Neuroprotective potential of a viral vector system induced by a neurological insult

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Gene transfer into neurons via viral vectors for protection against acute necrotic insults has generated considerable interest. Most studies have used constitutive vector systems, limiting the ability to control transgene expression in a dose-dependent, time-dependent, or reversible manner. We have constructed defective herpes simplex virus vectors designed to be induced by necrotic neurological insults themselves. Such vectors contain a synthetic glucocorticoid-responsive promoter, taking advantage of the almost uniquely high levels of glucocorticoids—adrenal stress steroids—secreted in response to such insults. We observed dose-responsive and steroid-specific induction by endogenous and synthetic glucocorticoids in hippocampal cultures. Induction was likely to be rapid enough to allow transgenic manipulation of relatively early steps in the cascade of necrotic neuron death. The protective potential of such a vector was tested by inclusion of a neuroprotective transgene (the Glut-1 glucose transporter). Induction of this vector by glucocorticoids decreased glutamatergic excitotoxicity in culture. Finally, both exogenous glucocorticoids and excitotoxic seizures induced reporter gene expression driven from a glucocorticoid-responsive herpes simplex virus vector in the hippocampus *in vivo*.

Because of their generally postmitotic nature, neurons are rarely replaced when killed by hypoxia-ischemia, seizure, or hypoglycemia. This vulnerability places pressure on developing interventions to save neurons after such insults. The development of viral vectors for delivering genes into neurons has promise in this realm. Numerous studies document the neuroprotective potential of viral vector-directed overexpression of genes targeting the degenerative cascade mediating necrotic neuronal injury [i.e., the excess of synaptic glutamate, free cytosolic calcium, calcium-dependent oxygen radical accumulation, and the triggering of apoptosis in a subset of neurons (1)]. The steps targeted include energetic components of damage (by overexpression of a glucose transporter) (2–4), calcium excess (with overexpression of a calcium binding protein) (5–8), protein malfolding (with hsp72) (9, 10), oxygen radical accumulation (with superoxide dismutase) (5), apoptotic elements (with apoptosis inhibitors) (11–16), excitatory components (by enhancing GABAergic tone) (17), and inflammation (18, 19).

These studies have used vectors with constitutive promoters, making it impossible to target transgene expression specifically to the time directly after onset of an insult. Obviously, in the case of some neuroprotective transgenes, it may be undesirable to have expression occur during other than the period immediately after an insult; such temporal control, however, would be virtually impossible with constitutive systems. One solution is to use an inducible promoter in which expression is regulated by manipulation of an exogenous signal [such as the tetracycline-responsive system (20–22)]. Even more advantageous from a clinical setting would be a promoter induced by the neurological insult itself, obviating the need for an exogenous signal.

Glucocorticoids are steroid hormones secreted by the adrenal gland in response to stress (23) and whose actions are mostly genomic. Positive transcriptional effects of glucocorticoids are accomplished by binding of hormone/receptor complexes to glucocorticoid response elements (GREs) in DNA (24, 25). Necrotic insults generate circulating glucocorticoid levels equaled only by severe physical stressors (e.g., sepsis) (26–29). Thus, the elevated glucocorticoid concentrations during necrotic insults may be harnessed as relatively unique signals in an insult-inducible system.

In this study, we assess the ability of a synthetic glucocorticoid-responsive promoter to act as a "smoke alarm" in responding to stress signals and inducing expression. Studies were carried out in hippocampal cultures and in the rat hippocampus, as this structure, with plentiful corticosteroid receptors, is highly glucocorticoid-sensitive (30). Our results suggest that this glucocorticoid-regulated vector meets the requirements of an insult-inducible system.

