Behavioral/Systems/Cognitive

Enhancing Cognition after Stress with Gene Therapy

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Hippocampal function is essential for the acquisition, consolidation, and retrieval of spatial memory. High circulating levels of glucocorticoids (GCs), the adrenal steroid hormones secreted during stress, have been shown to impair both acquisition and retrieval and can either impair or enhance consolidation, depending on experimental conditions. In contrast, estrogen can enhance spatial memory performance and can block the deleterious effects of GCs on such performance. We therefore constructed a chimeric gene ("ER/GR") containing the hormone-binding domain of the GC receptor and the DNA binding domain of the estrogen receptor; as a result, ER/GR transduces deleterious GC signals into beneficial estrogenic ones. We show here that acute immobilization stress, before acquisition and retrieval phases, increases latencies for male rats in a hidden platform version of the Morris water maze. This impairment is blocked by hippocampal expression of the ER/GR transgene. ER/GR expression also blocks decreases in platform crossings caused by acute stress, either after acquisition or before retrieval. Three days of stress before acquisition produces an estrogen-like enhancement of performance in ER/GR-treated rats. Moreover, ER/GR blocks the suppressive effects of GCs on expression of brain-derived neurotrophic factor (BDNF), a growth factor central to hippocampal-dependent cognition and plasticity, instead producing an estrogenic increase in BDNF expression. Thus, ER/GR expression enhances spatial memory performance and blocks the impairing effects of GCs on such performance.

Key words: **BDNF; behavior; dentate gyrus; estrogen (estradiol); glucocorticoid; hippocampal function; learning and memory; memory formation; spatial cognition; spatial memory; stress**

Introduction

Hippocampal-dependent spatial learning and memory can be separated into distinct phases, namely acquisition, consolidation, and retrieval (Roozendaal, 2003), and it is now recognized that hormones can modulate such processes. The effects of glucocorticoids (GCs), the adrenal steroid hormones released during stress, on spatial memory depend on GC levels and the time of GC administration relative to each phase (Roozendaal, 2003). Chronic stress (21 d) before acquisition impairs performance in the hidden platform version of the Morris water maze (MWM) (Bodnoff et al., 1995; Sousa et al., 2000; Sandi et al., 2003; Kitraki et al., 2004; Stewart et al., 2005). In contrast, 7–10 d of stress before acquisition may impair (Radecki et al., 2005) or enhance (Gouirand and Matuszewich, 2005) whereas stressors immediately before training worsen performance 24 h later in the MWM (Kim et al., 2001).

After acquisition, administration of GCs facilitate memory consolidation in MWM under low stress (25°C water) but not high stress (19°C water or predator exposure) conditions, suggesting that moderate stress levels of GCs are beneficial (Sandi et al., 1997; Diamond et al., 1999). In contrast, adrenalectomy impairs MWM acquisition (Oitzl and de Kloet, 1992; Conrad and Roy, 1993). Finally, stress or elevated GC exposure before memory testing interferes with memory retrieval, impairing performance (de Quervain et al., 1998; Roozendaal et al., 2003). Thus, collectively, whereas moderate increases in GCs can facilitate performance on spatial memory tasks, very low and high stress levels of GCs impair (Roozendaal, 2003).

In contrast with GCs, estrogen improves spatial performance (Luine and Rodriguez, 1994; Packard et al., 1996; McEwen et al., 1997; Packard, 1998; Sandstrom and Williams, 2001; Bowman et al., 2002; Frick et al., 2002) and spares stress-induced impairments (McEwen et al., 1997; Luine, 2002; Bisagno et al., 2003), effects shown in both female and male rats (Packard et al., 1996; Packard, 1998). Estrogen may exert these beneficial effects via brain-derived neurotrophic factor (BDNF) expression, because it is upregulated in the hippocampus by estrogen, is required for short- and long-term memory formation (Alonso et al., 2002), and blocks the adverse effects of stress on spatial memory (Radecki et al., 2004).

Estrogen and GCs are steroid hormones with intracellular receptors that, when activated, regulate gene expression by binding specific DNA-responsive elements (McEwen, 2001b). In the present report, we use gene therapy with a chimeric gene ("ER/GR") containing the hormone-binding domain of the GC receptor and the DNA binding domain of the estrogen receptor; this construct converts the actions of GCs at the receptor level into estrogenic

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effects at the transcriptional level, thereby converting deleterious GC effects into beneficial estrogenic ones (Kaufer et al., 2004).

