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Abraham, [No Value]; Veenema, AH; Nyakas, C; Harkany, T; Bohus, BGJ; Luiten, PGM; Ábrahám, I.

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Effect of Corticosterone and Adrenalectomy on NMDA-Induced Cholinergic Cell Death in Rat Magnocellular Nucleus Basalis

I. Ábrahám†, A. H. Veenema*, C. Nyakas*‡, T. Harkany*‡, B. G. J. Bohus* and P. G. M. Luiten*
*Department of Animal Physiology, Graduate School of Behavioural and Cognitive Neurosciences, University of Groningen, Haren, The Netherlands.
†Laboratory of Molecular Neuroendocrinology, Institute of Experimental Medicine and ‡Central Research Division, Haynal University of Health Sciences, Budapest, Hungary.

Key words: corticosterone, adrenalectomy, magnocellular nucleus basalis, cortex, astrocytes, neuroprotection.

Abstract

The present study demonstrates the effects of adrenalectomy and subcutaneously administered corticosterone on N-methyl-D-aspartate-induced neurodegeneration in the cholinergic magnocellular basal nucleus of the rat. NMDA was unilaterally injected into the nucleus basalis at different plasma corticosterone concentrations in adrenalectomized rats, in adrenalectomized animals with subcutaneously implanted cholesterol-corticosterone pellets containing 25% or 100% corticosterone, and in sham-adrenalectomized controls. The neurotoxic impact of the NMDA injection in the various experimental groups was assessed by the loss of cholinergic fibers stained with acetylcholinesterase histochemistry in the parietal neocortex. Reactive cortical astrocytes as a result of the treatments were detected by glial fibrillary acidic protein immunohistochemistry. Measurements of the densities of astrocytes and cholinergic fibers at the injected side of the brain were carried out by image analysis.

Adrenalectomy significantly potentiated the NMDA-induced neurodegeneration by 50%, while chronic administration of corticosterone significantly attenuated the NMDA-neurotoxicity in a dose-dependent manner. Compared to the ADX group, 25% corticosterone application reduced the NMDA damage by 37%, whereas the 100% corticosterone pellet dimished NMDA neurotoxicity by 75%. Both ADX and ADX + corticosterone implantation enhanced the NMDA-induced GFAP immunoreactivity. The increase of GFAP immunoreactivity was most pronounced in the adrenalectomized rats supplied with the 100% corticosterone pellets.

The results demonstrate that corticosterone exerts a potent neuroprotective effect on NMDA-induced neurotoxicity in the magnocellular nucleus basalis. The activated astroglia suggest that astrocytes may contribute to the benefical effect of corticosterone in the neuroprotective mechanisms against excitotoxic neuronal injury.

Corticosteroids (cortisol in human, corticosterone in rats) are secreted from the adrenal cortex in particularly high concentrations as a result of stressful experiences. Besides the peripheral sites of action, the brain is a major target organ for these hormones. Due to their lipophylic nature corticoids (CORT) easily pass through the blood–brain barrier and bind to intracellular mineralocorticoid (MRs) and glucocorticoid receptors (GRs) in neurons and glia as was shown by radioligand binding studies (1, 2) and immunohistochemistry (3, 4). The functions of glucocorticoids on brain processes are multifold, ranging from developmental effects to influencing complex emotional and cognitive processes (5–7). The impact of particularly high levels of glucocorticoids appear to be paradoxical, since both neuroprotective and neurodegenerative effects have been reported.

Glucocorticoids (GC) may exert a neurotrophic, protective role in the hippocampus (8). It was also demonstrated more recently that glucocorticoids are essential for the development of the rat cerebral cortex as these hormones play a role in the control of cellular differentiation and maturation (9). On the other hand, the same steroids can cause neuronal damage and increase the vulnerability of nerve cells in the hippocampus to metabolic insults (10), while specific effects of corticosteroids on cholinergic fiber degeneration by cholinotoxins were described by Hortangl *et al.* (11).

Chronic stress and stress evoked levels of corticosterone may cause shrinkage and atrophy of apical dendrites in the CA3 region in the hippocampus of rat and tree shrew (12, 13), while chronic exposure to cortisol reportedly induces damage to CA2 and CA3 neurons in monkey hippocampus (14). The neurotoxic mechanisms involved may include direct effects of glucocorticoids via intracellular receptors. However, it has been found that corticoids can increase the extracellular concentration of glutamate (15–17), possibly via inhibition of glutamate uptake by astrocytes (18), leading to neuronal excitotoxicity and subsequent neuronal injury. Glucocorticoids are also able to increase the Ca^{2+} influx via voltage gated Ca^{2+} channels further enhancing the Ca^{2+} mediated neurotoxic effects (19).