Materials and Methods

Construction of Amplicons. The GRE5 promoter and second rabbit β-globin intron was isolated from pGRE5–2 (31) as an *Xba*I–*Hin*dIII fragment, blunt-ended, and cloned into blunt-ended *Xho*I–*Eco*RI linearized pHSVGRE5GH.1 (32) to create pHLGRE. The firefly (*Photinus pyralis*) *luciferase* gene isolated from pJD281 (33, 34) as a *Bgl*II–*Bam*HI fragment was cloned into the *Bgl*II site of pHLGRE to create pHLGREluc. A small herpes simplex virus (HSV) *oriS* and "*a*" sequence isolated from pON812 (35) as a *Bam*HI fragment was cloned into the *Bam*HI site of pGEM2 (Promega) to create pGEMori-a. *Luciferase* and human cytomegalovirus (HCMV) *ie*1 poly(A) signal, were obtained as a *Bgl*II fragment from ptTAluc (21), blunt-ended, and cloned into blunt-ended *Hin*cII-linearized pGEMori-a to produce pLucori-a. A blunt-ended *Xba*I–*Hin*dIII fragment from pGRE5–2 containing the GRE5 promoter and β-globin intron was cloned into blunt-ended *Hin*dIII pLucori-a to create pGRE(L)luc. A blunt-ended *Xba*I-*Bam*HI fragment from pGRE5–2 containing only the GRE5 promoter was cloned into blunt-ended *Hin*dIII pLucori-a to create pGRE(S)luc. An *Xba*I fragment containing *lacZ* and the simian virus 40 (SV40) poly(A) signal was obtained from pON2 (a kind gift from Edward Mocarski, Stanford University). This fragment then was cloned into the *Xba*I site of pGEMori-a to produce pβgalori-a. A blunt-ended *Xba*I–*Hin*dIII fragment from pGRE5–2, containing the GRE5 promoter and β-globin intron, then was cloned into blunt-ended *Sal*I pβgalori-a to create pGRE(L)lacZ. pGRE(L)gtlacZ was constructed by removal of the *lacZ* coding sequences of pGRE(L)lacZ by *Xba*I digestion. The resulting fragment was ligated to an *Xba*I fragment of pα4gtβgal (2),

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Abbreviations: HCMV, human cytomegalovirus; HSV, herpes simplex virus; GRE, glucocorticoid response element; GT, Glut-1 glucose transporter; KA, kainic acid; SV40, simian virus 40; IRES, internal ribosomal entry site; X-gal, 5-bromo-4-chloro-3-indonyl-β-D-galactopyranoside.

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containing coding sequences for the GLUT-1 isoform of the rat brain glucose transporter (GT), the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (36–38), and *lacZ* coding sequences.

Cell Culture. Primary neuronal/glial cultures were prepared from hippocampi of embryonic day 18 Sprague–Dawley rats (Simonsen Labs, Gilroy, CA) (39). Cells were plated into poly-D-lysine-treated 48-well plates at a density of 3×10^5 cells/well and grown in MEM-Pak (modified MEM, University of California, San Francisco) with 10% (vol/vol) horse serum (HyClone). Cultures were used 10–13 days after plating. E5 (40) and Vero (ATCC CC81) cells were grown in DMEM (GIBCO/BRL) with 10% NuSerum (Collaborative Research). Cells were grown in 5% CO_2 at 37°C.

Generation of HSV Vectors. Defective HSV vectors were generated by using *d120* (40) as the helper virus; *d120* contains a deletion in the HSV-1 α-4 immediate early gene and requires the complementing E5 cell line for propagation. Our protocol for generating viral vectors has been described (41). Transfected E5 cells were superinfected with *d120* at a multiplicity of infection of 0.03 or 0.1, and all vector stocks were purified by centrifugation through sucrose (9). By convention, amplicon vectors are denoted with the prefix "p;" viral vectors generated from these plasmids are denoted with the prefix "v." Titers of luciferase-expressing vectors were determined by immunohistochemistry using a polyclonal rabbit antiluciferase antiserum (Cortex Biochem, San Leandro, CA), 12 h after addition of 10^{-6} M dexamethasone (Sigma) to transduced Vero cells. Titers of β-galactosidase-expressing vectors were determined by staining with X-gal (5-bromo-4-chloro-3-indonyl-β-D-galactopyranoside, Molecular Probes), 12 h after addition of 10^{-6} M dexamethasone to transduced primary neuronal/glial cells. All viruses had vector titers ranging from 1×10^5 to 2×10^7 particles/ml. Titers of helper virus were determined on E5 cells by using a standard plaque assay and ranged from 5×10^6 to 1×10^8 plaque-forming units/ml.

Infection and Steroid Induction of Cultures. For time course, dose response, and steroid specificity studies, primary hippocampal cultures were infected with vHLGREluc, vGRE(L)luc, vGRE(S)luc, or vGRE(L)lacZ, by addition of 1 μl of each directly to cells without media change; 8×10^3 to 2×10^4 particles/well of viral vector were used. Solutions of dexamethasone, corticosterone (Roussel-UCLAF), estradiol, progesterone, and testosterone (all from Sigma) were prepared by dissolving each in 100% ethanol. Two hours after transduction of cultures with viral vectors, 4 μl of stock solution was added directly to wells to yield the final appropriate steroid concentrations. For control wells, 4 μl of 100% ethanol was added to each well. Luciferase assays were conducted as described (43); cells were lysed with 100 μl of lysis buffer/well, and 50 μl of each lysate was measured by using a luminometer (Monolight 2001, Analytical Luminescence Laboratory, San Diego). Luciferase data were collected as light units.

