finding corresponds also to results from a study in which the infusion of ibotenic acid in the NBM led to loss of 55-60% of cholinergic neurons [Cossette et al. (1993)]. After lesion with quisqualic acid in the NBM of adult rats, reductions of ChAT activity by 40-75% have been described in various studies [for review see Dunnett and Barth (1991)]. This is in line with our results, where a decrease of 30-40% in the anterior and posterior cortex and of 23% in the hippocampus was observed 2 weeks post lesion. Zawia and coworkers (1992) also observed decreased ChAT activity levels 2 weeks after a lesion with another excitotoxic amino acid (i.e., ibotenic acid), which was more pronounced in old (-72%) compared to adult (-59%) rats. This could reflect a higher vulnerability of old animals. In our experiments, ChAT activity levels were also reduced 2 weeks post lesion in both adult and old animals, but in old animals this reduction did not reach statistical significance. This could be due to the small number of animals investigated and the high interindividual deviations observed in old animals. In contrast to this finding, the reduction of ChAT activity in the neocortex 3 months post lesion was significant in our study at both ages (15-20%) as compared with sham-lesioned animals of the same age. We also found indications for a higher vulnerability of old compared to adult animals. ChAT activity and NGF content in the hippocampus of old sham-treated animals were significantly reduced 2 weeks post lesion (up to –44%), although there was no significant reduction of both parameters in adult animals.

There is convincing evidence that the function of the cholinergic septohippocampal system is linked to the presence of endogenous NGF [Hörtnagl and Hellweg (1997), Van der Zee et al. (1995)]. This implies that damage to the cholinergic system is associated with a compensatory stimulation of neurotrophic factors to guarantee the survival of affected neurons [for reviews see Hellweg et al. (1998), Mattson and Scheff (1994)]. Taken together our data suggest that the compensatory