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Glucocorticoids Enhance Oxidative Stress-Induced Cell Death in Hippocampal Neurons *in Vitro*

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ABSTRACT

In patients with Alzheimer's disease, hippocampal cells are among the first neuronal cells of the brain to degenerate. Both rat primary hippocampal neurons and cells of the clonal mouse hippocampal cell line HT22 express endogenous functional glucocorticoid receptors (GRs), as shown by transient transfection of cells with a luciferase reporter plasmid containing GR-responsive elements. The influence of activated GRs on oxidative stress-induced neuronal cell death *in vitro* was investigated employing these hippocampal model systems. Two oxidative stressors were investigated, the free radical-inducing Alzheimer's disease-associated amyloid β-protein, which is toxic to hippocampal neurons, and the excitatory amino acid glutamate, which induces oxidative cell death in HT22 cells via an increase in intracellular peroxides. Cellular viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide test and trypan exclusion staining, followed by microscopical cell counting. Glucocorticoids strongly increased the vulnerability of the hippocampal cells to amyloid β-protein and glutamate. This increase could be blocked by the specific GR antagonist RU486. Our data suggest that changes in hippocampal GR homeostasis and regulation may render hippocampal neurons more vulnerable to oxidative stress-induced neuronal degeneration. (*Endocrinology* **138:** 101–106, 1997)

THE HIPPOCAMPUS is a primary target for neuronal degeneration in the brains of patients with Alzheimer's disease (AD). Because hippocampal cells express glucocorticoid receptors (GRs), they are the principal target sites for glucocorticoids, the adrenocortical hormones secreted during stress (1). The hippocampus is very sensitive to many types of neurological insults. Under certain conditions glucocorticoids exacerbate this sensitivity during pathological insults such as seizures, antimetabolite exposure, hypoxia-ischemia, and exposure to various neurotoxins (2–4). It has been reported that an altered response of the hypothalamic-pituitary-adrenal (HPA) system occurs in patients with AD and that these alterations may increase glucocorticoid levels (5).

Amyloid β-protein (Aβ) is a 40- to 43-amino acid peptide that is associated with plaques in the brains of patients with AD and is cytotoxic to neurons (6–10). Aβ-induced cytotoxicity has been shown to be caused by the intracellular accumulation of H_2O_2, ultimately leading to peroxidation of membrane lipids and cell death (6). In addition to its direct toxic effect, Aβ increases the vulnerability of rodent and human neurons to excitotoxins (8, 9). Excitatory amino acids, such as glutamate, may be directly toxic to cultured neuronal cells via two different processes. The classical pathway is mediated by specific glutamate receptors that can be blocked by specific glutamate receptor antagonists (11). Excitatory amino acid neurotoxicity may also be caused by the induction of an imbalance in antioxidant enzyme systems and a reduction in intracellular glutathione levels that ultimately lead to the intracellular accumulation of peroxides and cell death. The drop in glutathione levels has been shown to arise from the competition by glutamate for a glutamate/cystine antiporter, leading to an imbalance in the homeostasis of cystine, the precursor of glutathione formation (12). This second pathway can be blocked by the addition of antioxidants, which demonstrates the involvement of free radicals and oxidative stress (12, 13). In addition, several reports suggest that independently of the pathway, glutamate can induce the generation of several reactive oxygen species in neurons, such as the superoxide anion (14) and hydrogen peroxide (15). Glutamate has been implicated in various neurodegenerative diseases, such as AD, Huntington's disease, and Parkinson's disease (16). HT22 cells are sensitive to glutamate (13). Glutamate receptor antagonists do not protect HT22 cells from glutamate toxicity. This observation together with the finding that antioxidants, such as vitamin E, and high extracellular cystine levels protect these hippocampal cells from glutamate toxicity indicate that glutamate-induced cell death occurs via an oxidative pathway (13). Therefore, the toxicities of Aβ and glutamate were used as paradigms of oxidative stress-induced cell death in hippocampal neurons.

