

## IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTOR MEDIATION OF GLUCOCORTICOID-INDUCED APOPTOSIS IN HIPPOCAMPAL CELLS AND THE NEUROPROTECTIVE ROLE OF SYNAPTIC *N*-METHYL-D-ASPARTATE RECEPTORS

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**Abstract**—Glutamate receptors have been proposed to mediate the apoptotic actions of glucocorticoids in hippocampal cells. To further analyze the role of glutamate receptors in this process, we pretreated primary hippocampal cells from neonatal (postnatal day 4) rats with antagonists of ionotropic glutamate receptor (iGluR) and metabotropic glutamate receptor (mGluR) antagonists before exposure to the specific glucocorticoid receptor agonist dexamethasone (DEX) at a dose of 1  $\mu$ M. Dizocilpine (MK801; a general *N*-methyl-D-aspartic acid [NMDA] receptor antagonist, NMDAR antagonist) and ifenprodil (a specific ligand of the NMDAR 2B subunit, NR2B), were used to block iGluR; (*RS*)- $\alpha$ -ethyl-4-carboxyphenylglycine (E4CPG) and (*RS*)- $\alpha$ -cyclopropyl-4-phosphonophenyl-glycine (CPPG) were employed as I/II (E4CPG) and II/III (CPPG) mGluR antagonists. Blockade of iGluR resulted in a significant attenuation of DEX-induced cell death; the finding that ifenprodil exerted a similar potency to MK801 demonstrates the involvement of NR2B receptors in glucocorticoid-induced cell death. Apoptosis accounted for a significant amount of the cell loss observed, as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling histochemistry for the *in situ* labeling of DNA breaks; apoptotic cells were distinguished from necrosis on the basis of morphological criteria, including chromatin condensation, membrane blebbing and presence of apoptotic bodies. Treatment with E4CPG and CPPG completely abolished the apoptotic response to DEX, thus showing the additional contribution of mGluR to the phenomenon. Further, dose-response studies with NMDA revealed that whereas high (10  $\mu$ M) doses of NMDA themselves elicit cytotoxic responses, low (1–5  $\mu$ M) concentrations of NMDA can effectively oppose DEX-induced cell death. Interestingly, the neuroprotective actions of low dose NMDA stimulation were abolished when either synaptic or extrasynaptic NMDA receptors were blocked with MK801 in combination with the

GABA receptor antagonist bicuculline (synaptic) or ifenprodil (extrasynaptic). In summary, the present data show that both iGluR and mGluR mediate the neurotoxic effects of glucocorticoids on hippocampal cells and that pre-treatment with low doses of NMDA, by acting on synaptic and extrasynaptic receptors, render hippocampal cells less vulnerable to glucocorticoid insults. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** glucocorticoids, glutamate, NMDA, ionotropic glutamate receptor, metabotropic glutamate receptor, hippocampus.

Glucocorticoids (represented endogenously by cortisol and corticosterone) are hormones secreted by the adrenal cortex in response to stress. Acting through hippocampal receptors (glucocorticoid receptors [GR]), they play an important role in the physiological and behavioral adaptive responses to stress (Sapolsky et al., 2000). On the other hand, there is an abundant literature indicating that excessive glucocorticoid secretion can result in damaging effects on adaptive mechanisms as well as on the viability of structural integrity of neurons in the hippocampus. Notably, activation of GR leads to neuritic atrophy and death of neurons, a phenomenon that has been linked to disorders of mood and cognition as well as neuroendocrine dysregulation (De Kloet et al., 1998; McEwen, 2000; Sapolsky, 2000; Abraham et al., 2001; Sousa and Almeida, 2002). Despite some conflicting reports (Masters et al., 1989; Roy and Sapolsky, 2003), work from our laboratory has demonstrated that apoptosis is at least one of the forms of cell death resulting from exposure to glucocorticoids; that conclusion was made in light of the presence of specific morphological features (see Hassan et al., 1996) and the activation of key pro-apoptotic versus anti-apoptotic genes (Almeida et al., 2000). Several different, although not mutually exclusive, mechanisms have been implicated in these GR-mediated neurodegenerative effects; these include reduced glucose uptake by neurons, increases in the extracellular concentrations of glutamate with concomitant elevations in intracellular  $Ca^{2+}$  levels (Sapolsky, 2000).

Glutamate is the dominant neurotransmitter in the brain, being found in most excitatory synapses. Its actions are mediated by ionotropic glutamate receptors (iGluR) which are directly associated with ligand-gated ionophores permitting  $Ca^{2+}$  influx, and metabotropic glutamate receptors (mGluR); the latter are G protein-coupled receptors which can activate secondary messengers such as cAMP

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**Abbreviations:** CPPG, (*RS*)- $\alpha$ -cyclopropyl-4-phosphonophenylglycine; DEX, dexamethasone; E4CPG, (*RS*)- $\alpha$ -ethyl-4-carboxyphenylglycine; EBBS, Earle's buffered salt solution; GluR, glutamate receptor; GR, glucocorticoid receptor; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; MK-801, dizocilpine; MR, mineralocorticoid receptor; NMDA, *N*-methyl-D-aspartic acid; NMDAR, NMDA receptor; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; P, postnatal day; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

or diacylglycerol and phosphoinositides (Lipton and Rosenberg, 1994; Pin and Duvoisin, 1995). Both types of receptor can activate downstream pathways that determine cell survival or death. Their dichotomous actions appear to result from differences in the duration and magnitude of action and the subsequent levels of intracellular  $Ca^{2+}$ . Synaptic *N*-methyl-D-aspartic acid (NMDA) receptor (NMDAR), which respond to physiological (low dose) NMDAR activation, have been associated with the promotion of cell survival; in contrast, extrasynaptic NMDAR located on the cell body, axon and dendritic spines (Li et al., 1998; Tovar and Westbrook, 1999) have been associated with apoptosis (Hardingham et al., 2002; Riccio and Ginty, 2002). As compared with the synaptic NMDAR, the extrasynaptic NMDAR is characterized by a faster and more extensive rundown of peak current (Li et al., 2002). It is currently believed that while activation of extrasynaptic NMDAR is a rare event under normal conditions, it becomes more common during acute cellular insults or pathological situations when glutamate transporters operate in reverse, thereby increasing extracellular concentrations of glutamate (Rossi et al., 2000).