Toxicity Experiments. For glutamate toxicity studies, cultures were transduced with 1 μl of vGRE(L)lacZ or vGRE(L)gtlacZ. Two, 12, 24, or 48 h later, cells were rinsed with chloride-reduced media [80 mM Na_2SO_4, 4 mM K_2SO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM $CaCl_2 \cdot 2H_2O$, 15 mM dextrose, 5 mM glucose, and 27 mM $NaHCO_3$ (42)] and incubated in chloride-reduced media containing 30 μM glutamate (monosodium salt) and 10^{-6} M dexamethasone for 30 min. Cells were washed and overlaid with DMEM containing 5 mM glucose and 10^{-6} M dexamethasone. For controls, cells were: incubated with chloride-reduced media without glutamate for 30 min and not treated with dexamethasone, incubated with chloride-reduced media with glutamate for 30 min and not treated with dexamethasone, or treated with chloride-reduced media without glutamate and treated with 10^{-6} M dexamethasone. Cells then were incubated for an additional 12 h, fixed with 0.5% gluteraldehyde, and stained with X-gal. Cell survival was assessed by blind counting of X-gal-positive cells/well. Our previous studies have shown that typically 90% of infected cells are neurons (3). The ratio of X-gal-positive cells after glutamate exposure to those without glutamate was calculated for each vector and subtracted from 1 to determine the percentage of transduced cells that died as a result of glutamate toxicity. Equivalent amounts of vGRE(L)lacZ and vGRE(L)gtlacZ were not used; however, because data are presented as percentage of cell death within each group, differences in vector amount were controlled for internally.

***In Vivo* Studies.** Male Sprague–Dawley rats (200–250 g, Simonsen Labs) were anesthetized with 0.2 ml of ketamine/acepromazine/xylazine (10:2:1). Three microliters of vGRE(L)lacZ was deliv-

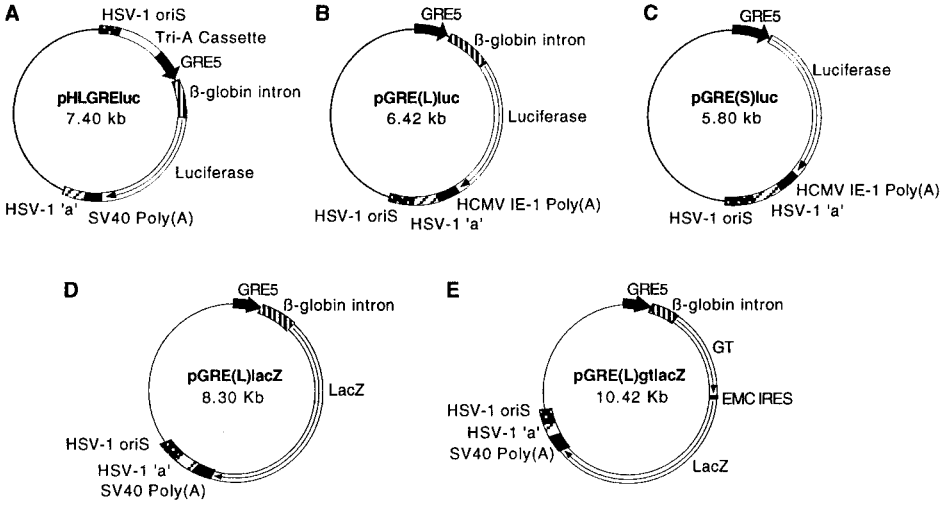

Fig. 1. Schematic diagrams of HSV amplicons encoding luciferase (*A–C*), β-galactosidase (*D*), or both β-galactosidase and GT (*E*) under transcriptional control of GRE5. Each amplicon includes an HSV origin of replication (*oriS*) and HSV "*a*" sequence required for replication and packaging of the vector.

