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# Targeting the FKBP51/GR/Hsp90 Complex to Identify Functionally Relevant Treatments for Depression and PTSD

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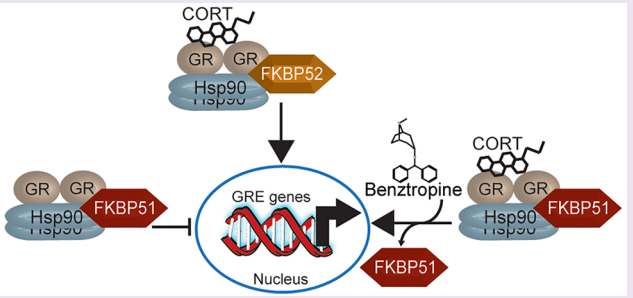
Supporting Information

**ABSTRACT:** Genetic and epigenetic alterations in FK506-binding protein 5 (*FKBP5*) have been associated with increased risk for psychiatric disorders, including post-traumatic stress disorder (PTSD). Some of these common variants can increase the expression of *FKBP5*, the gene that encodes FKBP51. Excess FKBP51 promotes hypothalamic-pituitary-adrenal (HPA) axis dysregulation through altered glucocorticoid receptor (GR) signaling. Thus, we hypothesized that GR activity could be restored by perturbing FKBP51. Here, we screened 1280 pharmacologically active compounds and identified three compounds that rescued FKBP51-mediated suppression of GR activity without directly activating GR. One of the three compounds, benztropine mesylate, disrupted the association of FKBP51 with the GR/Hsp90 complex *in vitro*. Moreover, we show that removal of FKBP51 from this complex by benztropine restored GR localization in *ex vivo* brain slices and primary neurons from mice. In conclusion, we have identified a novel disruptor of the FKBP51/GR/Hsp90 complex. Targeting this complex may be a viable approach to developing treatments for disorders related to aberrant FKBP51 expression.






The hypothalamic−pituitary−adrenal (HPA) axis is the central regulator of stress response in the body. The HPA axis is controlled by hormones called glucocorticoids (CORTs) that are produced by the adrenal cortex in response to other stress hormones secreted from the brain. Glucocorticoid receptors (GRs) are the main cytoplasmic receptors for CORTs.[1] These receptors are widely distributed throughout the brain. The 51 kDa FK506-binding protein (FKBP51) is ubiquitously expressed throughout the brain but is enriched in the hippocampus, cortex, hypothalamus, and amygdala[2−5] and is a central regulator of this stress response system.[6] Together with the 90 kDa heat shock protein (Hsp90), FKBP51 suppresses glucocorticoid receptor (GR) activity by decreasing its affinity for CORT.[7−12] Once bound to CORT, GR enters the nucleus and alters the expression of many genes including upregulation of *FKBP5*, the gene that encodes FKBP51, thus creating a short negative feedback loop.[9] Therefore, higher intrinsic levels of FKBP51 can prolong the duration of the stress response and, consequently, cause increased levels of circulating CORT.[8,13−15]

Dysfunctional and dysregulated GR signaling mechanisms have been associated with CORT resistance as well as mental health disorders.[16−18] Most, but not all, studies have shown a positive association between HPA hyperactivity in patients with depression and anxiety.[3−5] Since GR deficiency also correlates with HPA hyperactivity, it has been proposed that persistent elevated CORT in the limbic system contributes to the etiology of depression and anxiety. However, the inverse has been reported in individuals with PTSD,[6−10] that is, these patients commonly present with HPA hypoactivity. One possible explanation for the blunted cortisol response could be the desensitization of the HPA axis after prolonged stress. A recent study showed a positive correlation between HPA hypoactivity (low cortisol) and fear-potentiated startle.[14,15] These inconsistencies may also result from the high comorbidity with mood disorders, gender differences, types