Glucocorticoids and glutamate receptor (GluR) share an intimate relationship: (i) glucocorticoids can increase hippocampal cell vulnerability to GluR activation (Armanini et al., 1990); (ii) GR activation leads to an up-regulation of the expression of one type of GluR, the ionotropic NMDAR, strongly implicated in neurotoxicity (Weiland et al., 1997); (iii) elevated glucocorticoid levels have been associated with an increase in glutamatergic transmission (Moghaddam et al., 1994) and (iv) glutamate can enhance GR activation (Gursoy et al., 2001). In light of these interactions, and given the neurotoxic potential of each, glutamate and glucocorticoids, the many possibilities for mutual potentiation of each other's effects can be easily appreciated.

Blockade of the NMDAR was previously shown to mediate the inhibitory effects of stress (during which glucocorticoid secretion is increased) on neurogenesis in the hippocampus; this observation provides a clue as to the particular subtype of GluR that may mediate at least some glucocorticoid actions (Gould et al., 1997). The studies described here were designed to provide further insight into the role of GluR in dexamethasone (DEX)-induced cell death in the hippocampus. To do so, we resorted to an *in vitro* model, treating primary hippocampal cells from postnatal day 4 (P4) rats with ionotropic and metabotropic GluR antagonists, to evaluate the role of each of these receptors in DEX-induced apoptosis. A further objective of this study was to explore the possibility of an additive effect of DEX and NMDA; for this, hippocampal cells were exposed to a range of NMDA concentrations before subsequent treatment with DEX. Finally, the role of extrasynaptic versus synaptic NMDAR in these events was examined with the aid of ifenprodil, a selective antagonist of extrasynaptic NMDA-R, and a combination of MK-801 and bicuculline to block synaptic NMDAR.

## EXPERIMENTAL PROCEDURES

### Primary cell culture

Cultures were prepared from Wistar rats aged 4 days (P4; Charles River, Sulzfeld, Germany), following a protocol previously developed in our laboratory (Crochemore, 2000). Briefly, hippocampal slices were digested using the Papain Dissociation System from Worthington Biochemicals (Lakewood, NJ, USA) and the dissociated cells plated on poly-D-lysine-coated glass coverslips at a density of 400 cells/mm<sup>2</sup>. Cultures were maintained in Neurobasal A medium to which 2% B27 supplement, 1 mM GlutamaxI (Invitrogen, Eggenstein, Germany) and 0.1mg/ml kanamycin were added (all supplements from Invitrogen). Culture medium was half-renewed every 3 days. Experiments were started 6 days after plating. Immunocytochemical analysis of the cultures revealed that the cultures comprised ca. 90% neurons (neuronal markers used: NeuN, TuJ1 and doublecortin) and ca. 10% astro-glial cells (glial fibrillary acidic protein-positive).

### Drugs

DEX, obtained as a freely-soluble sodium salt from Merck (Darmstadt, Germany), was used at a concentration of 1  $\mu$ M in all experiments; DEX treatment was always added to cultures after 3–6 days *in vitro* for 72 h. In all experiments, the mineralocorticoid receptor (MR) antagonist spironolactone was added (10  $\mu$ M) in order to antagonize interfering effects from low (nanomolar range) MR-activating doses of corticosterone present in the culture medium (Crochemore, 2000). NMDA was obtained from Sigma Chemicals (Deisenhofen, Germany). All other drugs were purchased from Tocris (Bristol, UK). They included bicuculline (used at 50  $\mu$ M), MK801 (a general NMDAR antagonist; used at 10  $\mu$ M), ifenprodil (a specific ligand of the NMDAR 2B subunit; used at 10  $\mu$ M), and (*RS*)- $\alpha$ -ethyl-4-carboxyphenylglycine (E4CPG) and (*RS*)- $\alpha$ -cyclopropyl-4-phosphonophenyl-glycine (CPPG), antagonists of mGluR I/II and mGluR III, respectively (both used at 10  $\mu$ M). The precise treatment protocols used in individual experiments are detailed below.

### Experiment 1: attenuation of DEX-induced apoptosis by GluR blockade

Hippocampal cells were pretreated with either MK801, ifenprodil or a combination of CPPG and E4CPG for 15 min before addition of DEX (continued presence of antagonists) for 72 h after which cultures were analyzed for the incidence of apoptosis.

### Experiment 2: dose-dependent effects of NMDA on DEX-induced cell death

In the first part of this experiment, cells were treated with NMDA for 15 min at doses between 1 and 10  $\mu$ M; assessment of cell death (apoptosis and necrosis) was performed 72 h later. In order to maximize the NMDA effects, the usual culture medium (supplemented Neurobasal A) was replaced by and added to the cells in  $Mg^{2+}$  free Earle's buffered salt solution (EBSS; Invitrogen) containing 10  $\mu$ M glycine. The treatment schedule for the second part of the experiment was as follows: pre-exposure to NMDA (1–10  $\mu$ M in  $Mg^{2+}$  free EBSS plus 10  $\mu$ M glycine) for 15 min, followed by a washout step, and addition of DEX (1  $\mu$ M in supplemented Neurobasal A) for 72 h. At this point, the cultures were fixed and processed for the detection of apoptosis.