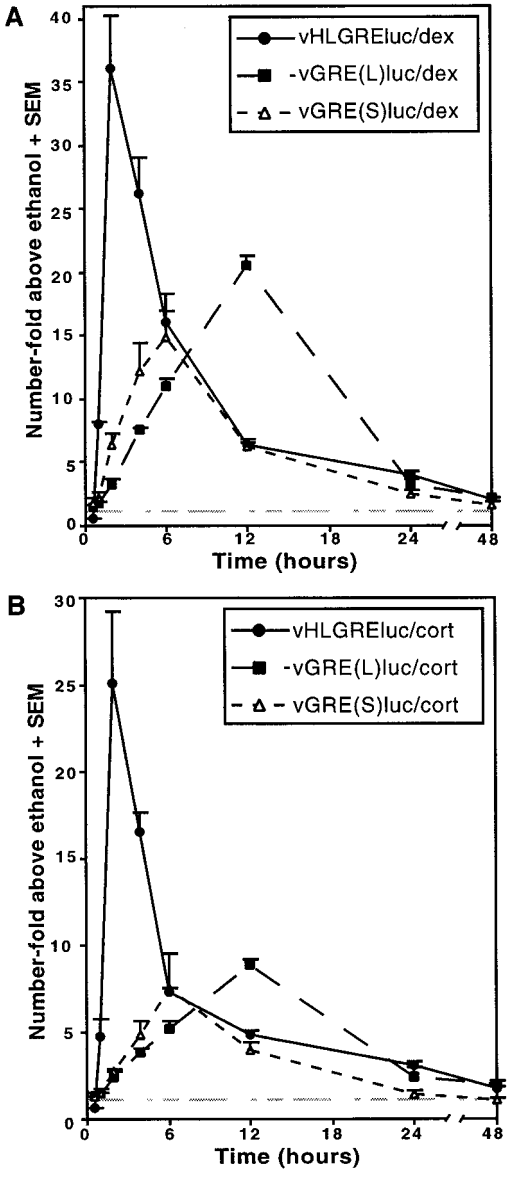

Fig. 2. Kinetic analysis of luciferase induction by glucocorticoids in cultures transduced with vHLGREluc, vGRE(L)luc, or vGRE(S)luc. Cells were assayed for luciferase activity 0.5, 1, 2, 4, 6, 12, 24, and 48 h after addition of 10^{-6} M dexamethasone (*A*) or corticosterone (*B*) to transduced cultures. Cultures were infected with virus 2 h before addition of steroid, and repeated measures were obtained from cultures over time. Data are number-fold induction above ethanol control and were obtained by dividing each value of luciferase activity by the average luciferase activity for the corresponding ethanol controls. For each time point, the number of wells assayed were for vHLGREluc, $n = 3$; vGRE(L)luc, $n = 4$; and vGRE(S)luc, $n = 6$. The gray dashed line represents the corresponding value of the ethanol controls (1); standard error of normalized ethanol controls for each time point and vector combination did not exceed 0.21. A slow rise in luciferase activity noted in some of the ethanol control cultures over time was likely caused by low concentrations of glucocorticoids in serum or by accumulation of luciferase protein in cultures. *P* values were obtained by Bonferroni/Dunn post hoc tests after one-way ANOVA of each steroid and vector combination as a function of time.