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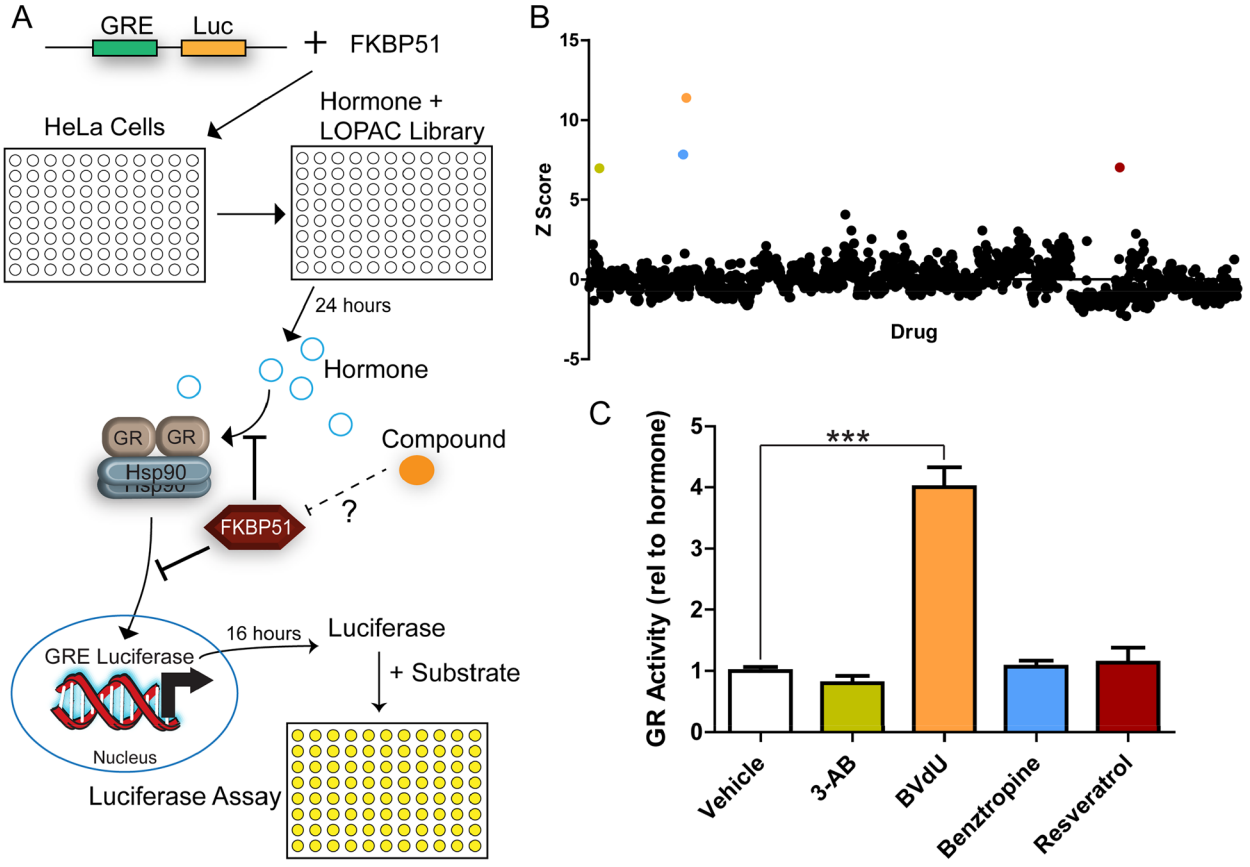