A previous study examined the beneficial effects of ER/GR in the realm of an excitotoxic insult (Kaufer et al., 2004). We now investigate whether ER/GR would also be beneficial in the cognitive realm.

Materials and Methods

Subject. Male Sprague Dawley rats (Charles Rivers Laboratories, Wilmington, MA), weighing between 200 and 400 g, were housed under standard laboratory conditions on a 12 h light/dark cycle with lights on at 7:00 A.M.

Vector construction and intracerebral infusion. The construction of a bipromoter herpes simplex virus-1 amplicon vector expressing the ER/GR chimeric receptor gene [along with green fluorescent protein (GFP) as a reporter] has been described previously (termed ER/GR) (Kaufer et al., 2004); the control vector expressed GFP alone (termed GFP). For stereotaxic infusion of vector, rats were anesthetized with 1 ml/kg of a mixture consisting of 100 mg/kg ketamine, 10 mg/kg acepromazine, and 100 mg/kg xylazine. The ER/GR or GFP vectors were infused (1 μl over a 5 min period with a titer of $\sim 1 \times 10^7$) dorsal to the apex of the dentate gyrus (DG) (coordinates: anteroposterior, −4 mm from bregma; mediolateral, 3.00 from midline; dorsoventral, −3.2 mm from dura). All injections for behavioral studies were bilateral. Rats were allowed 1 full day to recover before behavioral testing.

Histology. Transgene expression peaks 24–48 h after injection (Kaufer et al., 2004). Therefore, rats from behavioral studies were not used to determine ER/GR expression. A separate group of rats expressing ER/GR was perfused with heparinized saline, followed by a 4% solution of paraformaldehyde 36–48 h after injection. Because expression level of the GFP-tagged chimeric receptor itself is not stress inducible, nonstressed rats were used to demonstrate GFP expression for both stressed and nonstressed conditions. Brains were removed, placed in 30% sucrose overnight, sectioned at 30 μm, and visualized at 490 excitation to photograph GFP-positive neurons.

Morris water maze. This consisted of a black circular pool (1.7 m in diameter) filled with 25–27°C water. A light source and patterns on the walls surrounding the pool served as extramaze cues. Rats were trained for six blocks consisting of three (60 s) trials separated by 20 min interblock intervals. A different start location was used for each trial. Immediately after the sixth block, the hidden platform was removed and rats were scored during a 60 s probe trial for latency to and crossings over the previous platform location. Each swim was digitally recorded and tracked using MetaMorph software (Molecular Devices, Palo Alto, CA). For acute stress studies, the platform was returned and a final trial was given to reinforce platform presence. Another probe trial was run 24 h after training.

Acute stress manipulations for MWM studies. On day 1, rats were infused with control or experimental vector and were allowed 48 h recovery before undergoing MWM training on day 3. Rats received immobilization stress immediately before training, directly after the immediate probe trial, or immediately before the 24 h probe trial. Immobilization stress consisted of 90 min restraint in plastic decapicone bags secured at the base of the tail to prevent forelimb and hindlimb movement. This stressor produces circulating levels of corticosterone (the predominant GC of rats) in the medium stress range (i.e., ~30 μg/dl) (Sapolsky et al., 1995).

Chronic stress manipulations for MWM studies. On day 1, rats were infused with ER/GR or GFP. On days 2–4, rats received subcutaneous injection of estradiol (15 μg/kg) or vehicle and were immediately subjected to either gentle handling or rotating stressors (daily: 1 h restraint for two times, 1 h shaking for two times, 3 h cold room, and brief sedation with halothane, sequence rotated daily, with 30 min rest between each stressor). Such stressors produce circulating corticosterone levels in the moderate to medium stress range (i.e., 15–30 μg/dl) (Sapolsky et al., 1995). Immediately after the stressors on day 4, rats were trained on the MWM. Rats were carefully monitored during all stress procedures, which were performed per National Institutes of Health and Stanford University Department of Veterinary Services and Care guidelines.