Data are accumulating that the activities of different steroids can have an impact on neuronal function and the sensitivity of neurons to different toxic insults (4, 17–20). Recently, we showed that the sex hormone 17β-estradiol is an effective neuroprotector against oxidative stress-induced cell death (21). To investigate whether glucocorticoids can increase the sensitivity of hippocampal neurons to Aβ and glutamate, we studied the influence of glucocorticoids on the cell death caused by these neurotoxins using the clonal

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mouse hippocampal cell line HT22 and rat primary hippocampal neurons.

Materials and Methods

Cell culture and chemicals

The cell line HT22 is a subclone of the HT4 hippocampal cell line (22). HT22 cells were a gift from Dr. David Schubert (The Salk Institute, San Diego, CA). They were cultured in DMEM supplemented with 10% FCS under standard culture conditions. Rat primary cultures were prepared from embryonic day 19 (E19) hippocampi, as previously described (10). Primary cells were cultured on poly-L-lysine-coated dishes in 50% DMEM (4500 mg/liter glucose)-50% Ham's F-12 medium that contained N2 supplements. Using these minimal culture conditions, more than 95% of the cells were neuronal, as confirmed by staining with neuron-specific enolase and by the absence of glial fibrillary acidic protein-positive cells. $A\beta_{25-35}$ was obtained from Saxon (Hanover, Germany). All media, sera, and medium supplements were purchased from Life Technologies (Eggenstein, Germany). RU486 was a gift from Dr. M. Renoir (Baulieu Laboratory, Paris, France). Dexamethasone, corticosterone, and all other reagents were purchased from Sigma Chemical Co. (Germany).

Transfections and assays for luciferase and β-galactosidase activities

HT22 cells were grown in DMEM supplemented with 10% FCS. Transient transfections were performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, CA) after determination of the optimal electric field strength. Five micrograms of steroid-responsive luciferase reporter gene (MTV-LUC) were cotransfected with 5 μg pCH110 (Pharmacia LKB, Freiburg, Germany), a simian virus 40 promoter-driven β-galactosidase expression vector. Electroporated cells were replated in DMEM supplemented with 10% steroid-free (charcoal-stripped) FCS and incubated immediately with various concentrations of dexamethasone. Charcoal stripping of FCS was performed using Dextran T-70 (Pharmacia, Uppsala, Sweden) as described previously (23). After 24 h, cells were harvested, and extracts were assayed for luciferase and β-galactosidase activities as a control for transfection efficiency, as previously described (23–26).

Cell survival assays

Neuronal cell death was estimated by three different assays: 1) microscopical examination of the cells with phase contrast microscopy to monitor for morphological changes in the absence of knowledge of the condition, 2) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (6, 27), and 3) trypan blue exclusion staining followed by cell counting (6, 21). For all of these assays, culture medium was switched 24 h before hormone addition to DMEM (1000 mg/liter glucose) supplemented with 10% charcoal-stripped steroid-free FCS. Primary cultures were cultivated in FCS-free DMEM (1000 mg/liter glucose) 24 h before cell survival assessment.

The MTT assay was performed as described previously (6, 21). Briefly, 1000–3000 HT22 cells or 10,000 primary neurons were plated in 96-well microtiter dishes with 100 μl medium/well. Hormones were added the next day. The toxins were added 24 h later. After 6 h, 10 μl MTT (5 mg/ml stock) were added to each well, and the incubation was continued for 4 h. Finally, 100 μl solubilization solution (50% dimethylformamide and 20% SDS, pH 4.8) were added. Adsorption readings were performed at 570 nm. To assess cell lysis, trypan blue staining was performed as previously described (6, 21). Briefly, cells were plated in 60-mm dishes and left untreated overnight. Then hormones were added for 24 h, followed by the addition of glutamate. After an additional 24 h, trypan blue at a concentration of 0.12% was added, and the number of viable cells (trypan blue-excluding) per low magnification field was determined. Because dexamethasone, corticosterone, and RU486 stock solutions (10^{-2} M) were prepared in ethanol, a possible ethanol effect was also assessed. Cell viability was not influenced by 0.1% ethanol in the incubation medium. Also, there was no interference of corticosterone, dexamethasone, or RU486 with the colorimetric MTT assay. All MTT and trypan blue exclusion assays were repeated three to five times in triplicate, with similar results. An ANOVA with *post-hoc* tests was performed, and *P* values are presented.