**Figure 1.** Compounds that suppress FKBP51 repression of GR activity identified by a cell-based screen. (A) Schematic depicting the design of the cell-based compound library screen. HeLa cells were transfected as shown. Cells were then treated with hormone and compounds from the LOPAC library. Luciferase assays were performed to evaluate compound efficacy. (B) Quantification of luciferase activity following Lopac library screen as described in A. Chemiluminescence was normalized within each plate and converted to Z scores. Four compounds significantly increased GR activity: 3-aminobenzamide (3-AB; green), (E)-5-(bromovinyl)-2′-deoxyuridine (BVdU; orange), benztropine (blue), and resveratrol (red). (C) Quantification of luciferase activity in HeLa cells transfected with a glucocorticoid response element (GRE) luciferase reporter and treated with hormone and indicated drugs. ***$p < 0.001$ by one-way ANOVA. Rel = relative.

of trauma, and subtypes of PTSD profiles.[11] While there are discrepancies, overall these data suggest that there is a careful balance of HPA regulation and that disturbances in this regulation, such as FKBP51-mediated suppression of GR activity, will have negative consequences.

FKBP51 has been implicated in several deleterious processes and diseases including depression, PTSD, anxiety disorders, Alzheimer's disease, and even cancer[19−22] (see review for additional details[20]). FKBP5 has not been identified as a risk gene by genome-wide association studies (GWAS). However, chromosome 6, which houses the *FKBP5* gene, was associated with psychosis by GWAS.[23] Instead, candidate gene studies, which use a hypothesis driven approach, have identified common *FKBP5* single nucleotide polymorphisms (SNPs) that combine with environmental factors to increase risk for multiple neuropsychiatric disorders.[24−26] In PTSD, multiple studies have reported that *FKBP5* SNPs combine with early life stress to increase risk; however, GWAS studies have not supported this.[27] These discrepancies may be due to the limitations of either the candidate studies or the GWAS or both. Candidate gene studies are limited because they are based on what we know of the disease and have not often been replicated in follow-up studies.[28] Additionally, GWAS studies are limited because they often use DNA extracted from peripheral tissue,[29] require a large effect size to be above the limit of detection,[30] and do not sufficiently include gene-environment interactions.[20] Still, *FKBP5* may be one of the most well-studied candidate genes and, while there are discrepancies in certain SNPs being associated with specific psychiatric disorders, there is a growing amount of complementary data identifying repeatable SNPs with demonstrated mechanisms for the increased risk.[25,26,31−33]

Some of these disease-associated SNPs work by boosting the intrinsic levels of *FKBP5*,[31] suggesting that higher levels of *FKBP5* are deleterious in humans. These SNPs function through a process involving *FKBP5* demethylation, which allows for higher levels of FKBP51 protein to be made following stress.[31] This is a similar mechanism through which FKBP51 is upregulated in aging.[26,34] Thus, decreasing FKBP51 activity or levels may have therapeutic benefits for patients afflicted with affective disorders.

In fact, $Fkbp5^{-/-}$ mice are viable and present age-dependent antidepressive-like phenotypes, enhanced stress resiliency, and accelerated stress response feedback without impaired cognitive or immune function.[5,35−37] The viability of $Fkbp5^{-/-}$ mice suggests that inhibition of FKBP51 may be well-tolerated.

Herein, we performed a high-throughput assay to identify ligands that could block FKBP51-mediated suppression of GR activity. These studies revealed a single compound, benz-


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tropine mesylate, which disrupted the FKBP51/GR/Hsp90 heterocomplex *in vitro*. Follow-up analyses in cell culture models, primary neurons, and *ex vivo* brain slices confirmed the effects of this compound. These results demonstrate a novel mechanism for inhibiting FKBP51 by a small molecule. These findings also highlight the feasibility of high-throughput screening for modulators of steroid hormone receptor activity and suggest benztropine may be a useful scaffold upon which to develop a specific FKBP51 inhibitor.