In view of the growing evidence for a mechanistic link between

Correspondence to: P.G.M Luiten, Department of Animal Physiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

the disregulation of the intracellular Ca^{2+} homeostasis and glucocorticoid mediated neurotoxicity, it became of interest to investigate the effect of glucocorticoids on Ca^{2+} induced neurodegeneration in the brain. Furthermore, most of the studies on the effect of glucocorticoids on viability of nerve cells were carried out on the hippocampus, while little is known of the glucocorticoid-induced alterations of the neuronal survival in other brain regions. Such impact of corticosteroids appears to be particularly relevant to the proposed role of steroids in the pathogenesis of Alzheimer's disease (20, 21). In this view a deranged regulation of glucocorticoid balance and chronic exposure to high GC levels would accelerate the aging process of nerve cells and form a risk factor of neuronal damage in Alzheimer's disease (21). In this respect the vulnerability of cholinergic neurons of the basal forebrain to neurodegenerative mechanisms and GC is of special interest, because of the reported loss of this particular cellgroup in Alzheimer's disease (22–24).

In the present study we investigated the impact of corticosterone manipulation on neuronal degeneration of cholinergic cells in the magnocellular nucleus basalis (MBN) induced by the glutamate analog N-methyl-D-aspartate (NMDA). In previous reports it was demonstrated that injections of glutamate analogs like NMDA, AMPA and ibotenic acid in the MBN region caused cholinergic cell loss in the injection area and dose-dependently reduced the cholinergic fiber density in the somatosensory cortex via Ca^{2+} mediated neurotoxic processes (25–28).

An advantage of this neurodegeneration model is the easy and reliable quantification of the effect of the cellular lesion, since in contrast to cell loss, their fiber projections can precisely be measured by image analysis while omitting the hazards of cell counts (29). The same neurotoxicological model was used in the present study injecting NMDA into MBN of rats at different corticosterone plasma concentrations. The neurodegenerative experiments were performed in sham-operated controls, in adrenalectomized (ADX) animals and in ADX rats supplied with 25% (ADX + 25% CORT) or 100% (ADX + 100% CORT) corticosterone pellets. The neurotoxic effects of the NMDA infusions in the MBN after the corticosterone manipulations were established by measuring cholinergic fiber densities in the cortex visualized by acetylcholinesterase (AChE) histochemistry. In order to evaluate the putative role of an astrocytic reaction in these experiments the major intermediate filament protein of astrocytes, glial fibrillary acidic protein (GFAP), was visualized by immunocytochemistry.

Results

In the adrenalectomized animals the plasma corticosterone concentrations were below the detection levels indicating successful removal of the adrenals. The corticosterone level was 38 ± 8 nM (1.32 ± 0.3 μg/dl) in the ADX + 25% CORT animals and reached concentrations of 150 ± 16 nM (5.2 ± 0.55 μg/dl) in the ADX + 100% CORT rats.

The sham-ADX rats that received a vehicle injection into the nucleus basalis only showed a minor damage to MBN neurons expressed as a reduction of 4 ± 2.5% AChE-positive fibers in the ipsilateral neocortex. In the sham-ADX rats, the infusion of 60 nmol of NMDA in the MBN complex resulted in a profound decrease of 43.8 ± 2.9% of AChE fibers in the neocortex of the injected hemisphere (Figs 1 and 2). Adrenalectomy potentiated the neurotoxic effect of the 60 nmol NMDA injections in the MBN, in which case the percentage of cholinergic fiber loss in the somatosensory cortex was significantly increased ($P < 0.01$) from 43.8 to 66.0 ± 1.7% (Fig. 2). The increased vulnerability to NMDA neurotoxicity after adrenalectomy was prevented by implantation of 25% corticosterone pellets in the ADX rats. The NMDA injections in the ADX + 25% CORT group resulted in a fiber loss of 41.8 ± 1.5%, which was not significantly different from the fiber reduction caused by the NMDA injections in the sham animals. NMDA-induced fiber loss was strikingly attenuated by implantation of 100% corticosterone pellets in ADX rats. In this experimental group the cortical fiber reduction after NMDA injection was only 16.5 ± 2.9%, which was significantly less compared to the NMDA damage in the ADX and control groups ($P < 0.01$). Comparing fiber densities in the cortex of the non-lesioned control sides showed that ADX and the implantation of pellets with different corticosterone concentrations had no effect on the normal AChE fiber density (data not shown).

NMDA injection into MBN evoked a conspicuous increase of GFAP-immunoreactivity in the injection area in the basal forebrain when compared to GFAP-ir in the sham-ADX/vehicle injected animals. However, the GFAP-ir expression did not differ between the groups that received an NMDA injection (sham-ADX, ADX, ADX + 25% CORT and ADX + 100% CORT).