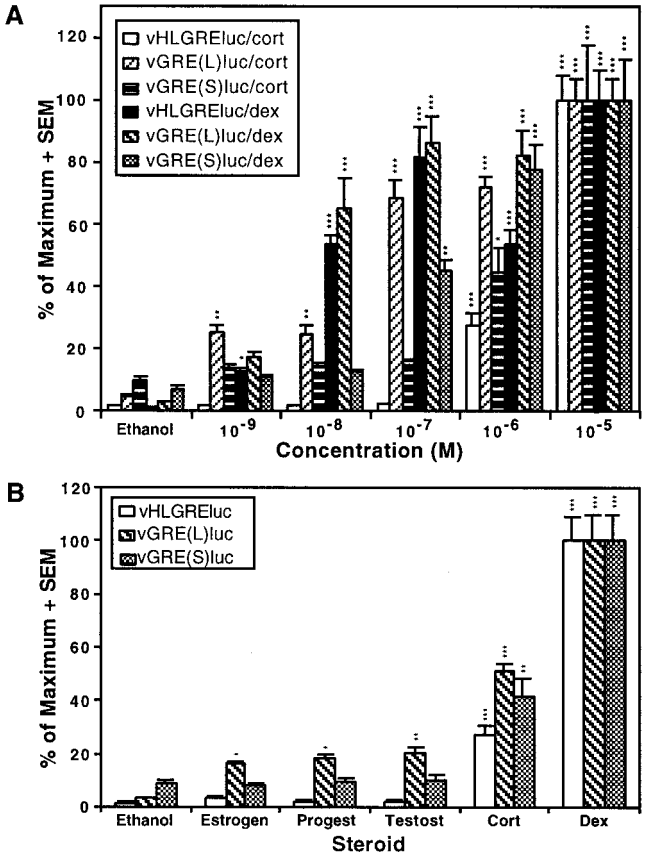

Fig. 3. GRE5 directs induced expression of *luciferase* in a dose-dependent and glucocorticoid-preferential manner. (*A*) Cultures transduced with vHLGREluc, vGRE(L)luc, or vGRE(S)luc were treated with indicated concentrations of dexamethasone or corticosterone and assessed for luciferase activity 2, 6, and 12 h later, respectively, at time points representing maximal induction. It should be noted that the culture media contained nM levels of glucocorticoids derived from serum; however, similar results were observed in serum-free media (data not shown). (*B*) Addition of dexamethasone or corticosterone resulted in significant induction relative to ethanol controls when cells were transduced with vHLGREluc, vGRE(L)luc, or vGRE(S)luc. Addition of the nonglucocorticoid steroids estradiol, progesterone (progest), and testosterone (testost) led to significant expression of *luciferase* above controls only in cells transduced with vGRE(L)luc. Data are percentage of the maximal average luciferase values [derived for the highest concentration for each steroid and vector (*A*) or for dexamethasone and each vector (*B*)]; statistical results obtained by Bonferroni/Dunn post hoc tests after one-way ANOVAs of each steroid and vector combination over all doses (*A*) or of each vector across all steroids (*B*). The luciferase values representing 100% were (in light units): (*A*) vHLGREluc/cort, 23181.0; vGRE(L)luc/cort, 1109.3; vGRE(S)luc/cort, 2159.5; vHLGREluc/dex, 43898.0; vGRE(L)luc/dex, 1886.3; vGRE(S)luc/dex, 3004.5; (*B*) vHLGREluc, 23529.3; vGRE(L)luc, 1556.2; vGRE(S)luc, 2339.0. Number of wells assayed were $n = 3$–4, vHLGREluc; $n = 4$, vGRE(S)luc; or $n = 12$, vGRE(L)luc for each dosage (*A*) or steroid (*B*). In this and subsequent figures, *, **, and *** = $P < 0.05$, 0.01, and 0.001, respectively, by the indicated statistical test.

ered unilaterally via stereotaxic injections to dentate gyrus at coordinates (from bregma): AP −3.0, ML +2.0, DV −3.5. Three milligrams of corticosterone dissolved in 0.3 ml of peanut oil was injected i.p. at the time of stereotaxic surgery. Control rats received equivalent injections of peanut oil. Kainic acid (KA)-treated rats received unilateral injections of 0.09 μg of kainic acid (Sigma), at the time of viral injection and at the same coordinates. Rats were perfused with 3% paraformaldehyde in PBS 24 h postsurgery, and brains were postfixed in 20% sucrose/3% paraformaldehyde solution for at least 24 h. Serial sections (25 μm) were collected with a cryostat and immediately reacted with X-gal solution. The total number of X-gal-positive cells was counted in every 12th section and multiplied by 12 to obtain an estimated total number/brain.

Statistics. Data are expressed as mean ± SEM and were analyzed with indicated tests using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA).

Results

Time Course, Dose Response, and Steroid Specificity of Glucocorticoid Induction in Hippocampal Cultures. HSV amplicons containing a synthetic glucocorticoid-inducible promoter (GRE5) were constructed (Fig. 1). GRE5 is composed of five high-affinity GREs placed upstream of the adenovirus 2 major late promoter "TATA" box/initiation site (31). The amplicons in Fig. 1 *A–D* differ with regard to placement of the HSV replication signal (*oriS*) relative to GRE5, inclusion or exclusion of a β-globin intron sequence, source of poly(A) signal (SV40 or HCMV *ie*1), and inclusion of either the firefly *luciferase* or bacterial *lacZ* gene as a reporter gene. pGRE(L)lacZ is a derivative of pGRE(L)luc; the vectors are identical, except *luciferase* and HCMV *ie*1 poly(A) signal of pGRE(L)luc were replaced with *lacZ* and an SV40 poly(A) signal to produce pGRE(L)lacZ. When compared by transient transfection of equal quantities of each amplicon DNA into Vero cells, a monkey kidney-based cell line, pGRE(L)luc exhibited lower levels of basal luciferase activity, relative to pHLGREluc and pGRE(S)luc (data not shown).

Hippocampal cultures were transduced with the viral vectors vHLGREluc, vGRE(S)luc, or vGRE(L)luc, and the time courses of induction after addition of a synthetic or endogenous glucocorticoid (dexamethasone or corticosterone, respectively) were determined (Fig. 2). All three vectors exhibited significant induction within 1 h of dexamethasone application (Fig. 2*A*). Significant induction was observed 1, 4, and 6 h after addition of corticosterone to cultures transduced with vHLGREluc, vGRE(L)luc, and vGRE(S)luc, respectively (Fig. 2*B*). Maximal induction with either glucocorticoid occurred at 2 h for vHLGREluc, 6 h for vGRE(S)luc, and 12 h for vGRE(L)luc and ranged from 15- to 36-fold above ethanol controls with dexamethasone and 8- to 25-fold with corticosterone. Induction returned toward baseline by 48 h after steroid application.