### Experiment 3: role of synaptic receptors in NMDA-associated neuroprotection

After pre-exposure to a mixture of bicuculline and MK801 (50  $\mu$ M and 10  $\mu$ M, both in  $Mg^{2+}$  free EBSS plus 10  $\mu$ M glycine) for 15 min, a washout step, NMDA (1 or 5  $\mu$ M) was added and incubation

continued for a further 15 min. A thorough washout step was then performed and cells were subsequently treated with DEX (1  $\mu$ M in supplemented Neurobasal A) and maintained for 72 h when they were processed for the detection of apoptosis.

#### Experiment 4: attenuation of NMDA-induced neuroprotection by ifenprodil

After pre-exposure to ifenprodil (10  $\mu$ M) and NMDA (1 or 5  $\mu$ M in  $Mg^{2+}$  free EBSS plus 10  $\mu$ M glycine) for 15 min, and a washout step, cultures were then transferred to supplemented Neurobasal A medium containing DEX at a concentration of 1  $\mu$ M for 72 h before being examined for apoptosis.

#### Cell death assay

Cell death was examined in 4% paraformaldehyde-fixed cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry or Hoechst 33342 staining. When TUNEL histochemistry was to be performed, permeabilization (0.1% Triton X-100) and peroxidase blocking (1%  $H_2O_2$ ) steps were carried out before application of the TUNEL assay as previously described (Almeida et al., 2000). Apoptotic cells were characterized by dark brown nuclear staining; only those nuclei showing evidence of DNA fragmentation without plasma membrane damage were taken to be apoptotic cells. Hoechst staining was used in a few cases to confirm TUNEL staining. Fixed cells were incubated with the dye (1:1000) for 15 min before examination under a fluorescence microscope. Apoptotic versus total cells (%) were quantified in at least five randomly chosen microscopic fields (0.072 mm<sup>2</sup>, magnification of 400 $\times$ ) across the long axis of the coverslips on which cells were grown; an average of 1000 cells were sampled on each coverslip and the results shown represent values from six to 10 coverslips per treatment.

#### Statistical analysis

All data are depicted as means  $\pm$  S.D. and represent the observations from three to five independent experiments, with three to four replicates for each data point. Data were analyzed for statistical significance using ANOVA and appropriate post hoc tests (Student-Newman-Keuls or Kruskal-Wallis multiple comparison procedures) in which  $P \leq 0.05$  was set as the minimum level of significance.

## RESULTS

Throughout, TUNEL-positive cells were considered to be apoptotic only if they showed the characteristic morphological features of apoptosis (see Hassan et al., 1996). Under control conditions (no drug treatment), basal rates of apoptosis were  $10.9 \pm 1.9\%$  (TUNEL-positive cells meeting morphological criteria of apoptosis expressed as a percentage of total number of cells; mean  $\pm$  S.E.M.). Representative microscopic fields from TUNEL-stained control and DEX-treated cultures are shown in Fig. 1.

#### Attenuation of DEX-induced apoptosis by GluR blockade (Fig. 2)

Apoptosis was significantly increased in hippocampal cultures exposed for 72 h to the GR-specific agonist DEX at a dose of  $10^{-6}$ M ( $P < 0.05$ ); the apoptotic effects of DEX were abrogated by the GR antagonist RU 38486 (mifepristone,  $10^{-5}$ M), indicating mediation by GR (data not shown). Subsequent experiments involved pretreatment of

cells with various GluR antagonists (at  $10^{-5}$ M for 30 min before the introduction of, and during exposure to, DEX at  $10^{-6}$ M). Concomitant treatment with the general NMDAR antagonist MK801 or the NR2B-specific antagonist ifenprodil rescued cells from DEX-induced apoptosis ( $P < 0.01$ ) to levels that were not significantly different from those observed under drug-naive conditions. In the absence of DEX, MK801 and ifenprodil did not exert significant effects on the rate of apoptosis in the cultures (data not shown). Application of the mGluR antagonists E4CPG (mGluR I/II) and CPPG (mGluR II/III), while not exerting any effects on their own (data not shown), abolished the apoptotic actions of DEX ( $P < 0.001$ ) and significantly improved basal cell survival ( $P < 0.05$ ), i.e. the protective effects afforded by the mGluR antagonists exceeded those provided by NMDAR blockade ( $P < 0.01$  or  $P < 0.001$ ).

The results of this experiment therefore conclusively demonstrate that induction of apoptosis by DEX is mediated by GR and, in turn, by iGluR and mGluR.