BDNF expression. Rats were stereotaxically infused with ER/GR into the dentate gyrus of the right hemisphere and GFP into the left. This was followed by 3 d of immobilization stress (3 h/d) or gentle handling, after which the hippocampus was removed. The effect of stress-dependent activation of ER/GR-mediated gene expression in the right hemisphere was measured as a percentage change in BDNF mRNA levels relative to levels in the GFP-expressing left hemisphere. Semiquantitative reverse transcription (RT)-PCR amplification was performed (ThermoScript

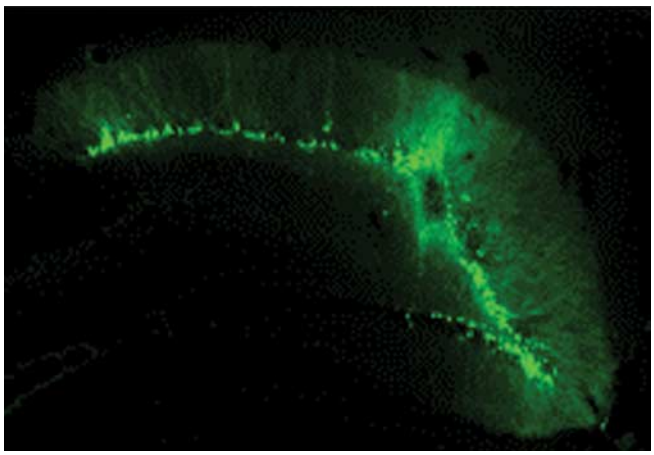

Figure 1. Typical expression of the GFP-tagged ER/GR transgene in hippocampus 48 h after infusion. Robust expression was observed for GFP and ER/GR vectors throughout the dentate gyrus of the dorsal hippocampus, with the vast majority in the dorsal blade.

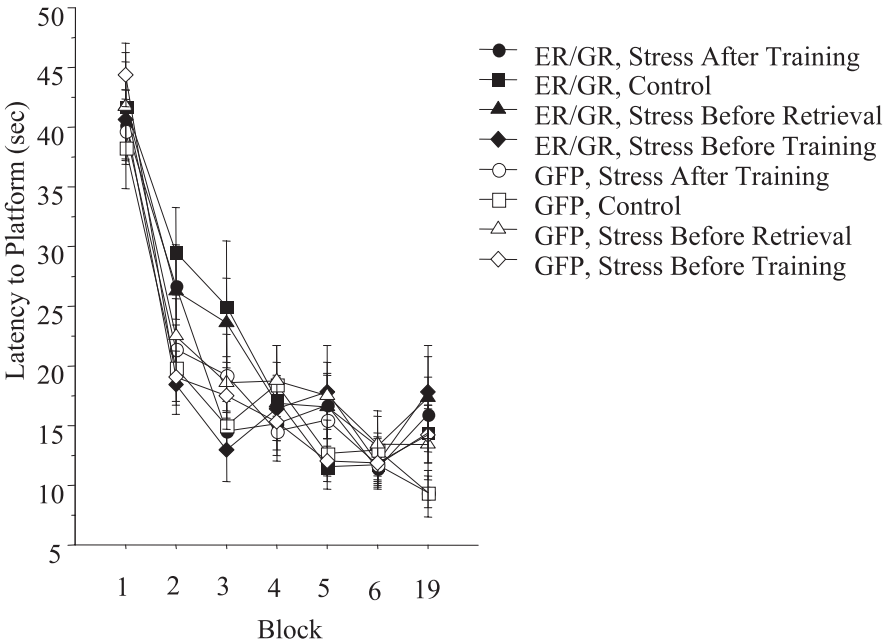

Figure 2. Learning curves for GFP- and ER/GR-expressing control rats and rats treated with stress before training, after training, and before retrieval are represented as latency to platform ($n = 9$–11 per group). There was an overall significant effect of block ($p < 0.001$) for all groups, with no difference between treatment groups. Mean ± SEM in all figures.