## RESULTS

**A Cell-Based Compound Library Screen Reveals Modulators of FKBP51.** We sought to identify compounds that mitigated the suppression of GR activity by FKBP51. A high throughput screen using the LOPAC library was performed in HeLa cells (see Figure 1A for schematic), which have high endogenous GR expression.[38] The ability of compounds to rescue this suppression was normalized within each plate and converted to Z scores. Four compounds were identified that restored GR activity in the presence of FKBP51 overexpression (Figure 1B). Each of these compounds was tested in a secondary screen for its ability to directly increase GR activity independent of FKBP51. Only one of these molecules, BVdU, was found to directly increase GR activity (Figure 1C), suggesting its effect was not specific to FKBP51 suppression. The effect of the other three compounds, 3-AB, resveratrol, and benztropine, was dependent on FKBP51.

**Benztropine Targets the FKBP51/GR/Hsp90 Complex.** Next, we used an *in vitro* flow cytometry protein interaction assay (FCPIA) to validate the disruption of the FKBP51/GR/Hsp90 complex (see Figure 2A for schematic) by 3-AB, resveratrol, or benztropine. A biotinylated recombinant ligand-binding domain of GR (GR-LBD) was complexed with full-length recombinant Hsp90$\alpha$ and a fluorescently labeled FKBP51 in the presence of dexamethasone. These complexes were then incubated with 3-AB, resveratrol, or benztropine. Benztropine, but not 3-AB or resveratrol, disrupted FKBP51 from the complex, as determined by measuring median bead-associated fluorescence (Figure 2B). To ensure the effects of benztropine were not unique to our screening model, we validated our initial findings in a neuroblastoma cell line, M17 (Figure 2C). Importantly, these data demonstrate that benztropine rescued FKBP51-mediated suppression of GR activity without affecting endogenous GR signaling pathways.

**Benztropine Selectively Inhibits FKBP51.** We next examined if benztropine was selectively binding FKBP51. To accomplish this, we synthesized benztropine with an exposed biotin group. In designing a benztropine probe compound (see Figure 3A), we wanted to retain the tertiary amine functionality, which precluded the use of an amide functionality. We chose propargyl analog **4**, which would fully retain the basicity of the parent benztropine. Demethylation of benztropine **2** (synthesized from tropine and benzhydryl chloride)[39] was achieved with chloroethyl chloroformate, isolated as the corresponding methyl carbamate. Following liberation of the secondary amine, alkylation with propargyl bromide gave the requisite acetylene, and click cyclization with a biotinylated azide afforded the probe compound **5**.

Streptavidin beads were incubated with fluorescently labeled FKBP51, BSA, or FKBP52 (*FKBP4*, an FKBP51 homologue) in the presence or absence of the biotinylated benztropine (see diagram in Figure 3B). Benztropine selectively interacted with