We next examined the dose responsiveness and steroid specificity of induction of vHLGREluc, vGRE(L)luc, and vGRE(S)luc at the time points of maximal induction of each. Primary cultures transduced with each vector and treated with dexamethasone or corticosterone demonstrated increased luciferase expression with increased hormone concentrations (Fig. 3*A*). Steroid specificity was assessed after addition of 10^{-6} M concentrations of corticosterone, dexamethasone, or nonglucocorticoid steroids (estradiol, progesterone, or testosterone) to transduced cells (Fig. 3*B*). For vHLGREluc and vGRE(S)luc, luciferase expression above ethanol controls was induced only by corticosterone or dexamethasone. In contrast, vGRE(L)luc was induced not only by glucocorticoids, but also was induced several-fold by estradiol, progesterone, and testosterone ($P < 0.05$). Induction by glucocorticoids, however, was still higher than by nonglucocorticoid steroids ($P < 0.001$). vGRE(L)lacZ exhibited glucocorticoid specificity, with no significant induction by 10^{-6} M nonglucocorticoid steroids after 12 h in transduced cultures (data not shown). The threshold of significant *lacZ* expression 12 h after addition of glucocorticoids to vGRE(L)lacZ-transduced cultures was 10^{-8} M for dexamethasone and 10^{-6} M for corticosterone (data not shown). Induction observed in vGRE(L)lacZ-transduced cultures averaged 804 ± 43-fold and 580 ± 6-fold above ethanol controls (based on number of X-gal-positive cells), 12 h after addition of 10^{-5} M dexamethasone and corticosterone, respectively (data not shown).

A GRE5 Vector Expressing GT Protects Against Excitotoxicity. The neuroprotective potential of GRE5 HSV vectors was tested by using GT as a transgene. To allow identification of cells expressing GT, pGRE(L)gtlacZ (Fig. 1*E*) was created by replacing *lacZ* in pGRE(L)lacZ with a bicistronic cassette containing GT, *lacZ*, and an IRES. The IRES ensures translation of both genes from the same transcript. IRES-directed translation is less efficient than cap-dependent translation in HSV amplicon vectors (2). Thus, when *lacZ* constitutes a second cistron as in pGRE(L)gtlacZ, *lacZ* expression is more conservative than that of GT, making *lacZ* expression a guarantee of GT expression. vGRE(L)gtlacZ decreased glutamatergic neurotoxicity, relative to vGRE(L)lacZ, even after periods of dormancy (Fig. 4).

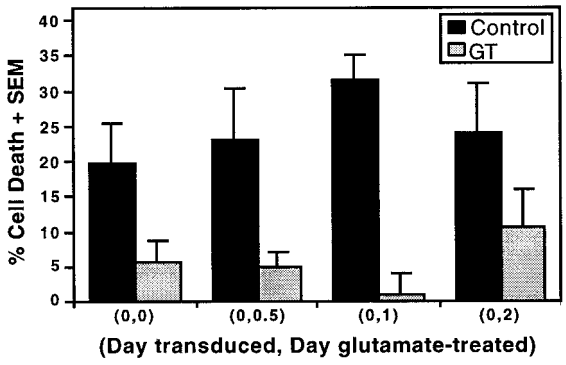

Fig. 4. The neuroprotective potential of vGRE(L)gtlacZ after a time delay between transduction of cultures and induction of transgenes was assessed. Cells were transduced with either vGRE(L)lacZ (control) or vGRE(L)gtlacZ (GT) at the same time and subsequently exposed to glutamate and dexamethasone 2, 12, 48, or 72 h later. Twelve hours after glutamate treatment and induction by steroid, the number of X-gal-positive cells remaining was assessed for both vectors. For comparison, transduced cultures were exposed to 10^{-6} M dexamethasone but did not receive glutamate treatment. At each time point, the percentage of cell death caused by glutamate was determined. GT-treated cultures had less glutamate-induced neuron death than control cultures ($P < 0.001$ by two-way ANOVA comparing GT and control against time). The number of wells assayed was $n = 10$ for controls and $n = 20$ for GT at each time point. Cultures exposed to dexamethasone and glutamate averaged 473.5 ± 52.2 and 5797.7 ± 307.6 X-gal-positive cells at time (0,0), and 3.5 ± 0.7 and 61.5 ± 7.7 at time (0,2) for vGRE(L)lacZ and vGRE(L)gtlacZ, respectively. Cultures exposed to dexamethasone but no glutamate had an average of 588.5 ± 56.2 and 6102.5 ± 208.6 at time (0,0), and 4.5 ± 1.0 and 68.8 ± 6.7 at time (0,2) for vGRE(L)lacZ and vGRE(L)gtlacZ, respectively. The average number of X-gal-positive cells in controls treated with neither dexamethasone nor glutamate at time (0,0) was 6.4 ± 1.1 and 7.6 ± 1.0 and at time (0,2) was 1.4 ± 0.5 and 5.8 ± 1.3, for vGRE(L)lacZ and vGRE(L)gtlacZ, respectively. Control cultures treated with glutamate but no dexamethasone showed a slightly lower number of X-gal-positive cells than controls that had received neither glutamate not dexamethasone.