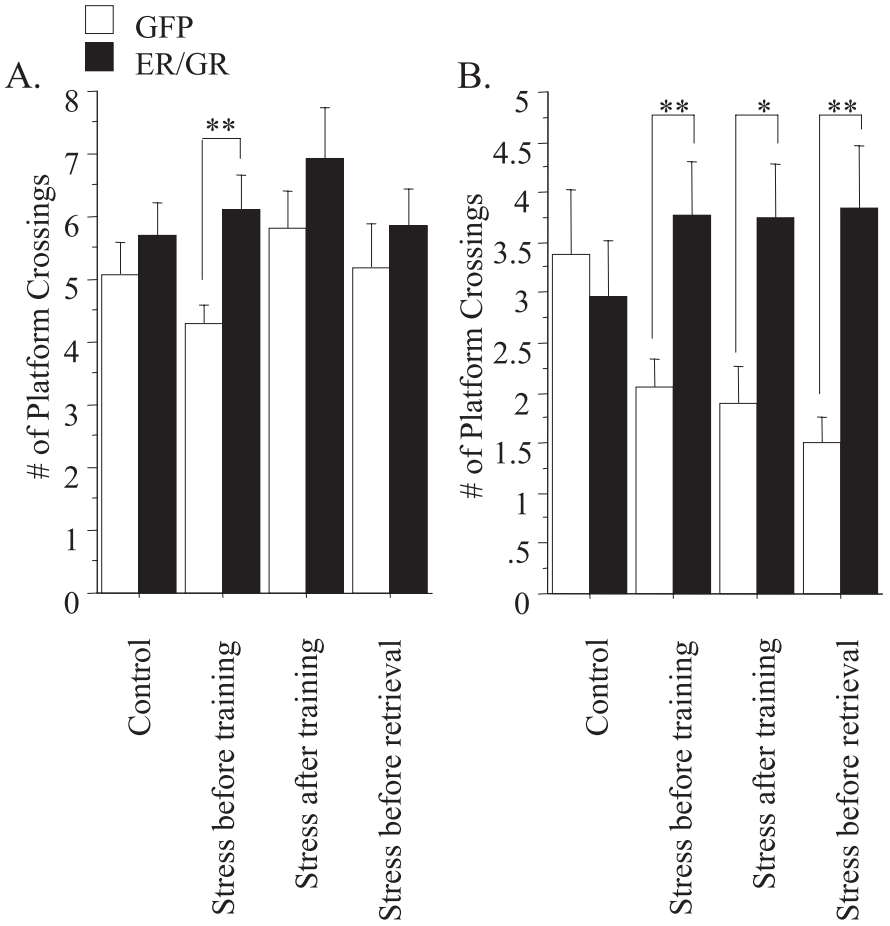

Figure 3. Platform crossing number during the immediate (***A***) and 24 h (***B***) probe trials of the MWM. ***A***, Crossings for GFP and ER/GR rats stressed before training, after training, or before retrieval ($n = 9$–11 per group). An overall enhancing effect of ER/GR on performance was observed ($p < 0.01$). *Post hoc* analysis revealed a trend toward significance in "Stress before retrieval" groups ($p = 0.09$). ***B***, An overall impairing effect of stress was observed ($p < 0.0001$) along with a significant interaction of vector and stress ($p < 0.05$). GFP rats stressed before retrieval made significantly fewer crossings than control rats ($p < 0.05$), whereas a similar trend was noted for GFP rats stressed after training ($p = 0.05$). ER/GR expression rescued the impairments in both treatment groups. Consequently, ER/GR rats made significantly more crossings when stressed after training ($p < 0.01$) and stressed before retrieval ($p < 0.001$) relative to GFP rats. A trend toward increased crossings for ER/GR rats was also observed for rats stressed after training ($p = 0.09$). Two-way ANOVA with *post hoc* analysis; *$p < 0.01$, **$p < 0.001$.

RT kit; Invitrogen, Carlsbad, CA) as per the instructions of the manufacturer. BDNF primers were 5′-ATG CTC AGC AGT CAA GTG CC-3′ (sense) and 5′-AGC CTT CCT TCG TGT AAC CC-3′ (antisense). Actin was amplified as an internal PCR control using the following primers: 5′-TGA AAC AAC ATA CAA TTC CAT CAT GAA GTG TGA C-3′ (sense) and 5′-AGG AGC GAT AAT CTT GAT CTT CAT GGT GCT-3′ (antisense). Photographs were scanned (HP 1200 series; Hewlett-Packard, Palo Alto, CA), and the optic density of the bands was quantified (NIH ImageJ software).

Statistics. Rats were scored for platform crossings, latency to platform, and time spent in goal quadrant. Data for MWM experiments were analyzed using two-way repeated-measures ANOVA and two-way ANOVA with *post hoc* Fisher's PLSD analysis and Bonferroni's/Dunn's adjustment when necessary. The percentage increase in BDNF mRNA levels was analyzed using a within-subject one-sample analysis (95% confidence interval). Data were analyzed with StatView (SAS Institute, Cary, NC).

Results

Expression of the ER/GR transgene

Robust expression was observed for GFP and ER/GR vectors throughout the dentate gyrus even 4 d after injection, with the vast majority in the dorsal blade (Fig. 1). Essentially all expression was confined to the dentate gyrus ~300 μm rostral and caudal of the injection site; expression in CA3 and CA1 was observed in one rat.