increase of NGF levels three months after an excitotoxic lesion of the NBM being observed in adult rats is impaired in old age.

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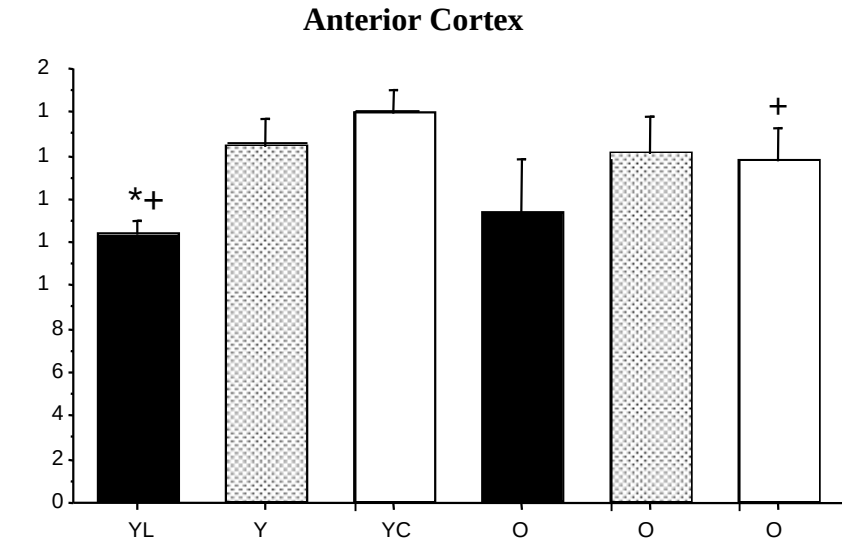
Figures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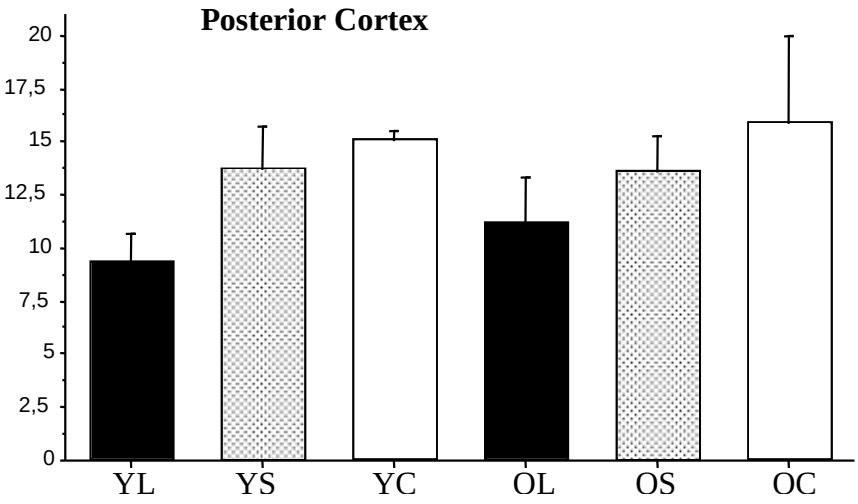
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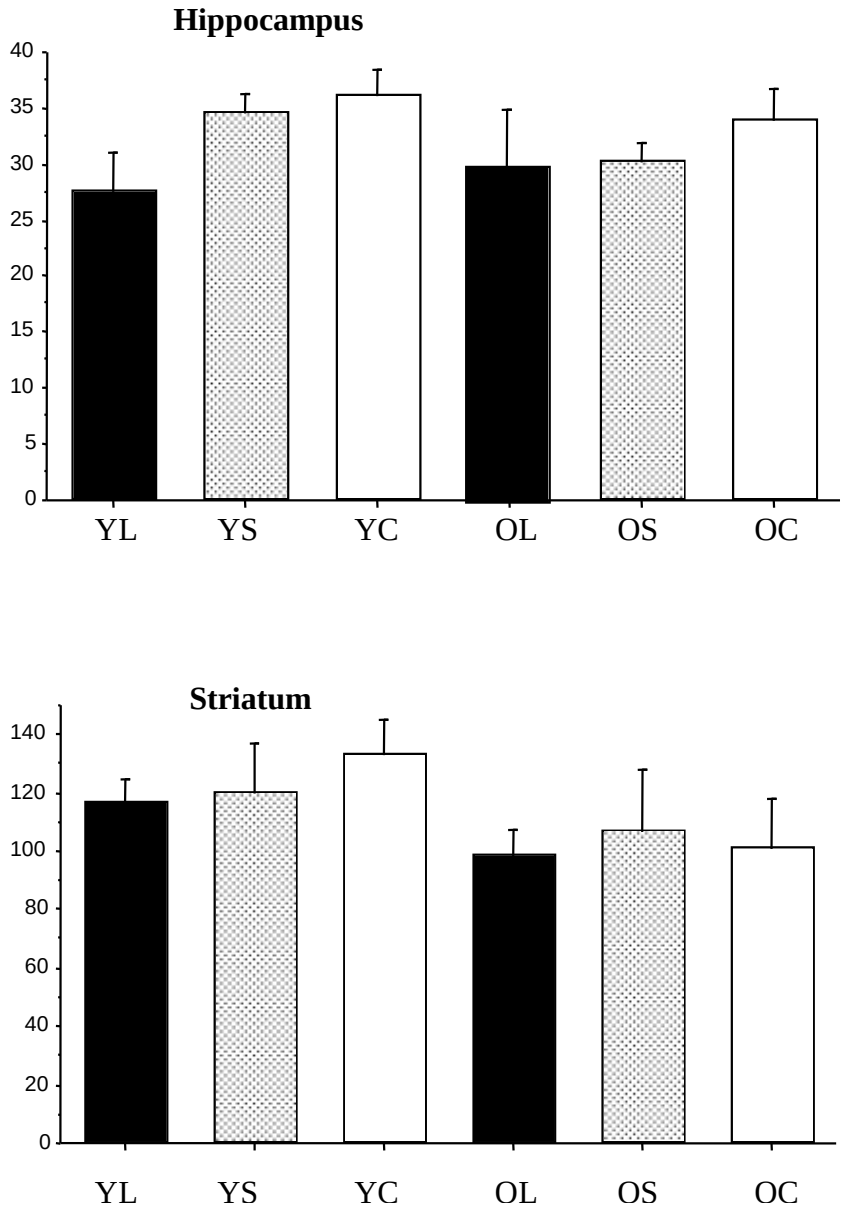
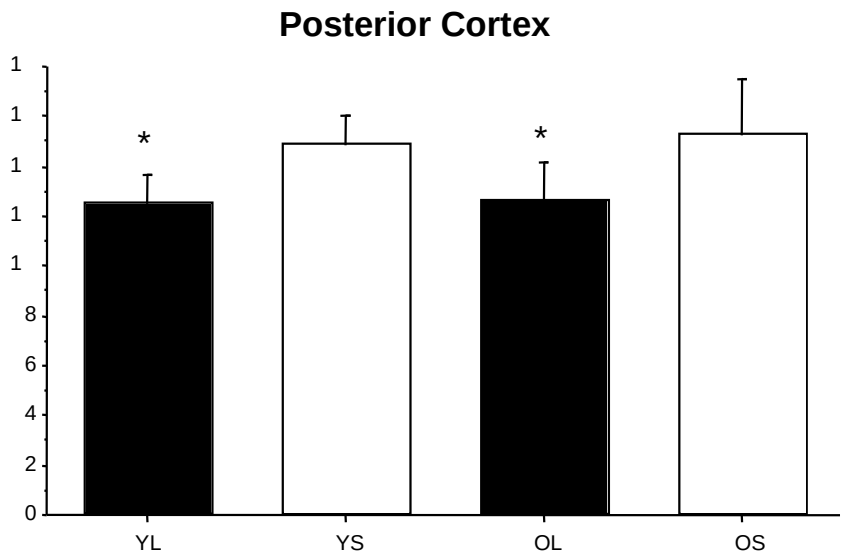


Figure 1. ChAT activity (μU/mg wet weight) 2 weeks after stereotaxic lesion of the basal forebrain shown as mean ± one standard deviation. Asterisks (∗) indicate a significant difference between respective control animals of the