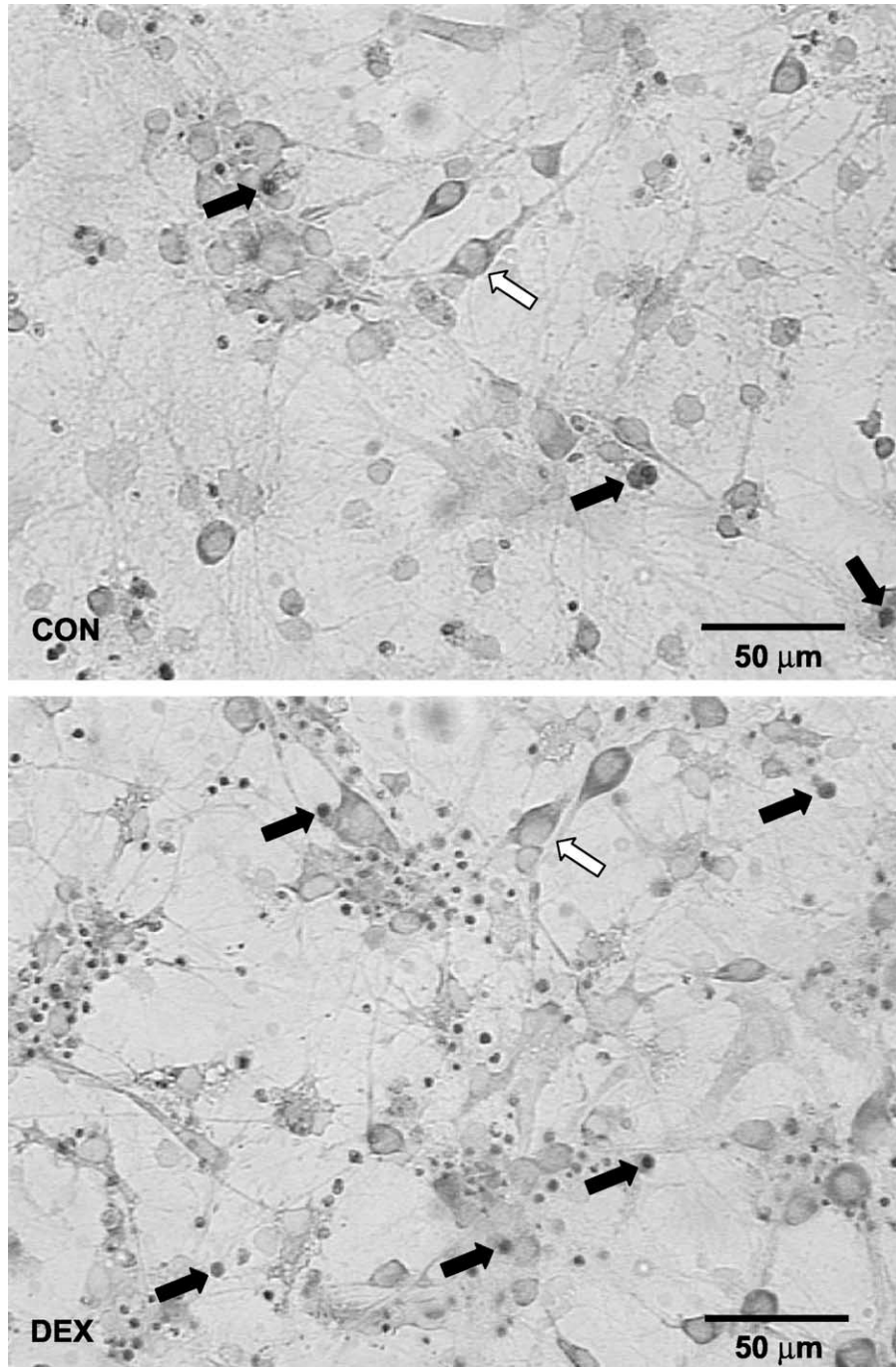
#### Dose-dependent effects of NMDA on DEX-induced cell death (Fig. 3)

In view of the above findings implicating NMDAR involvement in the apoptotic actions of DEX, we first conducted a dose-response study with NMDA. The NMDA was added for 15 min in  $Mg^{2+}$ -free medium containing 10  $\mu$ M glycine. As shown in Fig. 3A, the two lower doses of NMDA (1 and 5  $\mu$ M) did not influence hippocampal survival, as assessed 72 h after application of the drug. In contrast, the highest dose tested (10  $\mu$ M) led to a significant increase in cell death ( $P < 0.05$ ). Based on this result as well as reports that GR activation can increase the vulnerability of hippocampal cells to glutamatergic toxicity (Armanini et al., 1990), we subsequently examined whether pre-exposure to NMDA can influence the magnitude of DEX-induced cell death; in these experiments, cells were pre-treated with NMDA and then exposed to DEX (in the absence of NMDA) for 72 h. As shown in Fig. 3B, the highest dose of NMDA (10  $\mu$ M) resulted in cell death but did not potentiate the effects of DEX. On the other hand, and contrary to our predictions, NMDA at one and 5  $\mu$ M was found to significantly attenuate the apoptotic actions of DEX ( $P < 0.001$  and  $P < 0.01$ ).

These results demonstrate that low doses of NMDA can counteract the cell death-inducing effects of DEX.

#### Synaptic receptors mediate the neuroprotective effects of NMDA (Fig. 4)

Recent evidence suggests that extrasynaptic NMDAR may be responsible for triggering excitotoxicity; in contrast, synaptic NMDAR are thought to activate cell survival-promoting signaling cascades (Hardingham et al., 2002). In contrast to NMDA receptor subunit 2A (NR2A) subunits which are mainly found in synaptic NMDAR, NMDA receptor subunit 2B (NR2B) predominate in extrasynaptic NMDAR (Tovar and Westbrook, 1999). Because of this, and the fact that we observed that DEX-induced apoptosis could be attenuated by antagonism of the NR2B subunit with ifenprodil (Fig. 2), it was of inter-