Figure 3 shows the density of the GFAP expression in the neocortex, which significantly increased in the ADX animals (320 ± 38.3%) compared to sham-ADX animals (37.4 ± 10.9%) ($P < 0.01$). Cortical GFAP density values of the ADX + 25% CORT (307.0 ± 41.8%) and ADX + 100% CORT (634.5 ± 33.5%) groups were significantly different from sham-ADX rats ($P < 0.01$; Fig. 4). The GFAP reaction in the ADX + 100% CORT group was even significantly larger than the GFAP response of the ADX + 25% CORT rats. Adrenalectomy and the corticosterone implantation had no visible effects on GFAP expression in the cortex at the nonlesioned control side of the brain.

Discussion

The present experiments demonstrate that adrenalectomy increases the vulnerability of cholinergic neurons of the magnocellular nucleus basalis to excitotoxic neuronal injury. This potentiation of NMDA-induced cell loss in the MBN was absent in adrenalectomized rats in which the plasma corticosterone level was maintained at low serum levels of 38 nM by a 25% corticosterone pellet implantation. ADX and a 100% cortocosterone pellet leading to a serum concentration of 150 nM even produced a significant reduction of excitotoxic cell damage in the MBN. Accordingly, the NMDA-induced cortical GFAP expression was enhanced in adrenalectomized rats that had no corticosterone pellets and adrenalectomized rats implanted with corticosterone pellets leading to basal or higher CORT serum levels.

Corticosterone, neurodegeneration and neuroprotection

Corticosterone acting as a neuroprotective hormone against cytotoxic neurodegeneration appears to be in contrast to the reported effects of adrenalectomy and exposure to high CORT levels on cell damage in the hippocampus induced by neurotoxins or that occurring during stroke. Under such conditions in the rat, ADX decreases cell damage in the CA1 and CA3 regions of the hippocampus, whereas prolonged elevation to very high plasma