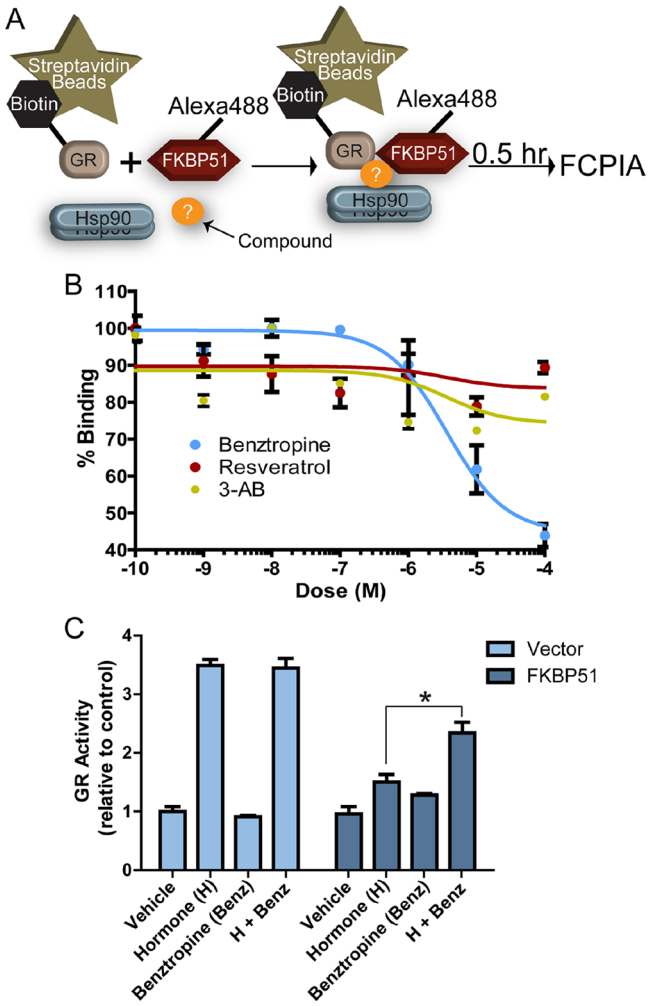


**Figure 2.** Disruption of the FKBP51/GR/Hsp90 complex and restoration of GR signaling by benztropine. (A) Schematic depicting the flow cytometry protein interaction assay (FCPIA) using labeled recombinant protein to measure disruption of the FKBP51/GR/Hsp90 complex. (B) Median bead-associated fluorescence was measured for each compound at the indicated range of doses. (C) GR activity was measured by luciferase assay in M17 cells treated as shown. *$p < 0.05$ by one-way ANOVA.

FKBP51, but not BSA or the highly homologous FKBP52 (Figure 3C,D). The lack of benztropine interaction with FKBP52 was surprising given the high homology with FKBP51. However, this specificity is preferred, since ablation of FKBP52 may have some negative impacts.[40,41] To learn more about how benztropine was interacting with FKBP51, we performed a peptidyl-prolyl isomerase (PPIase) activity assay (Supporting Figure 1). This assay confirmed that benztropine does not inhibit the PPIase activity of FKBP51 and therefore acts through a mechanism that is independent of this activity.

**Benztropine Restores GR Activity by Disrupting a Hormone-Dependent FKBP51/GR/Hsp90 Complex.** Since benztropine did not directly affect GR activity and was able to interact directly with FKBP51 and remove it from an Hsp90/GR complex, we speculated that benztropine was targeting FKBP51 or a transient FKBP51/GR/Hsp90 complex enriched in the presence of hormones. To assess this, we performed coimmunoprecipitations of GR, in the presence and absence of both a hormone (CORT) and benztropine. Though the addition of the hormone did not enhance the interaction