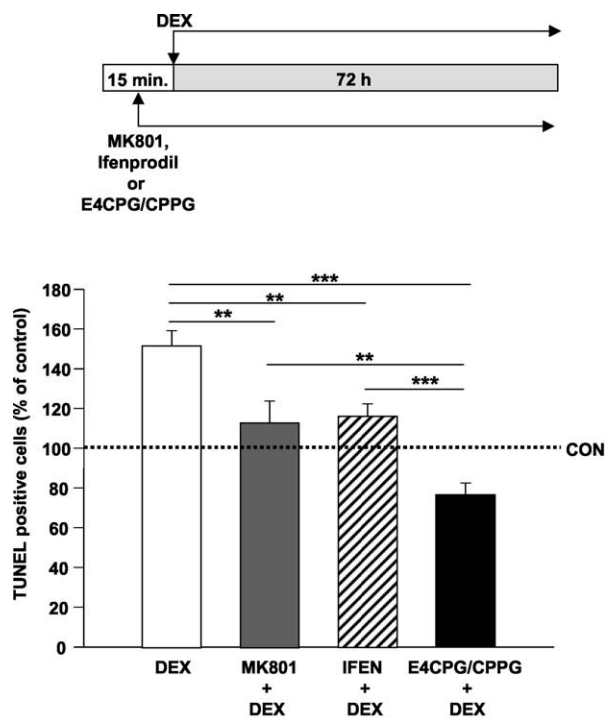


**Fig. 1.** DEX increases apoptosis in primary hippocampal cell cultures. Photomicrographs show TUNEL-staining in control (upper panel) and DEX-treated (lower panel) cells. Examples of TUNEL-positive cells displaying some of the morphological features used to designate apoptotic cells in this study (e.g. shrunken cell body or apoptotic bodies) are marked with black arrows. Note that the relative number of apoptotic versus healthy cells (white arrows) is increased after exposure to DEX (lower panel).

est to examine the role of extrasynaptic NMDAR in our paradigm. Following a recently described experimental paradigm (Hardingham et al., 2002), we applied the GABA receptor antagonist bicuculline (1  $\mu\text{M}$ ) together with MK801 (5  $\mu\text{M}$ ) in order to subsequently be able to activate extrasynaptic NMDAR selectively. This treat-

ment paradigm abolished the ability of the low doses of NMDA (1 and 5  $\mu\text{M}$ ) to counteract the apoptosis-inducing effects of DEX ( $P < 0.001$  and  $P < 0.05$ ).

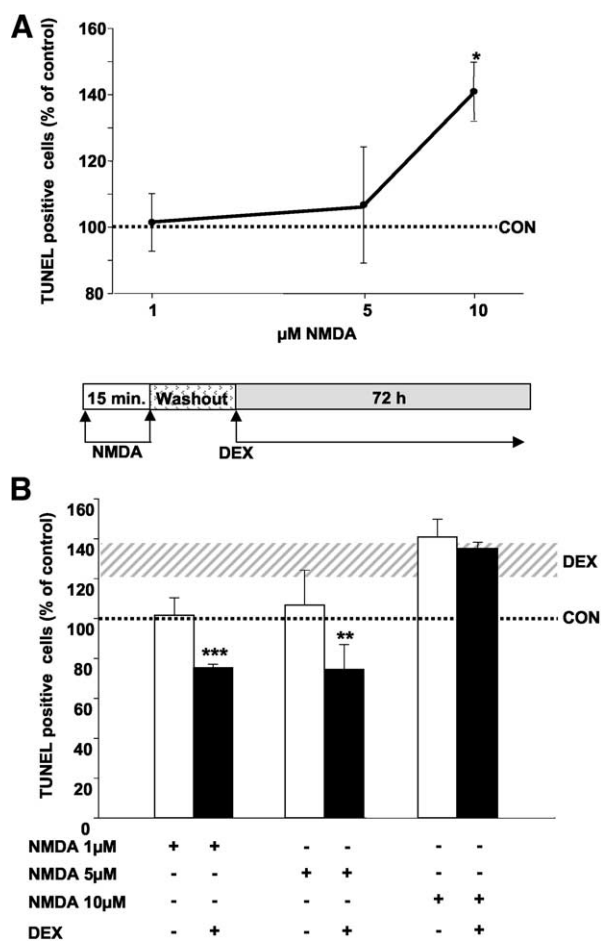
These findings conform with the view that synaptic NMDAR play a permissive role in the neuroprotective actions of low doses of NMDA.



**Fig. 2.** Attenuation of DEX-induced apoptosis by GluR blockade. Treatment of hippocampal cultures with MK801 (NMDAR antagonist), ifenprodil (IFEN; selective NR2B subunit antagonist) and E4CPG/CPPG (antagonists of mGluRI/II and mGluRII/III, respectively) for 15 min before and during application of DEX (1  $\mu$ M) resulted in a significant reduction in DEX-induced apoptosis, as measured by TUNEL histochemistry. All of the antagonists were used at 10  $\mu$ M. \*\* and \*\*\* indicate  $P < 0.01$  and  $< 0.001$ , respectively. Results represent means  $\pm$  S.D. from five experiments, with three replicates for each data point.

**Ifenprodil attenuates low dose NMDA-induced neuroprotection (Fig. 5)**

NR2B are predominantly expressed at extrasynaptic sites which have been associated with neurotoxicity (Hardingham et al., 2002). As mentioned, ifenprodil is a selective inhibitor of the NR2B subunit of the NMDAR (Gotti et al., 1988) and has proven neuroprotective efficacy in animal models of degeneration (Kemp and McKernan, 2002). The results depicted in Fig. 2 show that ifenprodil can reduce the cytotoxic effects of DEX. Since the results of the previous experiment (Fig. 4) indicated that synaptic receptors mediate the protective effects of low doses of NMDA, the question arose as to the impact of NR2B extrasynaptic receptor antagonism on DEX-induced cell death. Because ifenprodil is an activity-dependent NR2B antagonist, cells were pre-treated with NMDA (1 and 5  $\mu$ M) and ifenprodil (10  $\mu$ M) for 15 min, before wash-out and exposure to DEX (1  $\mu$ M) for 72 h. Contrary to our prediction, hippocampal cultures treated according to this paradigm displayed more apoptosis than those exposed only to the NMDA pulse followed by DEX ( $P < 0.01$  and  $P < 0.05$ ). Thus, these observations indicate that transient blockade of NR2B can prevent low doses of NMDA from exerting protective actions against DEX-induced apoptosis.