Influence of ER/GR expression and acute stress on platform crossings

Neither stress nor the infused vector altered learning of the MWM (Fig. 2). We then examined whether ER/GR expression altered the effects of acute immobilization stress, administered at different phases of the learning and memory process, on spatial performance in the MWM, in which better performance is shown as an increase in crossings over the region of the removed hidden platform.

Stress (before training) did not alter performance in the immediate probe trial in GFP rats (Fig. 3*A*). An overall significant influence of vector expression on numbers of platform crossings was observed (Fig. 3*A*), suggesting that rats that received ER/GR vector outperformed those that received GFP. *Post hoc* analysis indicated that ER/GR enhanced performance in pretraining stressed rats, suggesting an influence of ER/GR expression on immediate recall.

In the 24 h probe trial (Fig. 3*B*), all stress protocols (i.e., stress before or after training, or before retrieval) impaired performance in GFP rats compared with their nonstressed GFP controls. ER/GR expression completely rescued this stress-mediated impairment for all treatment groups.

Influence of ER/GR expression and acute stress on latency measures

We then tested whether ER/GR would spare and/or enhance latency performance in the face of stress. Latency to platform was scored as the amount of time taken to make the first platform crossing in either the immediate or 24 h probe trial; thus, shorter latencies indicate better performance.

Neither stress nor vector treatment altered performance in the immediate probe trial (Fig. 4*A*). In the 24 h probe trial, GFP groups that received stress before retrieval had significantly longer latencies relative to GFP control groups (Fig. 4*B*). ER/GR expression completely blocked this effect. For both immediate and 24 h experiments, there was no significant influence of stress ($p > 0.1$) or vector treatment ($p > 0.1$) on swim speed (data not shown), suggesting that observed differences in performance were not the result of differences in activity level.

Influence of ER/GR expression and repeated stress on MWM performance

Because ER/GR-mediated protection was observed for rats that received 90 min of stress immediately before training, we next investigated whether ER/GR expression altered the effects of 3 d of stress before training. An additional treatment group was

added to compare the effects of ER/GR activation with those of exogenous estrogen.

MWM acquisition data did not differ significantly between treatment groups (Fig. 5*A*). In GFP rats, 3 d of rotating stressors did not alter performance in the immediate probe trial, as assessed by numbers of platform crossings (Fig. 5*B*). Estradiol significantly enhanced performance under these conditions, as did stress in ER/GR-treated rats. No effect of stress or vector treatment was observed for latency or swim speed or platform crossings in the 24 h probe trial (data not shown).

Influence of ER/GR expression on the effect of stress on BDNF expression

We next examined the effects of stress and ER/GR at the genomic level, seeking a gene that is regulated in opposite directions by GCs and estrogen. We chose BDNF, which has been shown to protect against stress- and GC-dependent spatial memory impairment (Almli et al., 2000; Schaaf et al., 2000; Alonso et al., 2002; Radecki et al., 2004). To investigate ER/GR functioning *in vivo* at the transcriptional level, we measured intrahippocampal BDNF mRNA expression levels after stress and mild handling. Rats were injected with ER/GR and GFP into the dentate gyrus of right and left hemispheres of the hippocampus, respectively. In stressed animals, expression of BDNF mRNA was elevated in hippocampi expressing ER/GR relative to GFP, demonstrating an estrogen-like response (Fig. 6). In the absence of stress, no difference in BDNF mRNA expression was found between hemispheres expressing ER/GR or GFP.