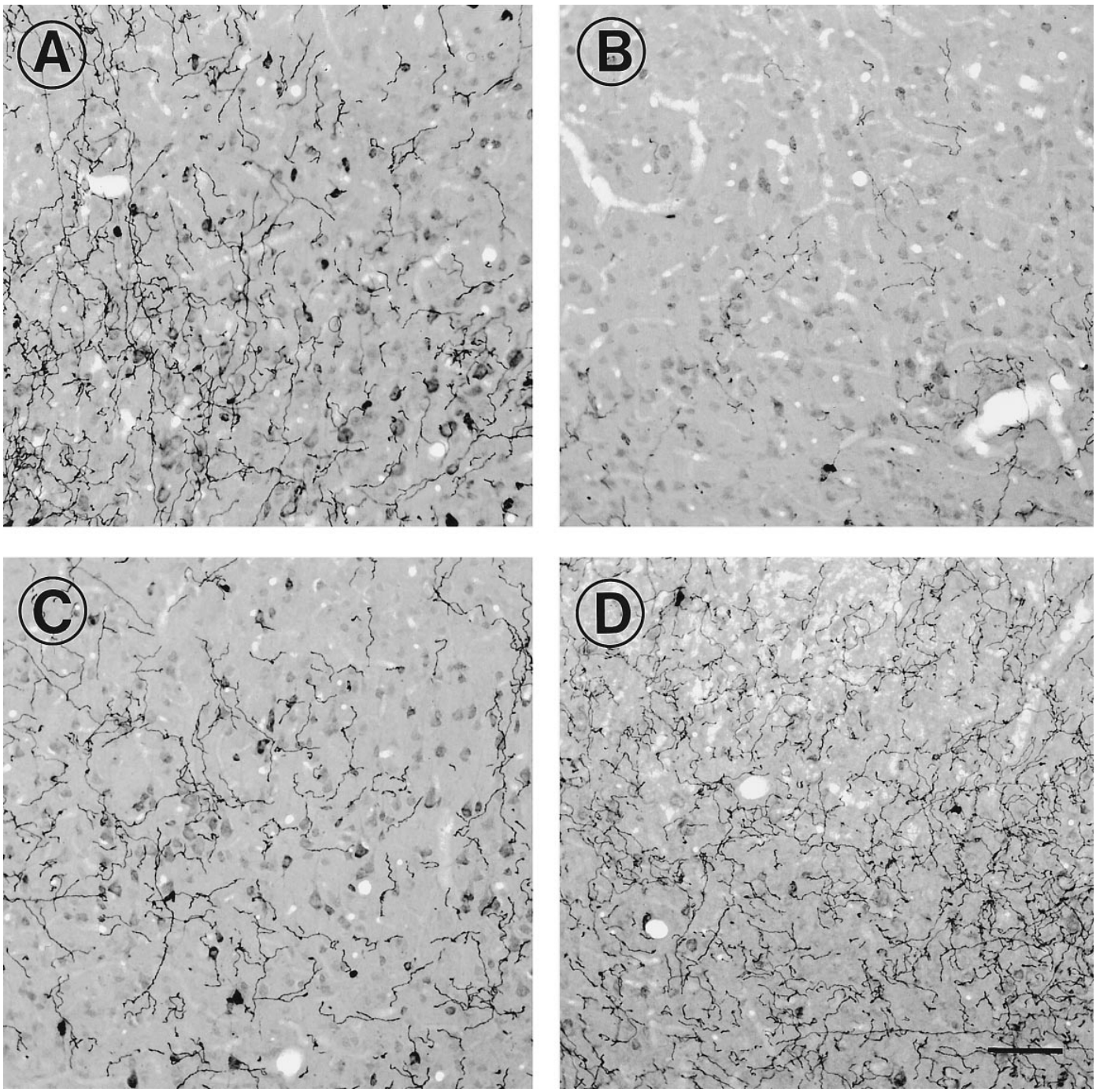

FIG. 1. Effects of N-methyl-D-aspartate (NMDA) infusion into the rat magnocellular nucleus basalis (MBN) on cholinergic, acetylcholinesterase-positive, innervation of the rat somatosensory cortex in combination with adrenalectomy and 25% or 100% chronic corticosterone implantation. (A) depicts parietal cortical section of an NMDA-lesioned animal, (B) that of an adrenalectomized rat with subsequent NMDA infusion into the MBN, (C) and (D) represent those of 25% or 100% corticosterone-implanted animals after NMDA lesions, respectively. Note the dramatic loss of cholinergic inervation after adrenalectomy in combination with acute NMDA injection (B), whereas chronic corticosterone administration dose-dependently prevented NMDA-induced loss of cortical cholinergic innervation (C and D). Representative photomicrographs depict layer V of the posterior somatosensory cortex. All panels have the same magnification, scale bar = 100 μm.

CORT levels can create a condition that increases the vulnerability of nerve cells to neurodegenerative processes as in aging, ischemia and possibly other neurodegenerative diseases (14, 20, 21, 30, 31). Several probably interacting mechanisms have been proposed to explain the neurodegenerative effects of high glucocorticoid exposure including derangement of calcium homeostasis, inhibition of glucose uptake and neurotrophin mobilization, and disturbance of ionic balance over the neuronal membrane (32).

Major factors that account for the differences between reported neurodegenerative action of high GC concentrations and the

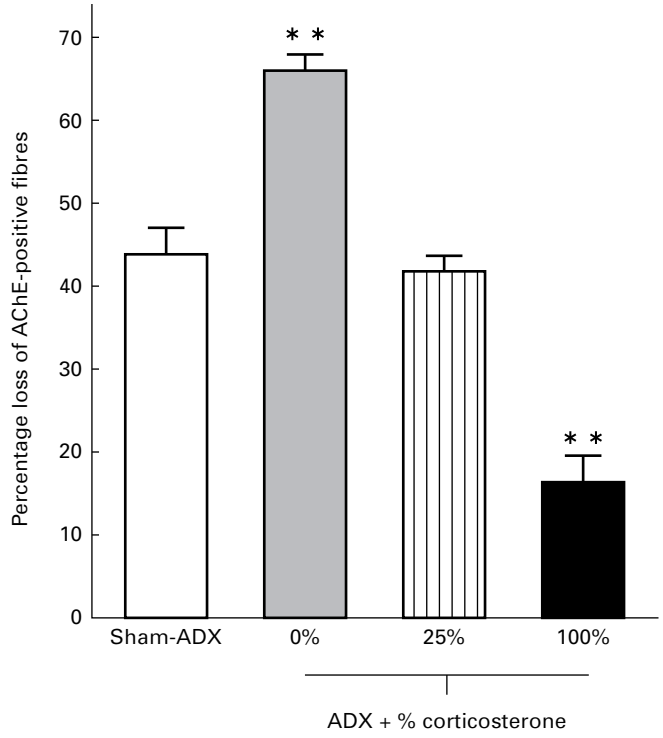

FIG. 2. Effect of ADX and corticosterone pellet implantation on percentages of area densities of cholinergic fiber loss in the cortex after unilateral NMDA injection in the MBN (mean ± SEM, *P < 0.005). The area densities of acetylcholine fibers at injected side were expressed as a percentage of area densities at control side for each individual.