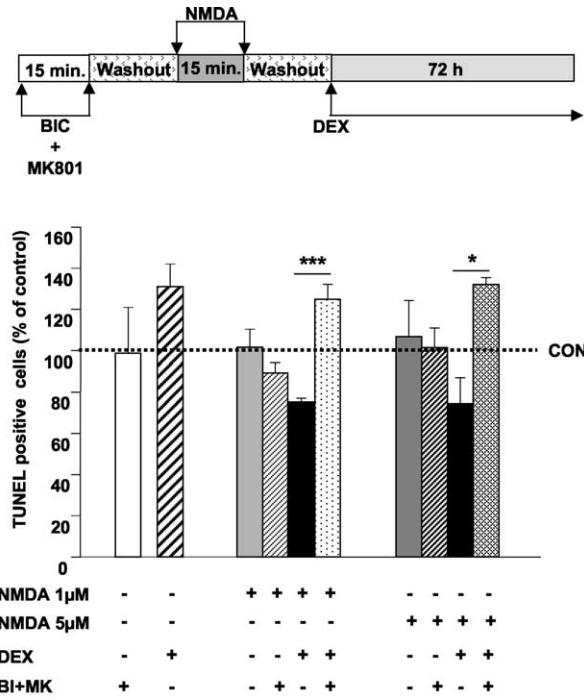


**Fig. 3.** Dose-dependent effects of NMDA on DEX-induced cell death. Cell death was assessed after treatment of cultures for 15 min with NMDA ranging from 1 to 10  $\mu$ M. A. Dose-response curve showing that 1 and 5  $\mu$ M NMDA did not have affect on cell survival, whereas 10  $\mu$ M of the drug significantly stimulated cell death. Note that data shown refer only to counts of TUNEL-positive cells, although after exposure to 10  $\mu$ M NMDA, there was an increase in the number of cells showing signs of necrosis. B. Demonstration that pretreatment with low, sub-toxic doses of NMDA (1 and 5  $\mu$ M) can attenuate DEX-induced apoptosis. The stippled area represents the effects of treatment with 1  $\mu$ M of DEX for 72 h. All values shown are means  $\pm$  S.D., derived from five experiments (in which each data point was replicated four times). \*, \*\* and \*\*\* indicate significant differences where  $P < 0.05$ ,  $< 0.01$  and  $< 0.001$ , respectively.

**DISCUSSION**

Glucocorticoids fulfill several important functions in the hippocampus, including cognition, mood and neuroendocrine regulation. Nevertheless, high levels of both endogenous and therapeutic glucocorticoids can affect hippocampal function deleteriously. These undesired effects have been proposed to result from either neuronal atrophy and, in extreme cases, neuronal cell death of certain principal cells of the hippocampus (for review, see Sousa and Almeida, 2002).

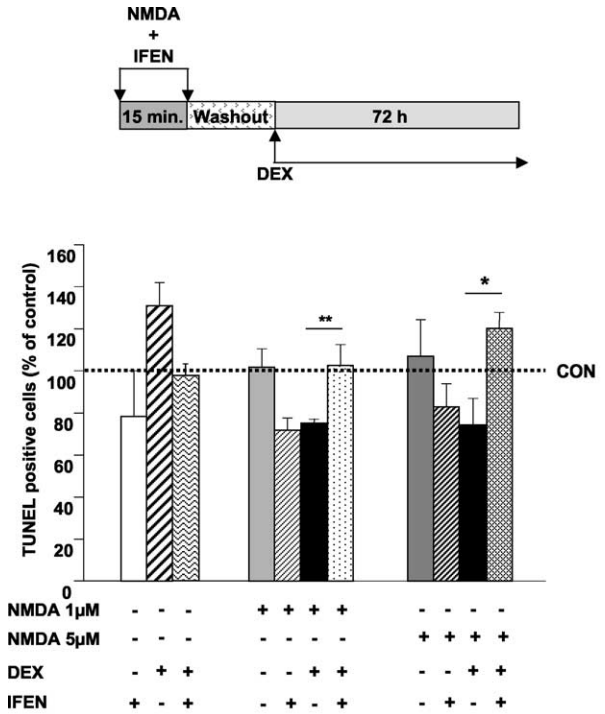
Glucocorticoids can exert rapid, transient effects on neuronal excitability involving increased cytosolic concentrations of  $Ca^{2+}$  (Nair et al., 1998). However, glucocorticoid actions are best known to be mediated through GR which are transcription factors and our group has previ-



**Fig. 4.** Synaptic receptors mediate the neuroprotective effects of NMDA. Synaptic NMDAR were blocked by initially exposing hippocampal cultures to the GABA antagonist bicuculline (50 µM) and the activity-dependent NMDAR antagonist MK801 (10 µM). Subsequently, cells were treated with NMDA (1 or 5 µM; 15 min), washed and exposed to DEX (1 µM) for a further 72 h when apoptosis was measured. Results (means±S.D. from four experiments, with four replicates for each data point) show that blockade of synaptic NMDAR prevents low doses of NMDA to counteract the apoptotic actions of DEX. \* and \*\*\* indicate significant differences where  $P < 0.05$  and  $0.001$ , respectively.

ously elucidated some of the cell death-related molecular pathways triggered by exposure to the potent GR agonist DEX (Almeida et al., 2000). Thus, glucocorticoid effects on hippocampal structure and function do not necessarily result from one exclusive mechanism (electrophysiological versus genomic) but rather may occur as a result of integrated signals arising from both the cell membrane and transcriptional activity. For example, by repressing the transcription of  $Ca^{2+}$  channel and  $Ca^{2+}$  extrusion pump genes (Bhargava et al., 2000), glucocorticoids contribute to long-term alterations in the dynamics of intracellular  $Ca^{2+}$  levels, including those originating at the plasma membrane (Kerr et al., 1992; Elliott and Sapolsky, 1993; Nair et al., 1998; Bhargava et al., 2000).