same age, crosses (+) indicate a significant difference between animals of different age and equal treatment. YL (young lesioned animals) (n=4), YS (young sham-lesioned animals) (n=5), YC (young control animals)(n=5), OL (old lesioned animals) (n=5), OS (old sham-lesioned animals) (n=4), OC (old controls) (n=5).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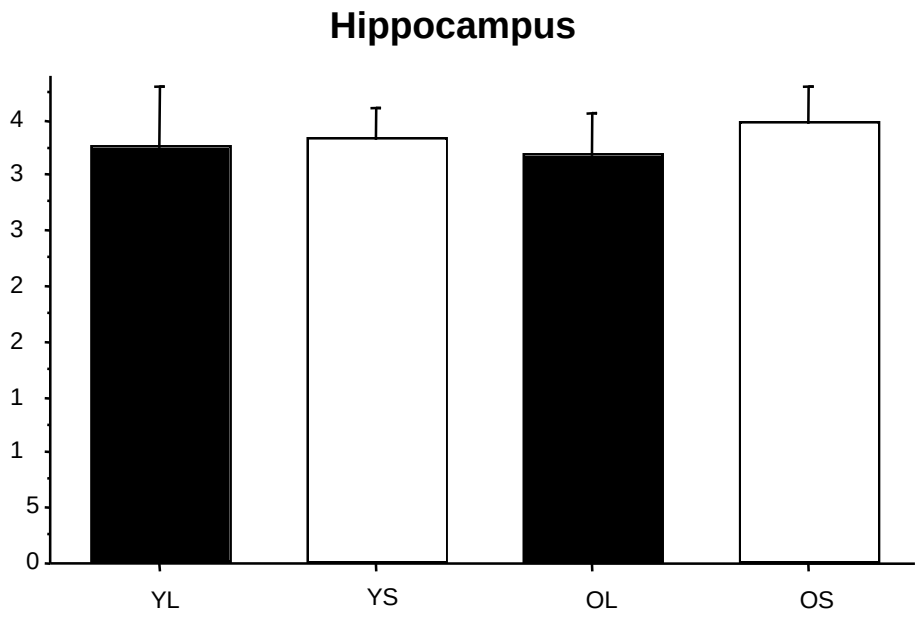
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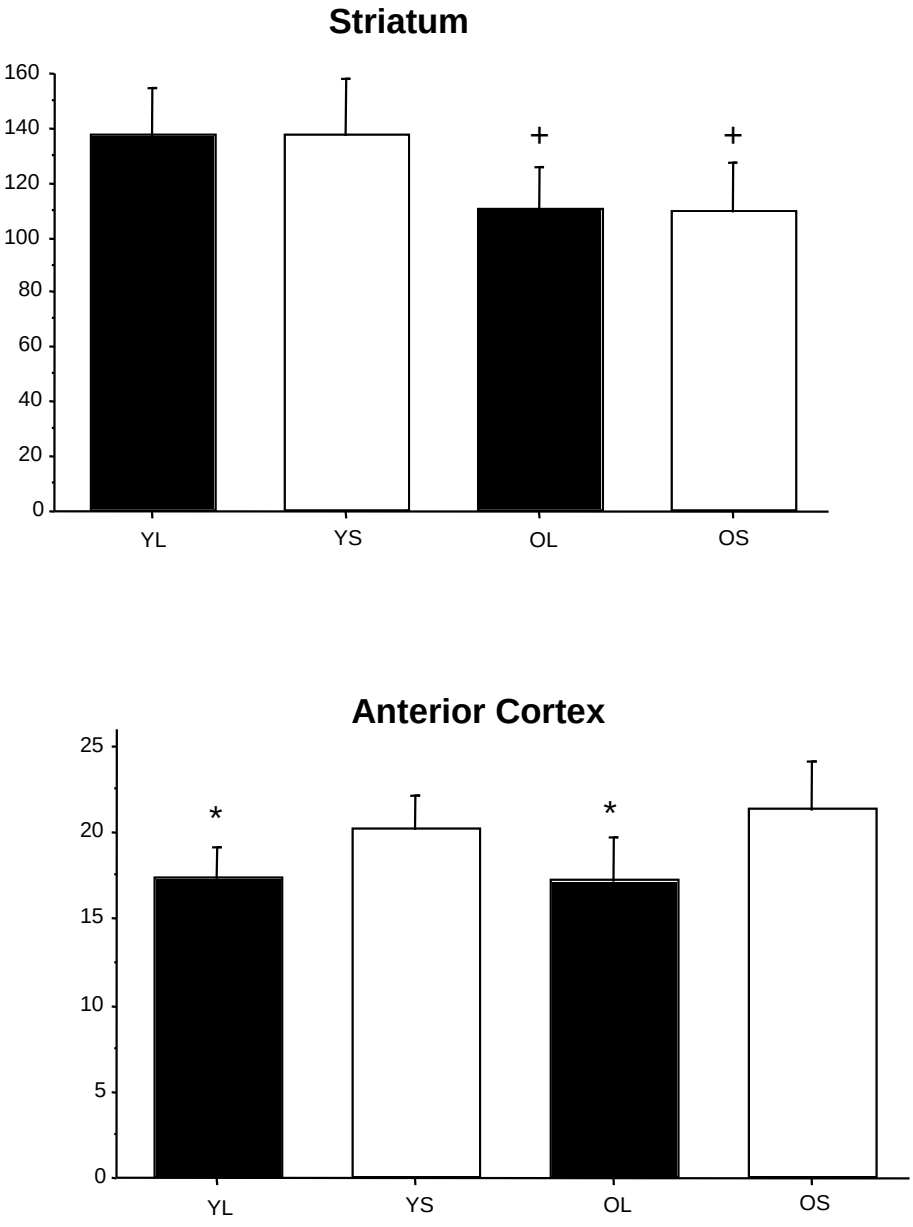
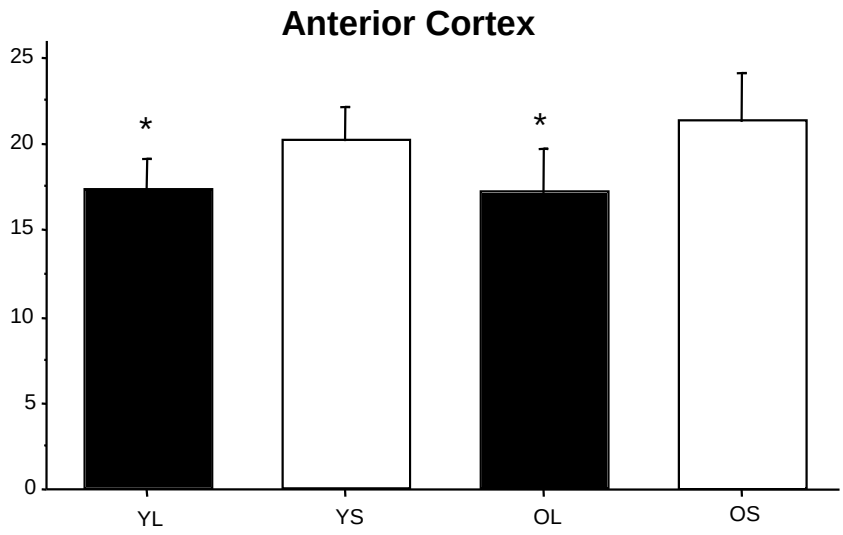


Figure 2. ChAT activity (μU/mg wet weight) 3 months after stereotaxic lesion of the basal forebrain shown as mean ± one standard deviation. Asterisks (∗) indicate a significant difference between animals of the same age, crosses (+) indicate a significant difference between animals of different age and equal treatment. YL (young lesioned animals) (n=12), YS (young sham-lesioned animals) (n=11), OL (old lesioned animals) (n=12), OS (old sham-lesioned animals) (n=10).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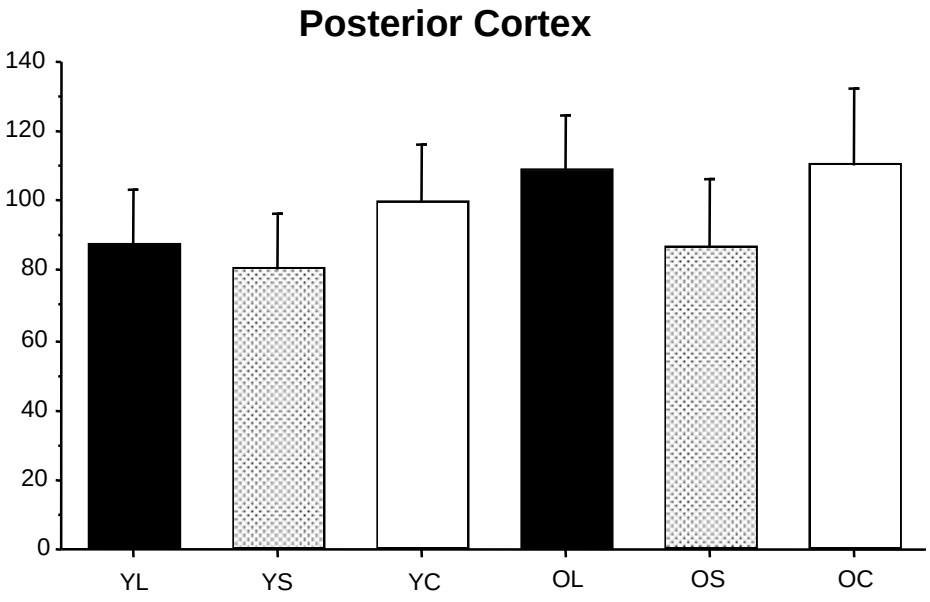
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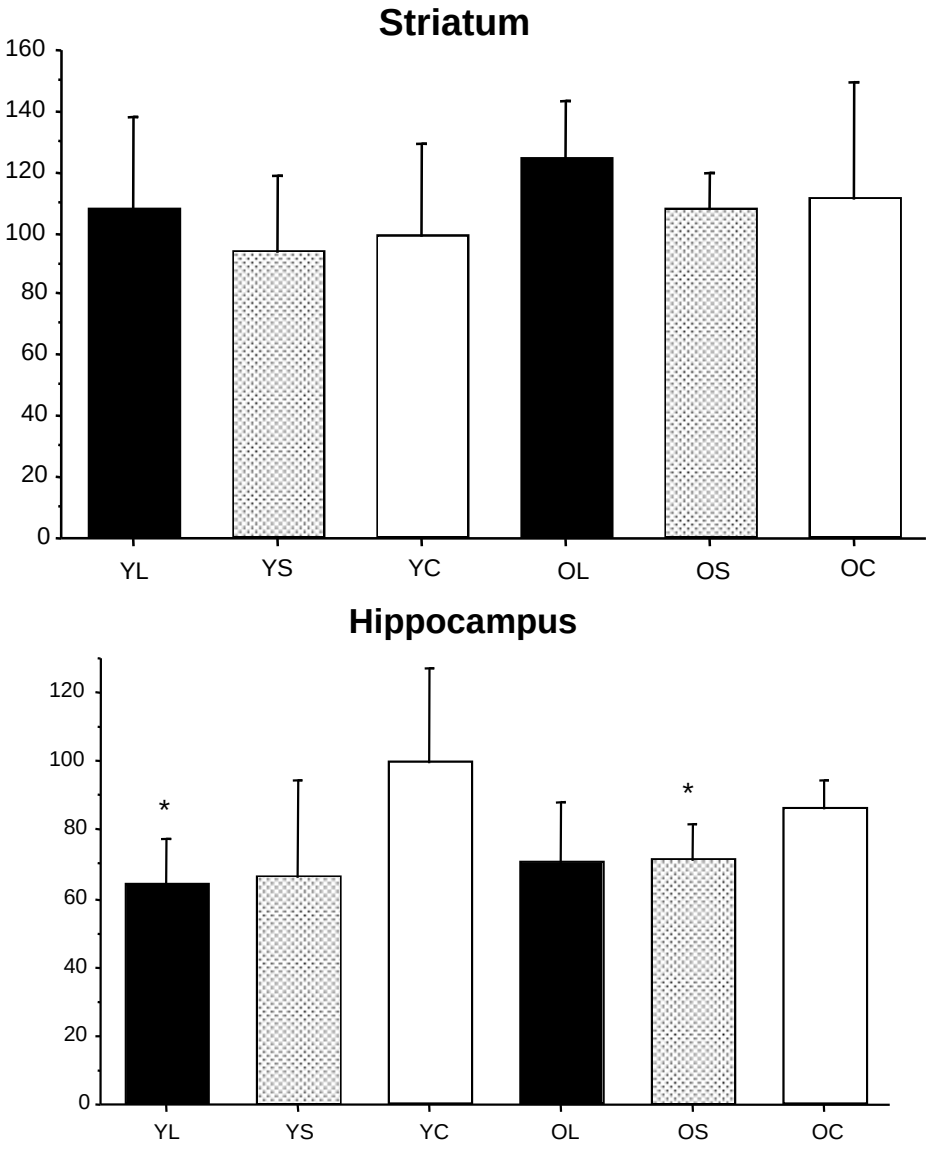
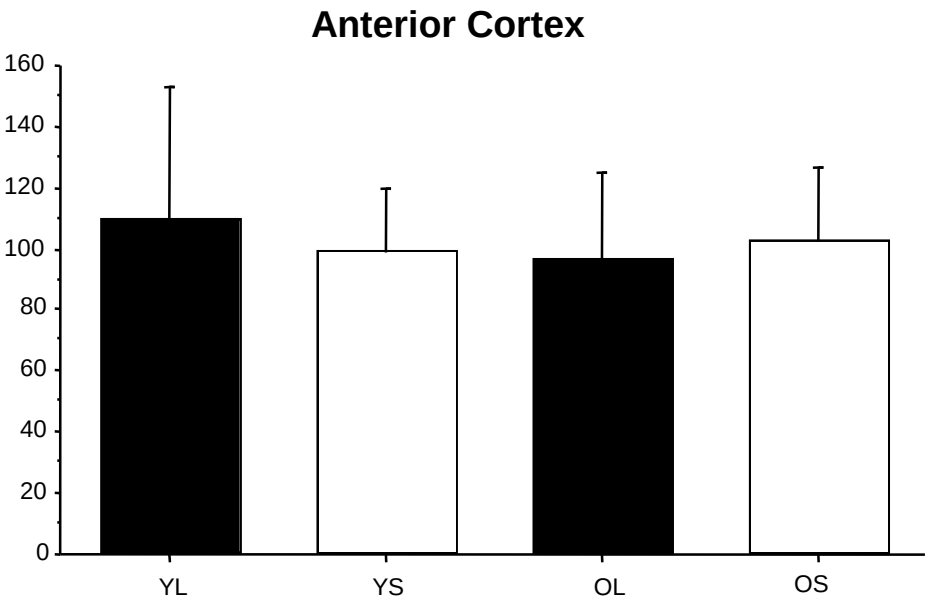
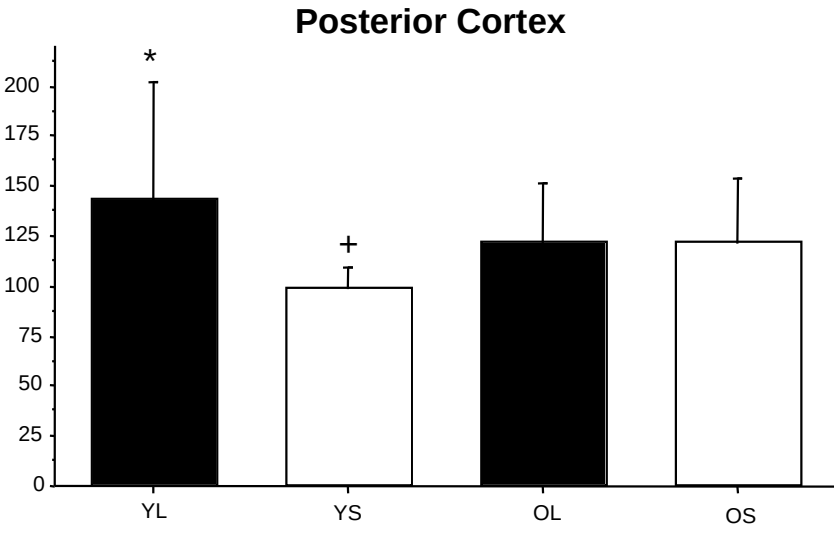


Figure 3. NGF concentrations expressed as percentage of young control animals 2 weeks after stereotaxic lesion of the basal forebrain shown as mean ± one standard deviation. Mean absolute NGF concentrations corrected for recovery of young controls in ng NGF/g wet weight were: 0.68 ± 0.09 in anterior cortex, 0.98 ± 0.16 in posterior cortex, 1.46 ± 0.39 in hippocampus, and 0.52 ± 0.16 in striatum. Asterisks (∗) indicate a significant difference between respective control animals of the same age, crosses (+) indicate a significant difference between animals of different age and equal treatment. YL (young lesioned animals) (n=4), YS (young sham-lesioned animals) (n=5), YC (young control animals)(n=5), OL (old lesioned animals) (n=5), OS (old sham-lesioned animals) (n=4), OC (old control animals) (n=5).