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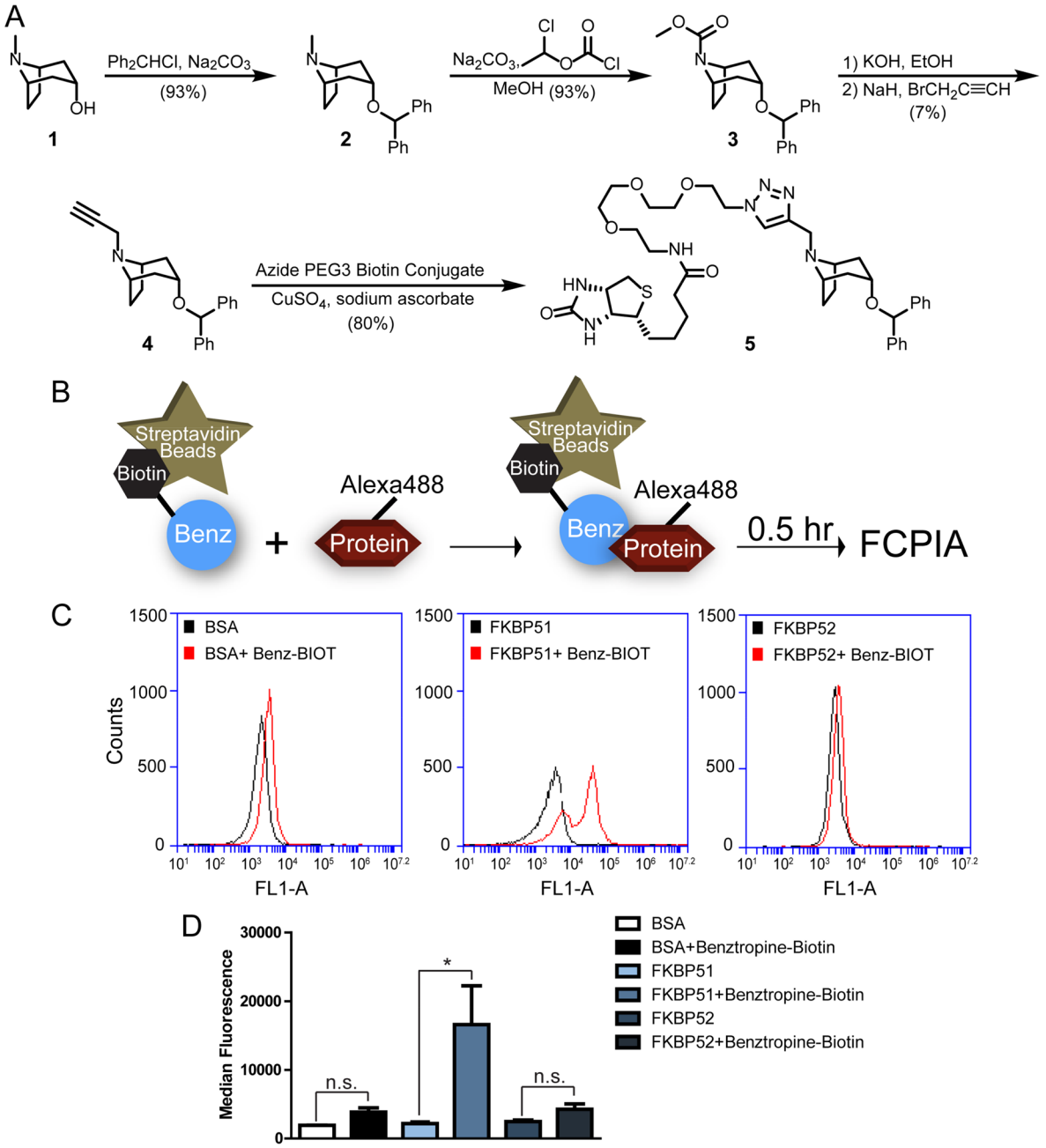


**Figure 3.** Benztropine, a selective inhibitor of FKBP51. (A) Scheme of biotinylated-benztropine synthesis. (B) Schematic depicting the flow cytometry benztropine-interaction assay using recombinant labeled protein to determine if benztropine interacts with FKBP51, FKBP52, or BSA. (C) Event counts and fluorescence intensity for bead associated BSA, FKBP51, and FKBP52, as indicated. (D) Quantification of the median fluorescence intensity. FCPIA = flow cytometry protein interaction assay; *Benz = biotinylated benztropine; Protein = BSA, FKBP51, and FKBP52. n.s. = not significant, $*p < 0.05$ by one-way ANOVA.

between GR and FKBP51, the amount of FKBP51 found associated with GR was significantly decreased exclusively in the presence of both the hormone and benztropine (Figure 4A,B). Importantly, changes were not observed in overall GR levels from the inputs (Figure 4A), indicating the interaction difference was not a result of increased GR translation. These findings suggest that, when in complex with Hsp90 and GR, the presence of hormone increases the effects of benztropine on disrupting the FKBP51/GR complex. To test this latter hypothesis, we used the FCPIA (see Figure 2A) to examine whether the inhibitory effect of benztropine was dependent on the hormone. Interestingly, benztropine was more effective at disrupting FKBP51 from the Hsp90/GR/FKBP51 complex in the presence of the hormone (Figure 4C,D). This increase may be due to a conformation change in the heterocomplex that has been previously described upon ligand binding.[42−45]