Exaggerated and chronic elevations in intracellular  $Ca^{2+}$  accompany glutamatergic excitotoxicity (Choi, 1991; Coyle and Puttfarcken, 1993) and have also been proposed to at least partially underlie the neurotoxic effects of glucocorticoids (Joëls, 2001). Increases in cytosolic  $Ca^{2+}$  concentrations result from the activation of both iGluR and mGluR, albeit through different mechanisms: iGluR stimulate the influx of  $Ca^{2+}$  from the extracellular space, whereas mGluR mobilize  $Ca^{2+}$  from intracellular reser-



**Fig. 5.** Ifenprodil attenuates low dose NMDA-induced neuroprotection. Pharmacological blockade of the NR2B subunit of the NMDAR was achieved by pre-incubating cells with ifenprodil (IFEN, 10 µM). Subsequently, the ability of low doses of NMDA (1 or 5 µM) to protect against the apoptotic effects of DEX (1 µM; 72 h) was examined. The data (means±S.D. derived from three experiments, with three replicates for each data point) show that the protective effects of NMDA are markedly attenuated when NR2B-containing NMDAR are rendered non-functional by IFEN. \* and \*\* indicate significant differences where  $P < 0.05$  and  $< 0.01$ , respectively.

voirs (Maiese et al., 1999; Otani et al., 2002). Glucocorticoids are known to increase NMDAR expression and glutamate synthesis and extracellular accumulation (Weiland et al., 1997; Moghaddam et al., 1994), and to potentiate glutamate-induced cell death (Goodman et al., 1996; Behl et al., 1997; Abraham et al., 2001; Johnson et al., 2002). To date, however, earlier suggestions that glutamatergic mechanisms may play an intermediary role in glucocorticoid-induced cell death have remained largely unsupported by firm experimental evidence. The data presented in this paper fill that gap by demonstrating that iGluR (here, only the NMDAR type was studied) and mGluR mediate at least some of the apoptotic effects of DEX in primary hippocampal cultures. With respect to the involvement of iGluR, our data show that general blockade of NMDAR with MK801 significantly attenuates DEX-triggered cell death (Fig. 2).

GluR are well-recognized triggers of neuronal cell death and may occur either acutely upon activation or after a period of delay (Choi, 1991). These two forms of neuronal death are distinguishable on the basis of their morphological characteristics and ionic dependence. The rapid-onset form is necrotic in nature, characterized by cell swelling, intact nuclei with diffuse nucleoplasm, disrupted cell membrane and ultimate cell lysis. In contrast, the delayed

form is  $\text{Ca}^{2+}$ -dependent, and is accompanied by cell shrinkage, membrane blebbing and nuclear condensation and fragmentation (karyoklasis); all of the latter features are typical of apoptosis (Choi, 1991). In the present work, the dominant form of cell death observed following treatment of hippocampal cells with both NMDA and DEX was of the apoptotic type.

The NMDAR has been the most intensely studied GluR, especially in the context of neurotoxicity. NMDAR are heteromeric in nature; they consist of a common NMDA receptor subunit 1 and one or more NR2 subunits (NR2A-D), whose insertion in the NMDAR complex varies during development and maturity of synapses (Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). For example, it is known that whereas NR2A is predominantly found in NMDAR located at the synapse, NR2B are localized almost exclusively in NMDAR at extrasynaptic (non-synaptic) sites. An important concept that has emerged from recent studies is that synaptic NMDAR can initiate neuroprotective mechanisms; in contrast, activation of extrasynaptic NMDAR results in cell death (Hardingham et al., 2002). The present experiments demonstrate that selective inhibition of NR2B with ifenprodil results in a suppression of DEX-induced apoptosis (Fig. 2); this finding is consistent with the documented efficacy of ifenprodil in retarding cell death in various animal models of neurodegenerative disease (for review see Chenard and Menniti, 1999).

Previous work which showed that GluR stimulation can result in excitotoxicity and that the latter can be amplified by pre-treatment with glucocorticoids (Goodman et al., 1996; Behl et al., 1997; Abraham et al., 2001; Johnson et al., 2002) prompted us to examine the effects of NMDA pre-treatment on DEX-induced apoptosis. To do this, we treated hippocampal cultures with NMDA at doses ranging from 1 to 10  $\mu\text{M}$  for a brief period (15 min) before exposure to DEX (1  $\mu\text{M}$ ) for 72 h. We observed a complete abolition of the apoptotic actions of DEX when cells were pre-exposed to NMDA at 1 and 5  $\mu\text{M}$ ; at a dose of 10  $\mu\text{M}$ , NMDA resulted in overt necrosis (Fig. 3). Subtoxic concentrations of NMDA are indeed known to elicit neurotrophic and anti-apoptotic mechanisms in neurons (Marini et al., 1998; Resink et al., 1996; Brandoli et al., 1998); moreover, one of the implicated neurotrophins, brain-derived nerve growth factor, has been found to stimulate (pro-survival) NR2A subunit expression and to suppress NR2B (death-promoting) expression (Glazner and Mattson, 2000). Because our experimental paradigm involved chronic exposure to DEX, it is pertinent to mention a study which showed that GR activation leads to an increase in NR2B subunit gene expression with a concomitant decrease in the expression of the gene encoding NR2A subunit (Nair et al., 1998).