Results

Clonal hippocampal HT22 cells express endogenous GRs

Rat hippocampal neurons express functional GRs (1). By using transient transfection assays with MTV-LUC-containing GR-responsive elements, we also detected functional endogenous GRs in the clonal mouse hippocampal cell line HT22. As demonstrated in Fig. 1, HT22 cells express endogenous functional GRs that can be stimulated by increasing the concentration of dexamethasone, a synthetic glucocorticoid. Therefore, in addition to rat primary hippocampal neurons, HT22 cells provide an ideal model system to study the influence of activated endogenous GRs on oxidative cell death in a highly reproducible manner with sensitive toxicity assays.

Aβ- and glutamate-induced oxidative cell death is specifically enhanced by glucocorticoids in HT22 cells and primary hippocampal neurons

$A\beta_{25-35}$, the toxic fragment of Aβ (10), is toxic to HT22 cells, as first evaluated using phase contrast microscopy. After a 24-h incubation with 20 μM $A\beta_{25-35}$, dead cells were clearly visible in HT22 cell cultures (Fig. 2B); cellular extensions were retracted, and cell bodies appeared rounded. To quantify these observations, the MTT assay was used (Fig. 3A).

HT22 cells are particularly sensitive to the excitatory amino acid glutamate (13). Glutamate-induced cell death was detected morphologically using phase contrast microscopy (Fig. 2C) or quantitatively with the MTT assay (Fig. 3B)

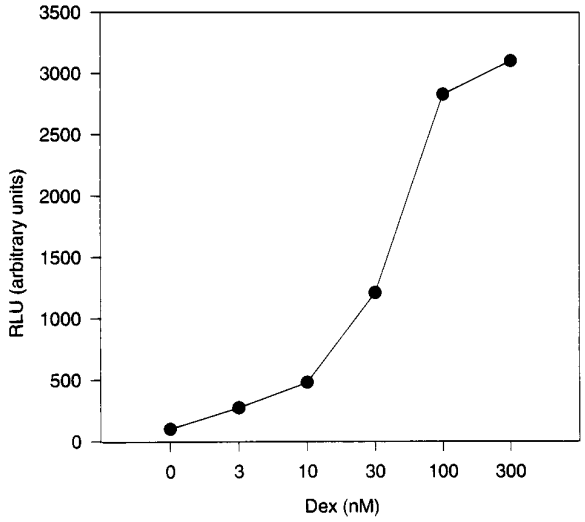

FIG. 1. Detection of endogenous GR in HT22 cells. HT22 cells were transfected with the glucocorticoid-responsive reporter plasmid MTV-LUC and incubated with increasing concentrations of dexamethasone (Dex). Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Results are shown in arbitrary units of luciferase activity (relative light units, RLU) corrected for transfection efficiency by the corresponding β-galactosidase activity and are presented as the average of at least five independent transfection experiments, with a variation from the mean of less than 25%.