**Benztropine Mechanism Is Structurally and Mechanistically Distinct.** Benztropine is a known antagonist of muscarinic acetylcholine (ACh) receptors, histamine receptors, and dopamine transporters (DATs);[46] interestingly, other compounds from the LOPAC library targeting these same mechanisms, including the M1 muscarinic antagonist, biperiden, and the H1 histaminergic antagonist, brompheniramine, did not ablate the FKBP51-mediated suppression of GR


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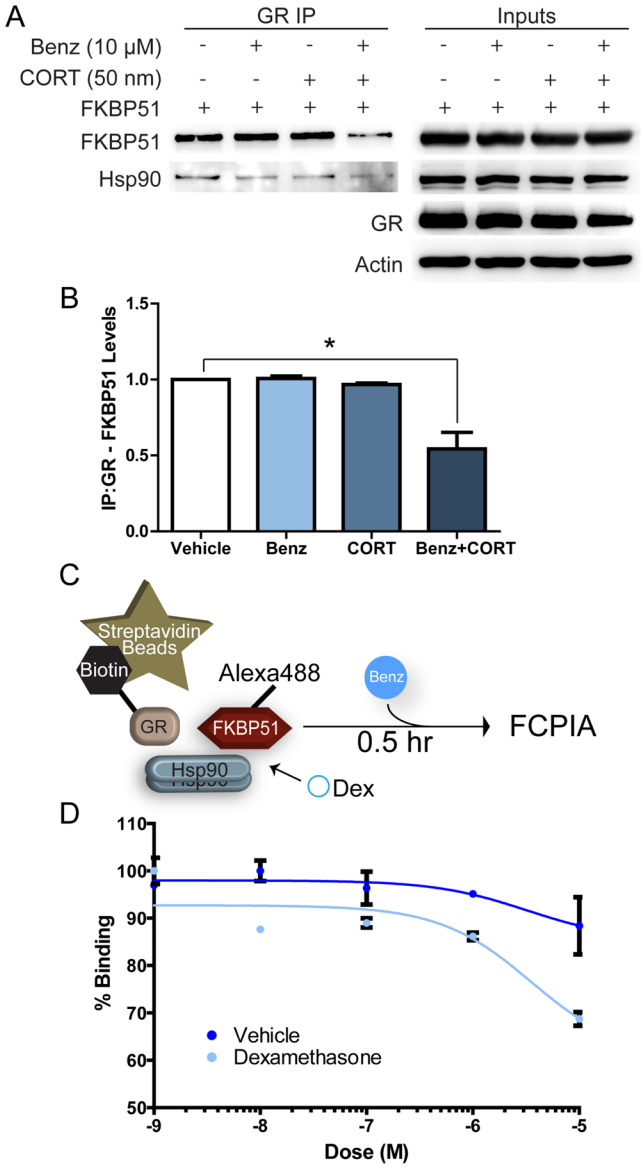


**Figure 4.** Hormone-dependent FKBP51/GR/Hsp90 complex disruption. (A) HeLa cells were transfected with FKBP51 and treated with the indicated doses of the hormone hydrocortisone (CORT) and/or benztropine prior to immunoprecipitation of GR and Western blotting. (B) Quantification of the Western blot is shown. $*p < 0.05$ by one-way ANOVA. (C) Schematic depicting the flow cytometry protein interaction assay (FCPIA) using labeled recombinant protein to measure disruption of the FKBP51/GR/Hsp90 complex in the presence or absence of dexamethasone. (D) Median bead-associated fluorescence was measured for benztropine at the indicated range of doses in the presence or absence of dexamethasone.