As noted above, NMDAR including the death-promoting NR2B subunit are predominantly localized at extrasynaptic sites; in contrast those comprising the pro-survival NR2A subunit have a synaptic location (Riccio and Ginty, 2002). Since the appearance of NR2A- and NR2B-containing NMDAR is correlated with the ontogeny of synapses

(Tovar and Westbrook, 1999; Li et al., 1998; Stocca and Vicini, 1998), it is pertinent to note that although the cultures used for the present studies were relatively young (experiments carried out after 6 days *in vitro*), cell–cell interactions were abundantly evident at the light microscopic level. In order to distinguish between synaptic and extrasynaptic NMDAR in the mediation of the protective actions of low doses of NMDA against DEX-stimulated apoptosis, we adopted the recently described elegant pharmacological paradigm described by Hardingham et al. (2002). Briefly, the paradigm which consists of pre-treating (15 min) hippocampal cultures with the GABA antagonist bicuculline (to activate synaptic GluR) and MK801 (to block active NMDAR) prevents NMDA activation of neuroprotective signaling cascades, leaving only extrasynaptic NR2B-containing NMDAR available for NMDA binding. Cultures pre-treated in this way were subsequently exposed to 1 or 5  $\mu\text{M}$  NMDA and DEX, after which they were analyzed for apoptosis. The observation that the bicuculline/MK801 pre-treatment abrogated the ability of NMDA to oppose the apoptotic actions of DEX (Fig. 4) is consistent with a synaptic site of NMDA-induced neuroprotection (cf. Hardingham et al., 2002).

In an experiment to examine the impact of NR2B subunit-containing NMDAR blockade on the neuroprotection afforded by subtoxic NMDA doses against DEX-elicited apoptosis, NR2B blockade was achieved by pre-treating cells with NMDA and ifenprodil. It was reasoned that this experimental design would ensure binding of ifenprodil, an activity-dependent antagonist, to extrasynaptic receptors and, at the same time, permit (or even enhance) the activity of synaptic NMDAR. Contrary to expectations, we observed that the NR2B-blocking procedure led to an amplification of DEX-induced apoptosis (Fig. 5). Only speculative explanations can be offered for this finding at present. Assuming similar affinities of NMDA for NR2A and NR2B, and that neuroprotective NR2A are predominantly occupied under basal conditions, a tenable explanation is that, as extracellular glutamate concentrations increase, neuronal fate is balanced by NR2A versus NR2B activity. Also, since the NMDA/ifenprodil pre-treatment was transient (15 min), it could be that the prolonged exposure to DEX resulted in increased sensitivity and/or up-regulation of the synthesis of death-promoting NR2B receptors, or equally, a down-regulation of pro-survival NR2A receptors; the likelihood of such a mechanism is supported by the observation that extended exposure to ifenprodil abrogated the neurotoxic actions of DEX (Fig. 2). In this context, it should be noted that the GR is a potent transcriptional factor. Thus, another plausible explanation would be that by employing transcriptional mechanisms to elevate glutamate synthesis (Ábrahám et al., 1996), DEX would effectively make more neurotoxic glutamate available for activating NR2 subunit NMDAR, thus positively driving a vicious circle. Further studies are required to clarify these issues.

A role for mGluR was demonstrated by the finding that E4CPG and CPPG, selective antagonists of mGluR I/II and II/III, respectively, can rescue hippocampal cells from

DEX-induced apoptosis; strikingly, it was observed that mGluR antagonism can also improve neuronal survival under basal conditions (Fig. 2). In general, the manifestation of mGluR-mediated actions are slow and involve gene activation. Type I mGluR have been associated with neuronal death (Snyder et al., 2001; Allen et al., 2001; Miskovich et al., 2002; Heidinger et al., 2002) while Type II/III mGluR have been shown to contribute to neuronal survival. The present results, using a cocktail of relatively non-selective antagonists because of the unavailability of more receptor type-specific drugs are interesting: they suggest the involvement of either complex regulatory interactions between the various mGluR or the up-regulation of neurotoxic type I mGluR by DEX.

The present paper represents the first attempt to understand the mechanistic and functional nature of interactions between GluR and glucocorticoids. Its results demonstrate that the apoptotic actions of DEX are at least partly mediated by GluR of the NMDA and metabotropic types. In addition, the results reported herein show that low doses of NMDA, acting via synaptic NMDAR can effectively block hippocampal cell death induced by DEX. Last, this work indicates that glucocorticoids can cause apoptosis in hippocampal cells by triggering rapid (NMDAR-mediated) as well as slow (mGluR- or GR-mediated) responses, and that the final outcome of glucocorticoid treatment on hippocampal cell survival depends on the convergence and integration of transcriptional signals (e.g. GluR and agonist availability; regulation of apoptosis-related genes) and signals originating at the cell membrane (e.g.  $Ca^{2+}$  conductance). Although the present findings are consistent with previous findings, it should be noted that the present experiments were carried out on cells obtained from neonatal hippocampal tissue; therefore, the mechanisms described here do not necessarily apply to the adult hippocampus which has been the focus of the majority of *in vivo* studies of the neurotoxic actions of glucocorticoids. A second caveat concerns the fact that the serum-free medium used for the cell cultures favored the survival of neurons rather than glia (neuronal–glial ratio approximately 10:1 versus 1:10 *in vivo*). Since astrocytic glutamate transporters are important for maintaining low extracellular glutamate concentrations (Nedergaard et al., 2002), the results obtained here more likely represent an extreme situation rather than the physiological norm. In this context, it is also important to remember that the dose of DEX (1  $\mu$ M) used in these *in vitro* experiments is high in relation to the nanomolar concentrations of bioavailable corticosterone that brain neurons are exposed to (Linthorst et al., 2000). Therefore, caution is required in directly extrapolating the present observations to the situation in the organism.

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