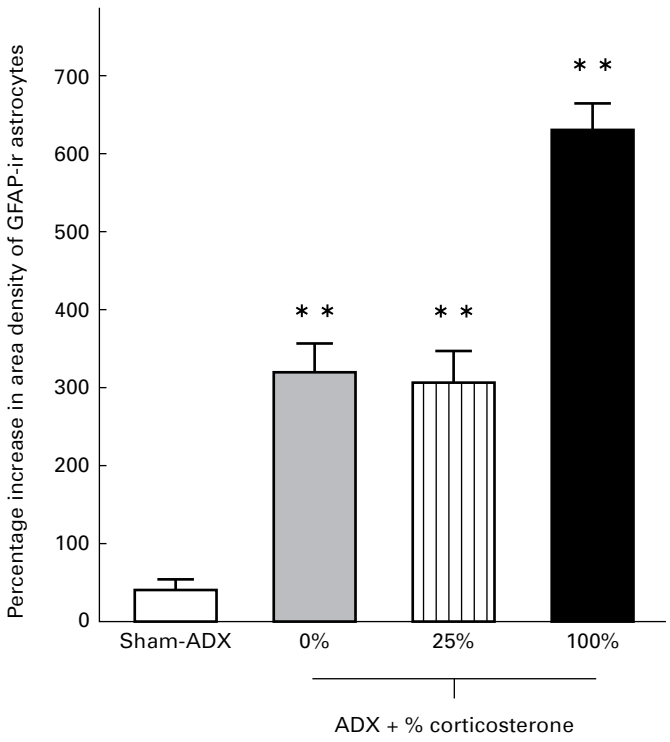

FIG. 4. Effect of ADX and corticosterone pellet implantation on percentages of area densities of GFAP expression in the cortex after unilateral NMDA injection in the MBN (mean ± SEM, *P < 0.005). The area densities of GFAP immunoreactivity at injected side were expressed as a percentage of area densities at control side for each individual.

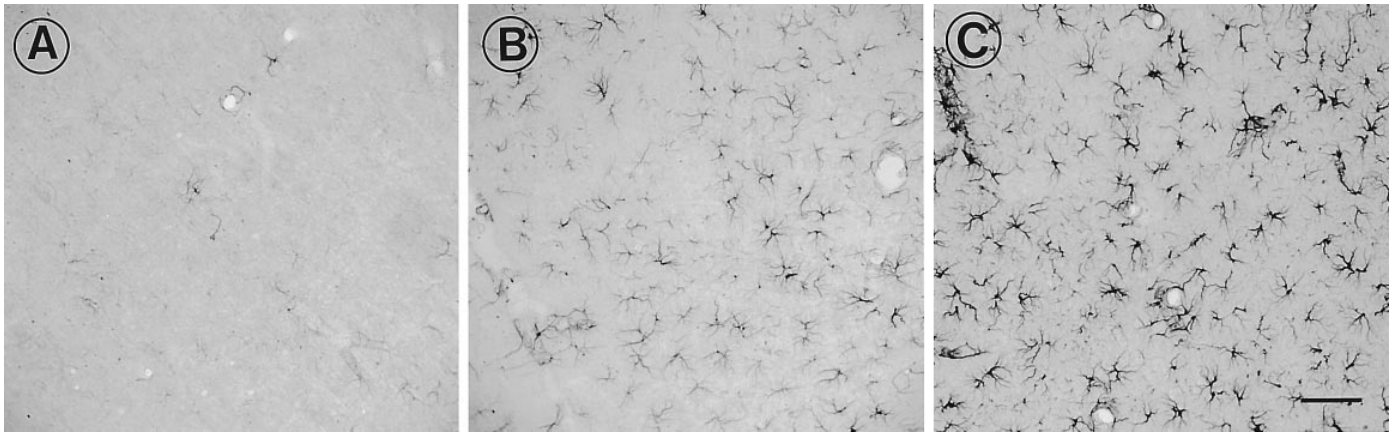

FIG. 3. Enhancement of glial-fibrillary acidic protein (GFAP) immunoreactivity (ir) after chronic corticosterone implantation and subsequent N-methyl-D-aspartate (NMDA) lesions in the magnocellular basal nucleus of rats (MBN). (A) depicts a parietal cortical section of an NMDA-lesioned animal, while (B and C) those of 25% or 100% corticosterone-implanted animals after NMDA lesions, respectively. Note the graded induction of GFAP-ir in astrocytes as the result of chronic 25% or 100% corticosterone treatment. All panels show layer V of the rat parietal cortex and have the same magnification. Scale bar = 100μm.