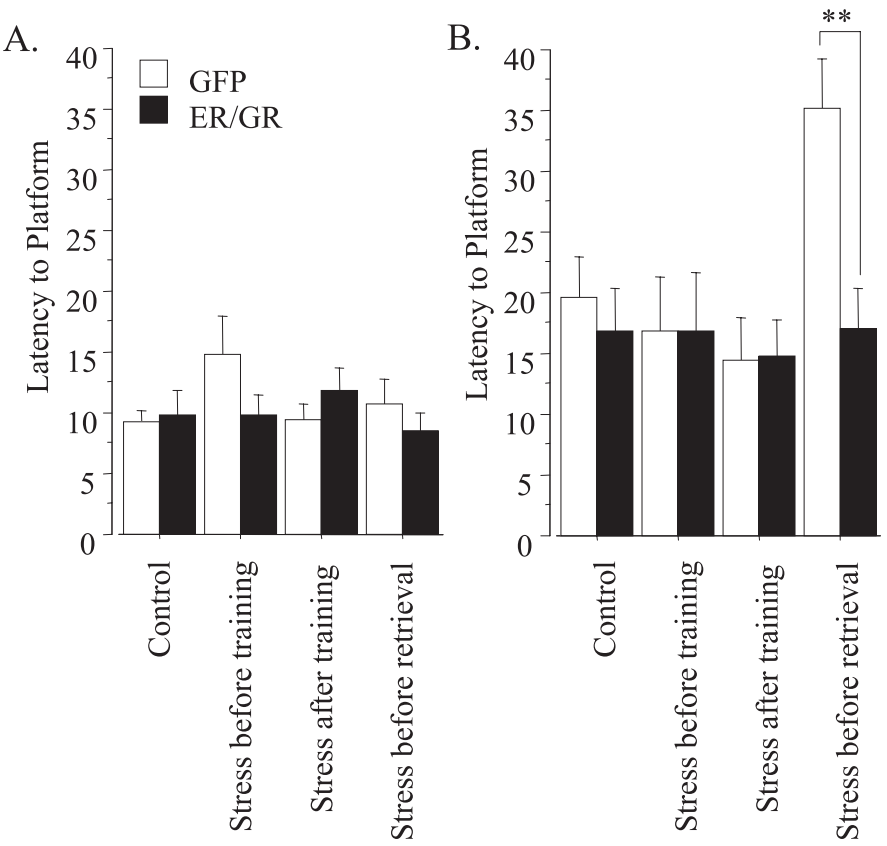

Figure 4. Latency to platform during the immediate (***A***) and 24 h (***B***) probe trials of the MWM. ***A***, Latency to platform for GFP and ER/GR rats stressed before training, after training, or before retrieval ($n = 9–11$ per group). No significant group differences were observed. ***B***, GFP rats stressed before retrieval in the 24 h probe trial displayed significantly longer latency to platform relative to all other GFP treatment groups ($p < 0.01$ relative to all groups). This stress-induced impairment was completely rescued by ER/GR expression ($p < 0.001$). Two-way ANOVA with *post hoc* analysis; $^{**}p < 0.01$.

Discussion

There is now ample evidence that hormones can alter facets of cognition. A number of studies have focused on blocking the often-deleterious effects of GCs. For example, this has been shown pharmacologically using GC-synthesis inhibitors or GR antagonists (Oitzl et al., 1997; Sousa and Almeida, 2002). In the present study, we use a gene therapy approach to not only block the deleterious effects of stress but to convert them into protective estrogenic actions. The construction of the chimeric ER/GR was contingent on the modular nature of steroid hormone receptors, something previously exploited in the construction of the opposite receptor chimera (i.e., one with a hormone binding domain from the ER and a DNA binding domain from GR) (Green and Chambon, 1987), as well as in our previous work with ER/GR, showing its capacity to protect hippocampal neurons from acute necrotic insults (Kaufer et al., 2004).

The actions of ER/GR in the present study reflect the array of ways that GCs and stress influence spatial and recognition task performance. Whereas severe and prolonged exposure to stress and/or stress levels of GCs impair many domains of hippocampal-dependent memory retrieval, milder exposure can actually facilitate it. This "inverse-U" pattern reflects the heterogeneity of receptors for GCs in the hippocampus. The salutary effects of mild GC elevations are mediated by the high-affinity mineralocorticoid receptor (MR), whereas the disruptive effects are mediated by the low-affinity GR that is only heavily occupied in the face of high stress levels of GCs. The balance between the opposing MR and GR effects varies with the sex of the animal, pattern of GC exposure, memory task, and context of exposure relative to the different phases of learning and memory, including acquisition, consolidation, and retrieval (Sapolsky et al., 2000; McEwen, 2001a; Roozendaal, 2003). It is an oversimplification to assume that only negative stress effects are mediated by GRs. Indeed, studies have shown that moderate increases in GCs and subsequent GR activation can also facilitate the learning and memory process (Sousa and Almeida, 2002; Roozendaal, 2003).

The present findings show the impact of immobilization stress on two distinct gauges of spatial memory performance. The first, platform crossings, or the repeated seeking of the platform in its absence, revealed an impairment of acute stress administered before training, after training, and before retrieval in the 24 h probe trial (Fig. 3*B*). This robust impairment in the 24 h probe trial likely results from the effects of stress on the consolidation and retrieval phases along with the increased difficulty of the 24 h memory task relative to the immediate recall task (Fig. 3*B*). ER/GR expression blocks this negative effect on spatial memory, suggesting that GC-mediated activation of the chimeric receptor effectively protects against stress.