Of concern to the clinical utility of an insult-inducible system is its ability to be activated after remaining dormant within host cells. Thus, we tested the inducibility of vGRE(L)lacZ by adding 1 μM dexamethasone after from 1 to 7 days of infection and dormancy (i.e., in the absence of glucocorticoids). Inducibility declined as a function of dormancy; nonetheless, there was still significant induction after 3 days of dormancy (data not shown). Induction above time-matched ethanol controls occurred after 3 days for vGRE(S)luc and after 5 days for vHLGREluc (data not shown).

Induction by Exogenous Corticosterone and KA-Induced Seizures *in Vivo*. The inducibility of vGRE(L)lacZ in rat hippocampus by high levels of exogenous corticosterone and by a neurological insult were examined. Rats injected unilaterally with vGRE(L)lacZ and that received corticosterone injections demonstrated large numbers of X-gal-positive cells centered around

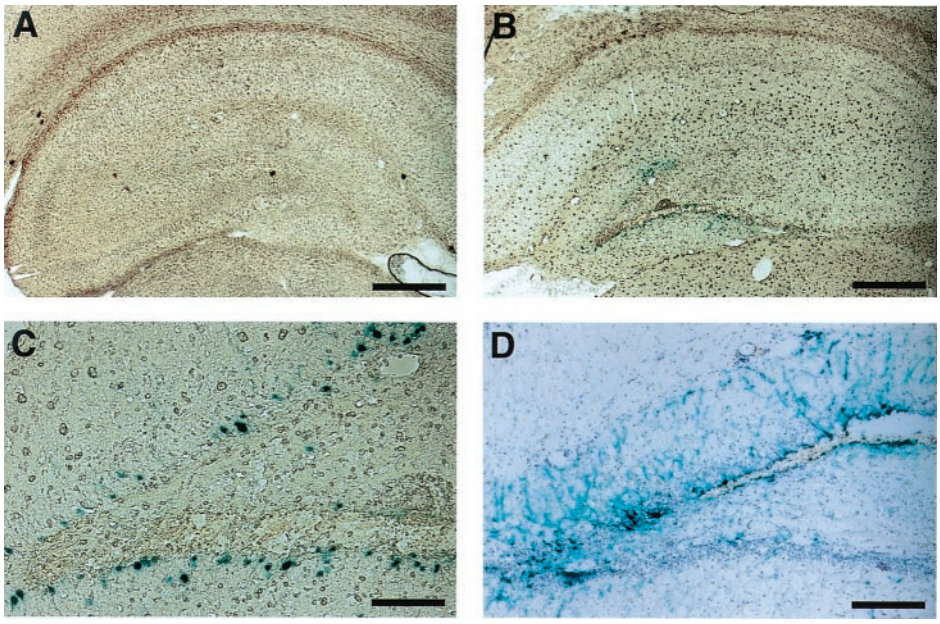

Fig. 5. The ability of the GRE5 promoter to be induced *in vivo* by exogenous corticosterone and KA-induced excitotoxic seizures was examined after injection of vGRE(L)lacZ into rat hippocampus. (*A*) In injected rats that received no corticosterone, no *lacZ* expression occurred after X-gal staining of sections taken from rats 24 h postsurgery. (*B*) Injected rats that received corticosterone showed a number of X-gal-positive cells surrounding the injection site in dentate gyrus. (*C*) Higher magnification view of X-gal-positive cells shown in *B*. (*D*) Rats that received KA infusion into the dentate gyrus exhibited high numbers of X-gal-positive cells surrounding the injection site. (Scale bars: *A* and *B* = 500 μm; *C* and *D* = 200 μm.)

the injection site in the dentate gyrus (n = 6, 1,816 ± 540 X-gal-positive cells/animal; Fig. 5 *B* and *C*). Rats that received both vGRE(L)lacZ and the glutamatergic excitotoxin KA exhibited an average of 3,117.6 ± 603.4 X-gal-positive cells/rat, centered around the dentate gyrus (n = 5, Fig. 5*D*). In contrast, control rats that received vGRE(L)lacZ and vehicle alone had no detectable X-gal staining (n = 4, Fig. 5*A*).