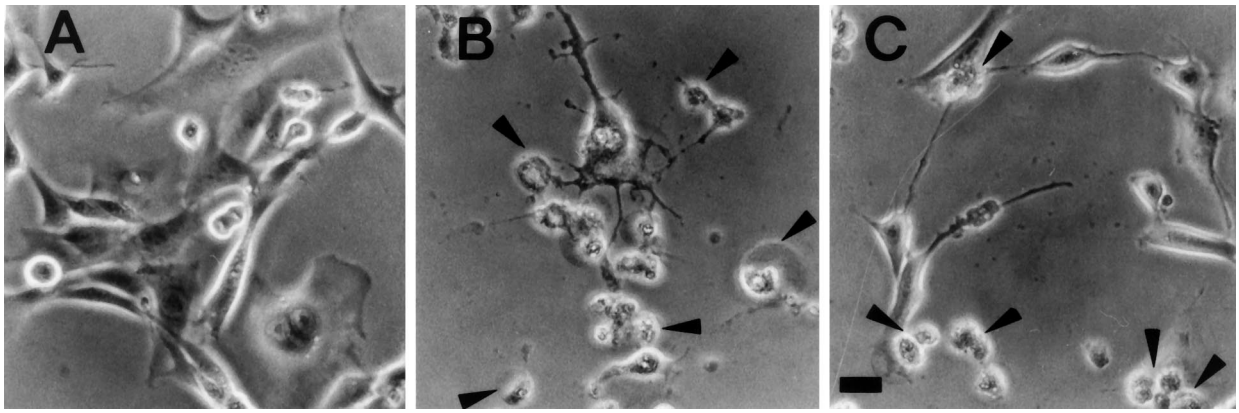

FIG. 2. $A\beta_{25-35}$ and glutamate toxicity in mouse clonal hippocampal HT22 cells as assessed by light microscopy. HT22 cells were plated and incubated with either 20 μM $A\beta_{25-35}$ (B) or 1 mM glutamate (C) for 24 h. Cell cultures were viewed with phase contrast microscopy and photographed. Degenerated cells are depicted by *black arrows*. A, Untreated control culture. *Bar* = 50 μm.

or trypan blue exclusion staining followed by cell counting (Table 1).

To investigate the influence of glucocorticoids on the course of cell death induced by the oxidative stressors Aβ and glutamate, HT22 cells and primary hippocampal neurons were treated with either different concentrations of corticosterone, the major naturally occurring glucocorticoid in rodents, or the synthetic glucocorticoid dexamethasone. A 24-h preincubation with these glucocorticoids further decreased the viability of HT22 cells after exposure to $A\beta_{25-35}$ or glutamate (Fig. 3, A and B). For Aβ, pretreatment with 10^{-5} M dexamethasone followed by a toxic challenge with 1 μM $A\beta_{25-35}$ led to a significant increase (20%; $P < 0.01$) in toxicity compared to that in cells incubated with Aβ alone. Preincubation of these cells with 10^{-5} M corticosterone had comparable Aβ toxicity-enhancing effects (Fig. 3A). HT22 cell viability after a challenge with 1 mM glutamate was also decreased, as only 18 ± 3% of HT22 cells survived after pretreatment with 10^{-5} M dexamethasone compared to 55 ± 4% of nonhormone-treated cells. Again, corticosterone had a similar effect (Fig. 3B). A significant increase in glutamate-induced cell death in HT22 cells could be detected after preincubation of the cells with lower glucocorticoid concentrations, such as 10^{-6} M corticosterone or dexamethasone (Tables 1 and 2). To demonstrate the glutamate toxicity-enhancing effect of glucocorticoids on cell lysis rather than on cellular viability and, therefore, to extend these MTT data, trypan blue exclusion assays were performed. As shown for HT22 cells, 10^{-6} M corticosterone or dexamethasone also increased the cell lysis induced by a 24-h incubation with glutamate (Table 1). When primary hippocampal neurons, which are equally sensitive to Aβ (Fig. 3) (10), were preincubated with 10^{-5} M dexamethasone or corticosterone, cell death induced by $A\beta_{25-35}$ was also significantly enhanced (Fig. 3C).

To demonstrate the specificity of this glucocorticoid effect, GRs were antagonized using the specific GR antagonist RU486. RU486 (10^{-6} M) was added together with corticosterone (10^{-6} M) for 24 h before the addition of 1 mM glutamate. With trypan blue exclusion assays followed by cell counting, a significant increase in cell survival could be detected (Table 1), indicating the specificity of this glucocorticoid effect. Interestingly, enhancement of Aβ and glutamate toxicity by glucocorticoids in our cellular hippocampal systems occurred under low glucose medium conditions only. When the cells were cultivated in high glucose conditions, no further increase in Aβ or glutamate toxicity after preincubation with corticosterone or dexamethasone was observed, as shown for HT22 cells in Table 2.