Anterior Cortex
160
140
120
100
80
60
40
20
0
YL
YS
OL
OS


Posterior Cortex
*
+
200
175
150
125
100
75
50
25
0
YL
YS
OL
OS

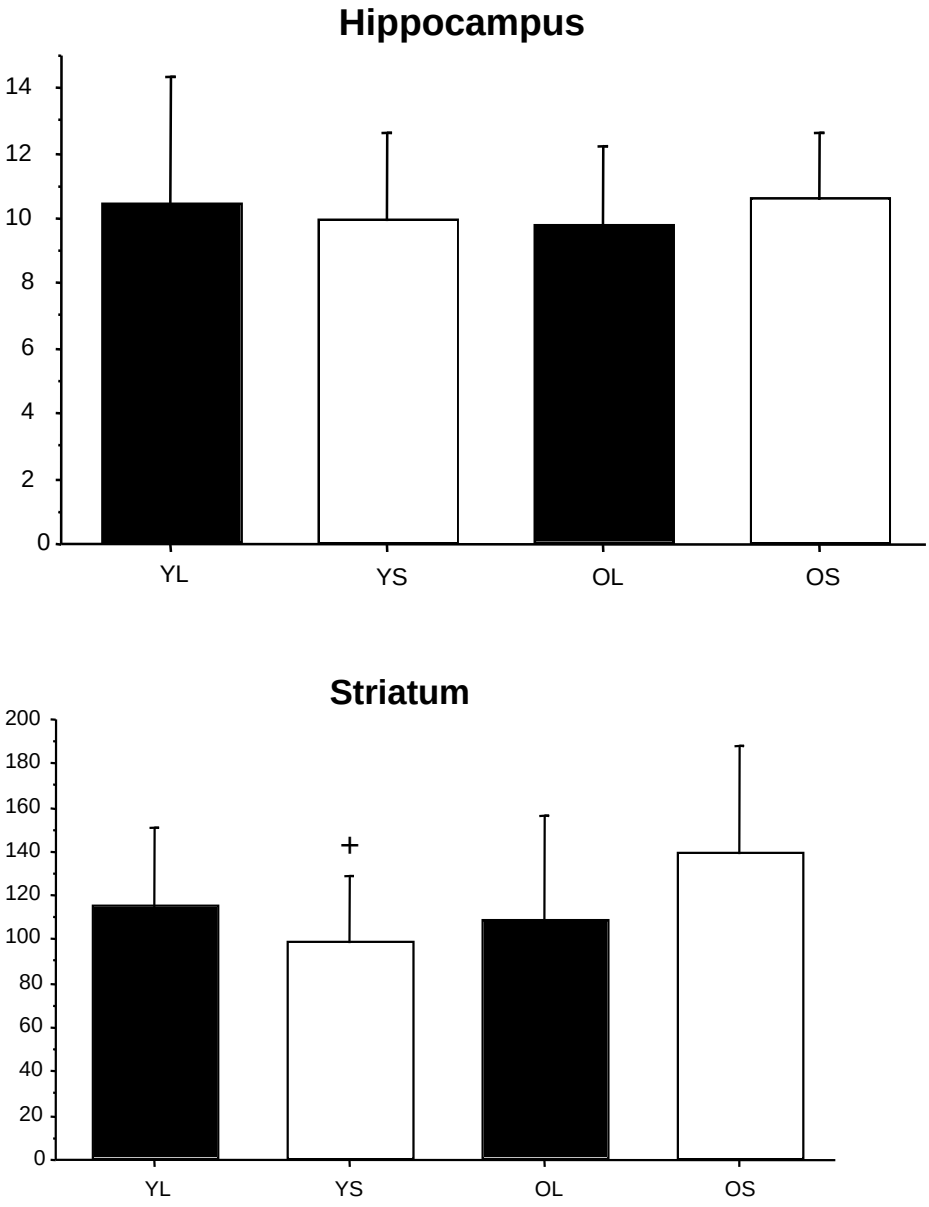


Figure 4. NGF concentrations expressed as percentage of young sham-lesioned animals 3 months after stereotaxic lesion of the basal forebrain shown as mean ± one standard deviation. Mean absolute NGF concentrations corrected for recovery of young controls in ng NGF/g wet weight were: 0.74 ± 0.12 in anterior cortex, 0.88 ± 0.19 in posterior cortex, 2.78 ± 1.17 in hippocampus and 0.49 ± 0.14 in striatum. Asterisks (∗) indicate a significant difference between animals of the same age, crosses (+) indicate a significant difference between animals of different age and equal treatment. YL (young lesioned animals) (n=12), YS (young sham-lesioned animals) (n=11), OL (old lesioned animals) (n=12), OS (old sham-lesioned animals) (n=10).