Discussion

Overexpression of various transgenes under the control of constitutive promoters in viral vector systems can protect neurons from necrotic insults. As reviewed, it would be advantageous to develop inducible promoters. Glucocorticoids, whose secretion is markedly stimulated by necrotic insults, are plausible candidates as the signal to be harnessed in an insult-inducible system with a GRE-containing promoter. The GRE5 promoter used in this study robustly induces chloramphenicol acetyltransferase gene expression in transiently transfected HeLa cells (31) and human growth hormone expression in 293 cell lines and primary hepatocytes when packaged into an HSV amplicon vector (32).

Circulating glucocorticoid levels rise within minutes of the onset of stress (23). Thus, after hypoxia-ischemia, seizure, or hypoglycemia, glucocorticoid secretion is likely to increase quickly enough to generate the levels necessary for inducing the GRE5 promoter within the vectors described. Reporter gene expression was induced *in vitro* in these vectors within a few hours of glucocorticoid exposure. Delivery of a constitutive HSV vector driving expression of GT is neuroprotective when delivered 1 h after KA infusion, which correlates with GT overexpression several hours after vector delivery (3). This delayed protection, along with the demonstration that protective pharmacological interventions are effective even after an excitotoxic insult (44), agrees with the emergence of mediators of excitotoxic neuronal death over the course of hours postinsult. Thus, the time required for induction of the glucocorticoid-inducible vectors described in this study after an insult is likely to be shorter than the time required for irreversible excitotoxic neuron death. Supporting this, GT expression from a glucocorticoid-responsive vector was of sufficient speed and magnitude to reduce excitotoxicity *in vitro*.

Glucocorticoid induction of vectors was dose-responsive. Dexamethasone induced higher levels of expression than did corticosterone. This result is explained by the presence in serum in media as well as in hippocampal neurons of transcortin, which binds corticosterone but not dexamethasone (45). Critically, significant induction by corticosterone did not occur in vHLGREluc, vGRE(S)luc, or vGRE(L)lacZ until levels were 10^{-6} M or higher, approximating those seen *in vivo* in response to major stressors. This relatively high threshold suggests that these vectors may be induced *in vivo* by the considerable glucocorticoid secretion associated with necrotic neurological insults and not by mild stressors. Preliminary data indicate this to be the case (unpublished observations). Induction of all but one of the GRE5 vectors tested was triggered by ligands of the glucocorticoid receptor (corticosterone and dexamethasone), but not by nonglucocorticoid steroids at high physiological concentrations; the less stringent steroid specificity exhibited by vGRE(L)luc may have been caused by the combination of particular DNA sequences in that vector. Obviously, steroid specificity is a prerequisite for harnessing a glucocorticoid signal for insult-induced neuroprotection.

These *in vitro* results suggested the feasibility of using this system *in vivo*. To that end, we observed no hippocampal expression from a vector in rats lacking glucocorticoids, but robust induction in rats with corticosterone concentrations in the upper physiological range. Moreover, KA induced robust expression in rat hippocampus. Expression was centered in the dentate gyrus, agreeing with prior reports (39). The cells that were transduced *in vivo* are likely to be those vulnerable to insult; we have observed that HSV vectors expressing neuroprotective transgenes delivered to the same region of hippocampus attenuates behavioral deficits after acute necrotic insults (unpublished observations). Ongoing work examines the parameters of

inducibility and neuroprotection of the glucocorticoid-inducible system *in vivo*.

The relatively short lifespan of transgene expression driven from HSV vectors within transduced cells is a prevailing problem in HSV amplicon-based gene transfer. Such declining transgene expression may be a function of nuclear peripheralization, extrusion, and/or degradation of amplicon DNA, rather than loss of transduced neurons (46). Obviously, further understanding of the mechanisms leading to loss of transgene expression over time is required for improving the utility of HSV amplicons for stable delivery of transgenes in gene therapy applications.

In conclusion, we report an insult-inducible gene therapy system within the nervous system. Such regulatable gene expression will be of heuristic value in gene therapy studies, allowing finer temporal control over expression. Although the hippocampus was chosen in this study because it is the preeminent glucocorticoid target tissue in the brain [because of its high concentrations of corticosteroid receptors (30)], such an insult-inducible system also would be useful in brain regions with lesser glucocorticoid sensitivity. In addition, an insult-inducible approach ultimately might be useful clinically for individuals at high risk for a necrotic neurological insult. These include persons with frequent seizures or transient ischemic attacks, or individuals about to undergo surgery whose fragility puts them at high risk of neurological complications. Such applications are, obviously, far in the future, but hopefully will prompt further research in this area.

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