Discussion

Brain cells are at particular risk of being damaged by free radicals because the brain has a high oxygen turnover, and central nervous system (CNS) neuronal membranes are rich in polyunsaturated fatty acids, which are potential targets for lipid peroxidation. An imbalance of the equilibrium between free radical generation and various antioxidant defense systems leading to the accumulation of free radicals is called oxidative stress (28). When cellular antioxidant defense systems are compromised, oxidative stress may occur, as demonstrated for glutamate toxicity in neuronal cell lines *in vitro* (12, 13). Free radicals and oxidative stress-induced neuronal cell death have been implicated in various neurological disorders, such as Parkinson's disease and AD (29, 30). Recently, we demonstrated that the AD-associated Aβ accumulating in CNS plaques of AD patient's brains induces the generation of oxygen-free radicals, ultimately leading to the peroxidation of membrane lipids and cell lysis (6). As age is the primary risk factor for AD, the investigation of age-associated physiological and pathophysiological changes that may potentially affect the vulnerability of neurons is of central importance.

It has been shown repeatedly that under certain conditions glucocorticoids exacerbate different types of neurological insults (2–4). As the hippocampus is a major target for neuronal degeneration in the brains of patients with AD, and as it is richly endowed with GRs, it is also a principal target site for glucocorticoids. The goal of our study was to investigate the influence of glucocorticoids on the sensitivity of hippocampal neurons to the potential oxidative stressors Aβ and glutamate. In addition to rat primary hippocampal neurons, we used cells of the clonal mouse hippocampal cell line HT22.

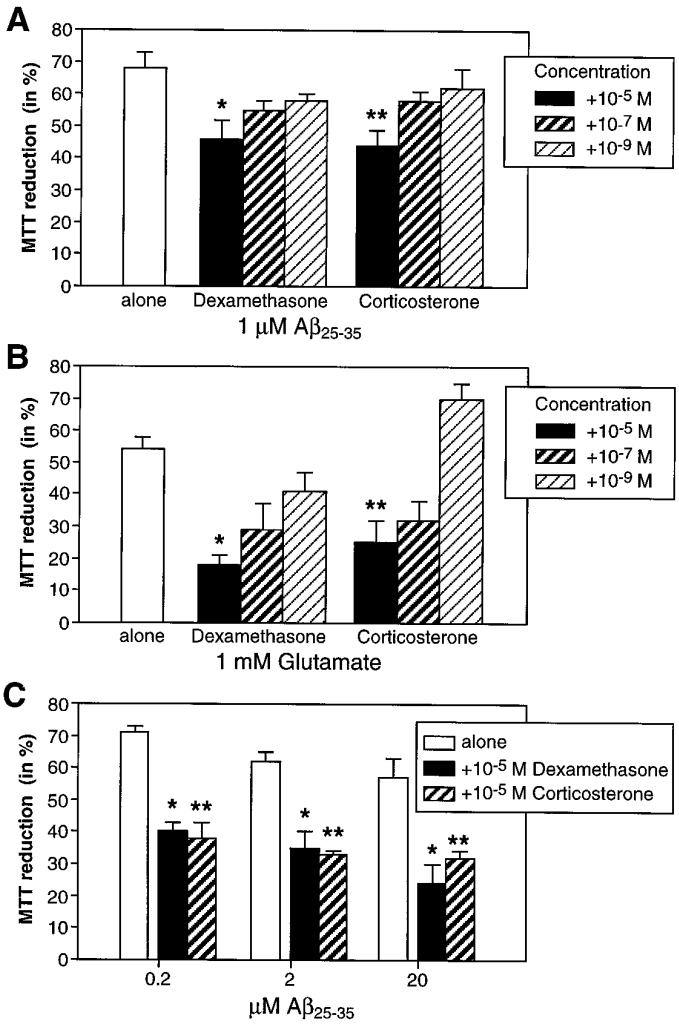

FIG. 3. Effects of glucocorticoids on $A\beta_{25-35}$ (A) and glutamate (B) toxicity in HT22 cells and on the toxicity of increasing concentrations of $A\beta_{25-35}$ in primary hippocampal neurons (C). The viability of cells with and without a 24-h preincubation with dexamethasone or corticosterone was assessed using MTT assays. Data are the mean ± SEM of triplicate determinations. *P* values were as follows: A, *P** and *P*** < 0.01; B and C, *P** and *P*** < 0.001. The viability of the HT22 cells was 93 ± 2% after incubation with 10^{-5} M dexamethasone alone and 89 ± 5% after incubation with 10^{-5} M corticosterone alone. The viability of primary hippocampal neurons was also unaltered, as the cell viability was 96 ± 5% after treatment with 10^{-5} M dexamethasone alone and 92 ± 4% after treatment with 10^{-5} M corticosterone alone. Lower glucocorticoid concentrations did not alter cell survival.