neuroprotective effects of GC observed in our experiments are the brain region involved, the time course and duration of treatments and the CORT concentrations used. Even within the hippocampus, CORT strongly enhanced ischemia-induced brain damage in CA1 but had only minor effects on the dentate gyrus (31). Conrad & Roy (33) showed long-term degenerative effects of ADX in the rat dentate gyrus but not in the ammon's horn regions. Likewise, Krugers *et al.* (34), three days after adrenalectomy, only observed degenerative changes that were limited to the dentate gyrus. Several other reports suggest that neuronal injury following stress and corticosterone administration is not a general phenomenon and depends on the type and age of the species under study and the duration of the treatment. Peripheral administration of corticosterone for 8 weeks in young Long Evans

rats did not affect the hippocampal cell structure (35), while anxiety stress for 6 months in young adult Fisher 344 rats failed to cause neuronal cell loss of hippocampal CA1 pyramidal cells (36). As argued above, neurotoxic effects of glucocorticoids were demonstrated mainly in the CA1 area of the hippocampus and not in other brain regions. Also *in vitro* experiments indicate that glucocorticoids exacerbate kainic acid-induced neuronal damage in cultures of hippocampal neurons but not of cerebellar or hypothalamic cells (37).

It should also be noted that very low CORT levels that occur after adrenalectomy in the present experiments exacerbate the neurotoxic action of NMDA infusions in the MBN region. In that sense the neurodegenerative impact of very low CORT levels corroborates the findings of Van Lookeren Campagne *et al.* (38) who reported massive NMDA induced apoptotic cell death in early postnatal rat brain, a period of life with very low CORT levels. Furthermore, in our experiments no conditions were investigated where CORT concentrations reached levels that are reported after prolonged chronic stress.

It may well be concluded that the effects of glucocorticoids depend on the hormone concentration and on the anatomical region, which may be the consequence of the presence or absence of the two corticosteroid receptor types and the changed balance between MR and GR (39).

Possible mechanisms of neuroprotection by glucocorticoids

An obvious conclusion from the extensive literature on this subject is that complex interacting mechanisms underly the impact of glucocorticoids on neuronal survival. Glucocorticoids exert a profound influence on ionic conductances as was demonstrated in a number of electrophysiological studies notably on hippocampal pyramidal cells (40). Low levels of corticosteroids result in reductions of calcium currents and reduced sensitivity to several transmitters, mediated by CORT effects on MRs (40, 41), which was partly confirmed recently in transgenic mice lacking GRs (42). In contrast, CORT activation of the GR enhances calcium influx and reduces the effects of excitatory amino acid stimulation (19). It should be noted, however, that simultaneous activation of both MR and GR is essential to induce large Ca^{2+} currents (41). As a result of such CORT-evoked changes in calcium conductance, high CORT levels enlarge after hyperpolarization of pyramidal cells (19, 40), which probably renders these cells less sensitive to excitatory amino acids (19). It may be clear from these observations that CORT effects will be highly dependent on the presence of MRs and GRs. Presence of GR but probably not MR in MBN neurons (43) may thus yield decreased sensitivity to transmitters like glutamate and in this way antagonize the process of NMDA-triggered degeneration of MBN neurons.

A predominant role for GRs in the neuroprotective effects of relatively high CORT titers against NMDA-induced neurotoxicity is also supported by differential CORT binding dependent on the hormone concentration. Administration of 25% CORT pellets results in a saturation of the MR and minor occupation of the GR (44, 45). Implantation of 100% CORT pellets, which produced a significant neuroprotective condition, leads to a half maximal occupation of the GRs (45), suggesting a GR mediated protective mechanism. Similar mechanisms of CORT action were reported by Zoli *et al.* (46), who found significant antagonistic effects of glucocorticoids against glutamate neurotoxicity in the arcuate nucleus, an area highly enriched in GRs.

It should be realized, however, that the putative neuroprotective potential of GC against excititoxic cell damage cannot only be explained on GC effects on locally applied NMDA in the nucleus basalis. GC manipulation not only affects the MBN neurons but has also considerable influence on the cortical regions that receive the MBN projections, as is indicated by the cortical glia response of GFAP expression. In this respect the changes in the reductions of cholinergic fiber innervation after NMDA injections in the MBN might be influenced as well by CORT effects on degenerating axonal projections in the cortex.

Besides changes in ionic conductance several additional synergistic mechanisms may be involved in the neuroprotective effect of high concentrations of corticosterone against the NMDA-induced cell death. Glucocorticoid receptor activation promotes the synthesis of the protein lipocortin-1 (47), which can act as a neuroprotective agent in ischemic insults (48) and equally well inhibits cell damage induced by NMDA receptor agonists (49). Furthermore, GC can potently inhibit glucose transport and thus the supply of energy to the nerve cell, which becomes of particular importance when the nerve cell is under threat of overload by Ca^{2+} as is the case after NMDA exposure. However, the inhibitory effect of GC on glucose transport was only demonstrated in cultured hippocampal neurons (32).