ER/GR expression also resulted in an overall enhancement of performance in the immediate probe trial (Figs. 3*A*, 5*B*). ER/GR-expressing rats that received either acute immobilization stress

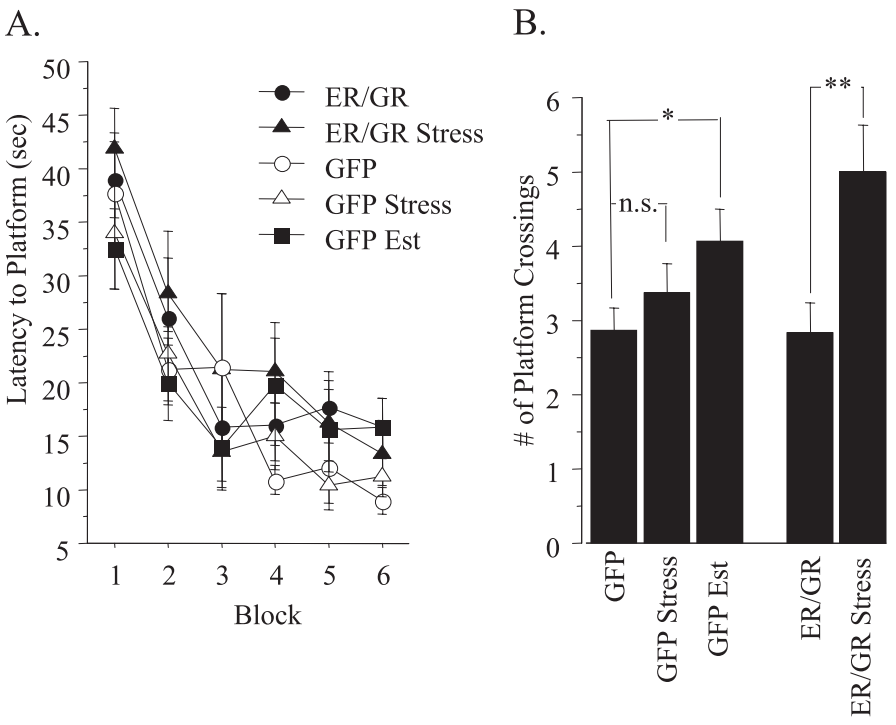

Figure 5. Learning curves (***A***) and platform crossing number during the immediate probe trial of the MWM for rats stressed for 3 d. ***A***, Learning curves for GFP- and ER/GR-expressing rats exposed to 3 d of rotating stressors are represented as latency to platform ($n = 9$–11 per group). There was an overall significant effect of block ($p < 0.001$) for all groups, with no significant difference between treatment groups. ***B***, Platform crossings for GFP and ER/GR rats in response to stress or 17b-estradiol (Est) treatment ($n = 6$ per group). Stress had no effect on GFP rats but boosted performance in ER/GR rats. Estradiol treatment also significantly boosted performance ($p < 0.05$). Consequently, stressed ER/GR animals differed from all groups ($p < 0.05$ in all cases) except estradiol. Two-way ANOVA with *post hoc* analysis; n.s., not significant. *$p < 0.05$, **$p < 0.01$.

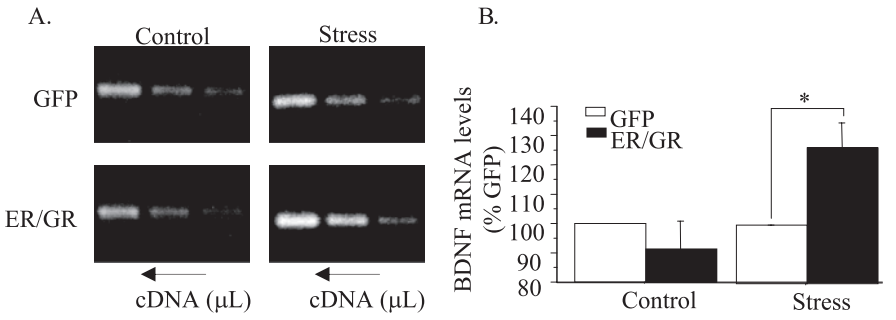

Figure 6. Effects of ER/GR chimera on BDNF mRNA expression. ***A***, RT-PCR analysis performed on RNA extracted from left and right hippocampi of a control and a stressed rat was run with decreasing volume of cDNA to show linear range of product accumulation. ***B***, Intensity of BDNF bands as a percentage of GFP. After stress, mRNA levels in ER/GR tissues ($n = 5$) were higher than in GFP tissues ($n = 5$). One sample analysis with 95% confidence interval; *$p < 0.05$.