Here we report that the glucocorticoids corticosterone and dexamethasone enhance cell death induced by the oxidative stressors Aβ and glutamate in HT22 cells and cell death induced by Aβ in rat primary hippocampal neurons. The concentrations of glucocorticoids used are consistent with those used in a previous study, showing that 10^{-5}-10^{-7} M corticosterone can increase the vulnerability of hippocampal neurons (19). This increased vulnerability of hippocampal neurons was quantified with the sensitive MTT assay to detect rather early mitochondrial disturbances and changes in cellular viability, and the trypan blue exclusion assay was used to describe cell lysis. The MTT assay measures the reduction of MTT to a colored formazan (27). As the major

TABLE 1. Enhanced glutamate toxicity in HT22 cells after pretreatment with corticosterone and dexamethasone and block by RU486, as assessed by trypan blue exclusion staining followed by cell countings

Control cells	100
1 mM glutamate alone	65 ± 5
$+10^{-6}$ M corticosterone	29 ± 2[a]
$+10^{-6}$ M RU486	63 ± 4
$+10^{-6}$ M corticosterone + 10^{-6} M RU486	89 ± 4
10^{-6} M corticosterone alone	95 ± 7
10^{-6} M RU486 alone	98 ± 3

HT22 cells were plated at low density in 60-mm dishes. Culture medium was switched as described in *Materials and Methods* 24 h before hormone addition. Cells were incubated with corticosterone with or without RU486 for 24 h before the addition of 1 mM glutamate. Cultures were observed after additional 24 h, and trypan blue exclusion stainings were performed. Data presented are the means ± SEM for one representative triplicate determination and are expressed as the percent survival compared to the corresponding controls. The viability of untreated control cells was defined as 100%.

[a] $P < 0.05$ (cell survival after incubation with corticosterone and glutamate compared to cell survival after incubation with glutamate alone) was considered significant.

TABLE 2. Enhanced Aβ protein and glutamate toxicity in HT22 cells after pretreatment with corticosterone and dexamethasone, as assessed by MTT assays: effects of low and high glucose culture medium

	1 μM Aβ	1 mM glutamate
Control cells	100	
Toxin alone	65 ± 4	58 ± 5
Low glucose conditions		
$+10^{-6}$ M corticosterone	48 ± 2[a]	26 ± 2[a]
$+10^{-6}$ M dexamethasone	43 ± 4[a]	21 ± 4[a]
High glucose conditions		
$+10^{-6}$ M corticosterone	70 ± 5	50 ± 8
$+10^{-6}$ M dexamethasone	63 ± 4	52 ± 5

HT22 cells were plated in DMEM supplemented with 1000 mg/ml glucose (low glucose conditions) or with 4500 mg/ml (high glucose conditions). Both media contained 10% charcoal-stripped FCS. After 12 h, cells were incubated with 10^{-6} M corticosterone or 10^{-6} M dexamethasone for 20 h before the addition of 1 μM Aβ or 1 mM glutamate. After 6 h, MTT assays were performed. Data presented are the mean ± SEM for triplicate determinations. The viability of untreated control cells was defined as 100%.

[a] $P < 0.05$ (MTT reduction after incubation with glucocorticoids followed by the toxin compared to MTT reduction after incubation with toxin alone) was considered significant.

site of MTT reduction is thought to be at two stages of electron transport (31), the MTT assay is a very sensitive first assay of changes in mitochondrial activity and, therefore, of cellular viability. Moreover, this assay has been shown repeatedly to be a very sensitive indicator of the cell death induced by Aβ and other amyloidogenic peptides as well as by other oxidative stressors (6, 32–35). Cell lysis induced by Aβ has been demonstrated previously (6, 10, 15). The excitotoxin glutamate can induce the generation of several reactive oxygen species in neurons independently of the pathway (receptor-mediated or nonreceptor-mediated) of toxicity (12–15).