A third neuroprotective mechanism stimulated by CORT appears to be the production of neurotrophic factors of which nerve growth factor (NGF) is highly relevant in view of the specific localization of its receptors on cholinergic basal forebrain neurons. Previous *in situ* hybridization studies revealed that systemic corticosterone administration elicited a temporal induction of basic fibroblast growth factor (bFGF) and NGF mRNA in the cerebral cortex (50). Conversely, adrenalectomy leads to a decreased expression of neurotrophins including NGF, which is directly associated with attenuated neuronal survival in ischemic conditions and hypoglycemic stress (51, 52). The association of NGF and its receptor on cholinergic neurons is obvious. Selective cholinergic cell destruction can be induced by immunotoxins that are directly acting on the NGF receptor (53), whereas nerve growth factor can be protective or regenerative to excitotoxic MBN lesions (54). Interestingly, intracellular elevation of calcium concentrations by ischemia and NMDA channel stimulation by glutamate, which is thought to underlie ischemic cell damage, can be dose-dependently prevented by neurotrophins like NGF (55). However, we are only starting to understand the complex interactions of glucocorticoids and the temporal and GC concentration-dependent regulation of neurotrophin expression and the neurotrophin receptor linked signal transduction pathways.

Corticosterone and GFAP expression

The present experiment revealed that all NMDA injected rats showed abundant amounts of GFAP immunoreactive astroglia in the area surrounding the NMDA infusion site in the MBN probably as a result of mechanical and chemical injury induced by excitotoxin. The expression of GFAP in the neocortex was only slightly enhanced as a result of the MBN lesion and subsequent cortical cholinergic fiber loss. However, adrenalectomy followed by the MBN lesion strongly increased GFAP immunoreactivity in the cortex of the lesioned hemisphere.

The impact of ADX and corticosterone on astrocyte activation were previously studied by O'Callaghan *et al.* (56). These authors reported an increase of GFAP positive astrocytes and GFAP

mRNA in the neocortex after ADX, while glucocorticoids suppressed the level of GFAP protein and mRNA. Such results were not confirmed in the present study where we failed to find evidence for changes in GFAP expression in the neocortex at the control side of the brain. This implicates that the effects of ADX and corticosterone on GFAP-ir were most likely mediated by mechanisms associated with the NMDA exposure to MBN neurons and the subsequent degeneration of cortical cholinergic fibers. The functions of GFAP are not entirely clear. The protein appears to be essential for maintenance of the structural integrity (57), while GFAP positive astrocytes have the capacity to support the neurons during neurological insults. Since GFAP-ir cells were only observed in the neocortex of the lesioned side of the brain this suggests that the observed GFAP increase can only be attributed to the neurodegenerative events that occur as a result of the cholinergic cell lesion.

The present results provide evidence that glucocorticoids can exert neuroprotective effects cholinergic cells in the MBN against cell death evoked by excitotoxic neurodegenerative mechanisms. Adrenalectomy and suppletion of corticosterone potentiate astrocyte activation in the areas where the cholinergic fiber undergo degeneration. These findings indicate that corticosterone can be beneficial for neuroprotective and possibly neuroregenerative processes after brain insults involving damage to the basal forebrain cholinergic cell system. The functional consequences of this neuroprotective action, and its relation to the CORT-related alteration in hippocampal neuronal survival remain to be determined.

Materials and methods

Animals

Young adult male Wistar rats (weighing 300–350 g) were used for all experiments bred in our own facilities under SPF conditions. The animals were housed at 24 °C and 55–75% humidity with light-dark cycles (12/12 h) beginning at 06.00 h. Animals had standard rat chow and tap water available *ad libitum*. All procedures in this study were approved by the Committee on Animal Bio-Ethics of the University of Groningen.

Surgeries

All surgeries were carried out under deep anaesthesia with sodium pentobarbital (30 mg/kg, i.p.) and Hypnorm (Janssen, 0.4 mg/kg, i.m.). Adrenalectomy (ADX) was performed using a dorsal approach. During the same surgery corticosteroid-cholesterol pellets (crystalline CORT, Sigma, St. Louis, MO, USA) weighing approximately 200 mg were inserted subcutaneously 2 cm caudal to the skin incision made for the adrenalectomies (58). The animals were divided into four groups: (1) sham-adrenalectomized control rats implanted with pellets consisting of 100% cholesterol (n = 12); (2) ADX rats (n = 6); (3) ADX rats with pellets of 75% cholesterol-25% corticosterone (n = 6); and (4) ADX animals provided with a 100% corticosterone pellet (n = 6).