(Fig. 3*A*) or 3 d of rotating stressors (Fig. 5*B*) before training both showed an increase in platform crossings relative to their GFP controls, suggesting that stress-dependent activation of the ER/GR chimera enhances recall of the platform location. Because no stress-mediated impairment was observed in the immediate probe trial, acquisition and recall may be less sensitive to short-term GC effects than consolidation and retrieval.

Like platform crossings, latency (Fig. 4), the time needed for a rat to recall the exact location of the hidden platform and execute escape, is not impaired by stress in the immediate probe. Impairment is observed only when acute stress occurs before the 24 h probe trial, demonstrating an adverse effect on retrieval of spatial memory (Fig. 4). Intrahippocampal expression of ER/GR blocks this impairment, suggesting that the GC-dependent protective effects of the gene therapy can occur immediately after stress (Fig. 4).

There is a wide range of mechanisms that may underlie the protective effects of ER/GR expression. After stress, an estrogenic-like transcriptional upregulation of BDNF mRNA was observed in hippocampi expressing ER/GR, showing that the chimeric receptor effectively converts a GC signal into a genomically mediated estrogenic event *in vivo* (Fig. 6). BDNF itself is necessary for memory formation and protects against stress-dependent impairment of spatial memory (Alonso et al., 2002; Radecki et al., 2004). Additional studies are underway to investigate whether the observed behavioral results may involve ER/GR-dependent stimulation of dendritic remodeling or synapse formation, a known effect of estrogen (Woolley, 1998).

Ultimately, ER/GR is activated by GCs via stress and functions effectively at both the behavioral and genomic levels. Although there is a strong literature supporting the theory that stress-dependent increases in GCs can modify hippocampal function and cause spatial memory impairments (Sapolsky et al., 1985; Roozendaal et al., 1997; McEwen, 1999; McGaugh and Roozendaal, 2002), we cannot state that the stress-mediated behavioral impairments observed here are definitively GC dependent. Supporting the idea of a GC role, our current work demonstrates similar protective effects using a vector that exclusively blocks GR-mediated effects, without causing estrogenic ones (data not shown). However, even if the underlying cause of the impairments does not involve GCs, the GC-mediated activation of the estrogenic effects of ER/GR, like BDNF upregulation, are still protective. If, conversely, the behavioral impairments are GC dependent, ER/GR-mediated protection may occur via its estrogenic effects and/or through direct competition with endogenous GR receptors for circulating GCs, thereby lessening GR-mediated events in DG. In that case, the fast block ER/GR provides against the stress-induced increase in latency before retrieval may depend more on its ability to compete with endogenous GRs rather than its estrogenic genomic events. In support of this, previous work has shown latency to be a GR-dependent measure (Oitzl et al., 1997; de Kloet, 2000).

These studies are of heuristic value for understanding endocrine modulation of cognition. In addition, these studies are commensurate with those suggesting a therapeutic potential for gene therapy in the CNS. Although the majority of such studies have been oriented toward preventing neuron death after an excitotoxic insult, a few have altered behavior (for example, addic-

tive behavior, affiliative behavior or, in the present case, the cognitive response to stress hormone exposure) (Carlezon et al., 1997; Landgraf et al., 2003). Stereotaxic microinfusion of herpes amplicon vectors at the titers used in the present study typically heavily infect cells (with a marked preference for neurons over glia) up to a few millimeters from the infusion site (Fig. 1). Although this represents only a small volume of the hippocampus, previous studies with this same vector system have demonstrated that this is sufficient to alter hippocampal-dependent behaviors (McLaughlin et al., 2000). Thus, the capacity for this vector to influence a behavior dependent on the entire hippocampus as well as other neural structures becomes that much more noteworthy.

The vectors used in this study preferentially infect granule cells of DG, and previous studies have shown the infection itself to have no impact on DG function (Dumas et al., 1999, 2000). In addition, the DG is highly responsive to stress (Bear and Abraham, 1996; Camodeca et al., 1998; Huang et al., 1999; Fujikawa et al., 2000; Ahmed et al., 2006), and modulation of DG function can readily influence spatial performance in the MWM (Conrad and Roy, 1993, 1995; Vaher et al., 1994; Xavier et al., 1999). We have shown here that gene delivery into DG can protect against stress-mediated impairment of spatial performance. This suggests that, should techniques for the safe delivery and spread of such vectors improve, gene therapy approaches might ultimately be useful for diminishing the adverse neurobiological consequences of stress.

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