The increase in sensitivity after GR activation could be prevented by the GR antagonist RU486, indicating the receptor specificity of this effect. Hence, the results of the present study support and extend our initial observations

(36). In addition, the present data are consistent with those of a very recently published report showing that corticosterone can exacerbate oxidative neuronal injury (37). Taken together, this evidence clearly suggests, as also proposed for other neurotoxic insults, an important role for glucocorticoid homeostasis in degenerative processes resulting from the oxidative challenges imposed by glutamate and Aβ.

The hippocampus is almost always damaged in patients with AD (38–40). This area of the brain is important in regulation of the stress-adaptive and stress-responsive HPA system (41), and its activity is controlled mainly by corticosteroid receptors (1). An altered response of the HPA system may lead to increased glucocorticoid levels (5). Although additional mechanisms cannot be ruled out, the ability of glucocorticoids to increase the vulnerability of cells has been reported to be mediated via broad catabolic disruptions of cell biology, such as energy depletion, rather than through the activation of relatively discrete mechanisms of cell death (42–45). We agree with the hypothesis that GR activation can induce an energy crisis that resembles glucocorticoid-induced cellular stress (19, 43–45), as the enhancement of glutamate and Aβ toxicity through glucocorticoids in our cellular systems could be observed only after reducing glucose levels approximately 4-fold. The use of reduced glucose concentrations in the cell survival assays is consistent with previous studies (19). Hippocampal neurons may, therefore, be left glucose deprived after activation of the GRs, rendering them more vulnerable to subsequent insults.

In addition to the increased glucocorticoid levels that may occur in patients with AD (5), it is well known that the human hippocampus loses neurons with aging (38) and that there is an age-related increase in HPA system activity (46). Because the primary risk factor for AD is age, any model designed for the study of neuronal cell death in AD must account for age-related physiological changes. These changes also include a drop in antioxidant defense system expression and activity, a reduced plasticity of hippocampal GR regulation, and, ultimately, a hypersecretion of glucocorticoids (47–49). A decrease in some antioxidant enzyme activities can be directly caused by high levels of glucocorticoids in the adult rat brain, as there is a 50% decrease in glutathione peroxidase activity reported for the hippocampi of glucocorticoid-treated rats (50). Glutathione peroxidase is the major H_2O_2-detoxifying enzyme in the brain and a decrease in its activity has been shown to be involved in glutamate toxicity in neuronal cell lines (12, 13). Recently, we have been able to show that increased expression and activity of cellular antioxidant enzymes, such as glutathione peroxidase and catalase, are sufficient to afford resistance of neuronal cells to Aβ and that neuronal cells stably double transfected with glutathione peroxidase and catalase are significantly less sensitive to H_2O_2 and Aβ than are control transfectants (35). The important role of an antioxidant defense for overall cellular protection is further strengthened by several other *in vitro* investigations (6, 12, 13, 15). All of these *in vivo* and *in vitro* data are complemented by the well documented fact that one major change in the CNS that is associated with aging is free radical-induced oxidative damage (see Ref. 49 for review). Therefore, we hypothesize that oxidative stressors, such as Aβ and glutamate, can add to accumulating oxidative stress and bring additional oxidative challenges into play, thus increasing the neurodegeneration observed in AD in age-compromised neurons that may be additionally compromised by a preexisting hypercortisolism.

In summary, our data show that the neurotoxic challenges induced by the oxidative stressors Aβ and glutamate, two neurotoxins that have been implicated in AD (6–9, 11, 15, 16), are enhanced by pretreatment of the hippocampal neurons with glucocorticoids. Our findings add to the growing body of evidence that supports the idea that physiological interactions between the brain and the HPA system play a crucial role in brain aging and possibly also in the etiology of AD and other neurodegenerative and age-related neurological diseases.

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