After four days the animals were anesthetized with halothane, mounted and 60 nmol NMDA (Sigma) in 1 µl 0.1M phosphate buffered saline (PBS, pH7.4) was stereotactically injected into the right MBN at the coordinates (from bregma and dura: A:-1.8, L:3.2, V:7) according to the atlas of Paxinos and Watson (59). Half of the sham-ADX group was injected with vehicle alone (n = 6). The injection procedure was described in detail elsewhere (29). After the NMDA injection the animals survived for 11 days until sacrifice and histological processing.

Hormone measurement

Before perfusion blood samples were taken from all animals by cardiac puncture. The samples were collected in pre-chilled tubes containing 20% K-EDTA, centrifuged and the plasma stored at -20 °C until hormone measurement. Plasma corticosterone was measured by radioimmunassay (RIA) without extraction as described previously (60). The antiserum was raised in rabbit against corticosterone-carboxymethyloximine-BSA. ^{125}I-labelled corticosterone-carboxymethyloximine-tyrosine-methylester was used as the tracer. The interference of plasma transcortin was eliminated by inactivating transcortin at low pH. The sensitivity of the assay was 0.1 pmol. Intra- and interassay variations were 6.4% and 23.8% respectively.

Tissue processing

Fixation of the brains was carried out by transcardial perfusion with 300 ml cold fixative composed of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 27 ml/min, which was preceded by a short prerinse of heparinized saline. The brains were removed from the skull and stored for 3 h in the fixative followed by 48 h storage at 4 °C in 0.1 phosphate buffered saline (PBS) with 30% sucrose for cryoprotection. Subsequently the brains were coronally sectioned on a cryostat microtome at a thickness of 25 µm and series of adjacent sections collected in PB for parallel staining for cholinergic fibers by AChE and glia activation by GFAP immunoreactivity.

Histochemistry

AChE staining was performed to visualize the intact cholinergic fibers. Free floating brain sections were postfixed by overnight immersion in a 2.5% glutaraldehyde solution in 0.1M PB at 4 °C. The AChE staining was carried out according to Hedreen *et al.* (61) using a $AgNO_4$ intensification procedure.

Immunocytochemistry

The tissue sections were rinsed in 0.01 M PBS (pH 7.4) and preincubated for 15 min with 0.1% H_2O_2. Next the sections were rinsed for 30 min with 5% normal sheep serum (NSS) and incubated for 24 h at 4 °C with the first antibody (monoclonal anti-GFAP IgG, Amersham, 1 : 200). 5% NSS and 0.2% Triton-X-100 were added to the PBS in all incubation and rinsing steps. The sections were rinsed and exposed for 2 h to biotinylated sheep anti-mouse IgG(1 : 200, Amersham) and for 1.5 h to HRP-streptavidin (1 : 200, Zymed). The HRP label was visualized by reacting with 3,3′-diaminobenzidine(60 mg/100ml Tris-HCl buffer, pH 7.6) and 0.01% H_2O_2. The reaction was stopped by rinsing in Tris buffer.

Quantifications

Quantification of AChE fiber- and GFAP cell densities was performed in the MBN projection areas in the parietal somatosensory cortex using an automatic image analysis system (IBAS) as described in detail in a previous report (62). Briefly, in each experimental animal four sections of the parietal cortex with intervals of 200 µm were measured and the values averaged. The area densities of the cholinergic fibers were measured in layer V of the ipsilateral and contralateral somatosensory cortex. Because of the strictly unilateral organization of MBN projection, the contralateral intact side served as a control within each individual subject and the fiber reduction calculated from the fiber densities at the injected site as a percentage of the densities of the intact control side. Measurements of cholinergic fiber reductions were limited to layer V since this cortical layer in the neocortex normally contains the highest density of innervation. Besides, layer V can easily be delineated from the adjacent layers IV and VI and thus yield a reproducible and reliable region for quantitative measurements. With the magnification used, a column width of 1 mm was measured. The densities of GFAP-positive astrocytes were measured in an area of 1.6 mm width of layers II, III, IV and V of the same somatosensory cortex regions of both hemispheres. Layers II-V were measured since changes in GFAP expression were limited to these cortical layers. Area densities of astrocytes at injection side were expressed as a percentage of area densities at the non-lesioned control side for each individual.

Statistical analysis

Data were expressed as the mean ± SEM. The Mann–Whitney nonparametric test was used with downward adjustment of α level with the Bonferoni t method for calculating significant differences between the groups ($P < 0.05$).

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