(Figure 5A–C). This suggested that the effects on GR signaling were not related to the activity of a muscarinic receptor antagonist, possibly through a novel mechanism. We also assessed the selectivity of the benztropine scaffold by comparing small molecules with moieties and structural similarities to benztropine. Neither atropine nor diphenhydramine rescued FKBP51-mediated suppression of GR activity, despite being the component scaffolds of benztropine (Figure 5A,D). Thus, benztropine uniquely rescued the suppression of GR by FKBP51, which correlates with restored expression of GRE regulated genes (Supporting Figure 2).

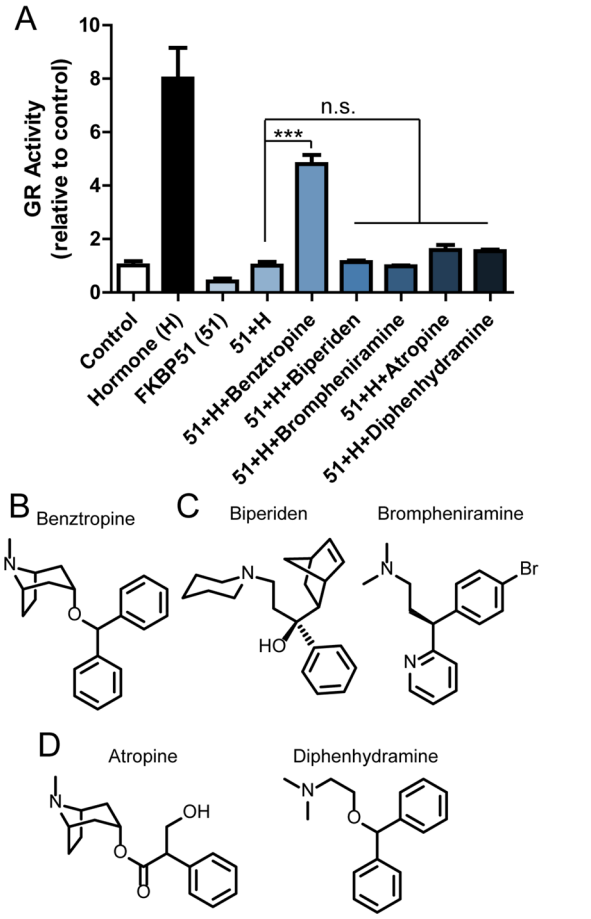


**Figure 5.** Unique structure of benztropine responsible for its inhibitory effect. (A) HeLa cells were transfected with GRE luciferase and FKBP51 or 6TR. Cells were treated with benztropine, biperiden, brompheniramine, atropine, or diphenhydramine as well as a hormone. GR activity was measured using a luciferase assay. $***p < 0.01$, n.s. = not significant by one-way ANOVA. The structures of (B) benztropine, (C) biperiden and brompheniramine, and (D) atropine and diphenhydramine are shown. GR = glucocorticoid receptor, GRE = glucocorticoid response element.

**Benztropine Reverses FKBP51 Inhibition of GR Nuclear Trafficking.** Next, we measured GR localization using a RFP-GR reporter plasmid in HEK293T cells, to determine if benztropine was regulating GR activity by altering GR localization. As expected, overexpression of FKBP51 significantly reduced GR translocation (Figure 6A). Benztropine rescued this effect (Figure 6B), demonstrating that benztropine can abrogate FKBP51-mediated suppression of GR translocation, an important step in GR activation.[47] Benztropine did not affect GR nuclear localization in the absence of hormones. Moreover, benztropine only affected hormone-mediated GR translocation when FKBP51 was overexpressed, reinforcing its specificity for FKBP51.

To corroborate these results, we prepared acute *ex vivo* brain slices from aged, wild-type mice to examine whether benztropine enhanced GR translocation in the brain. We used aged mice since we have previously shown that FKBP51 levels increase with age in both mouse and human brain.[34,48] Therefore, *ex vivo* slices from aged mice were treated for 4 h with benztropine and/or dexamethasone prior to subcellular fractionation to isolate nuclear and cytosolic fractions. Western blot analysis revealed that benztropine enhanced dexametha-


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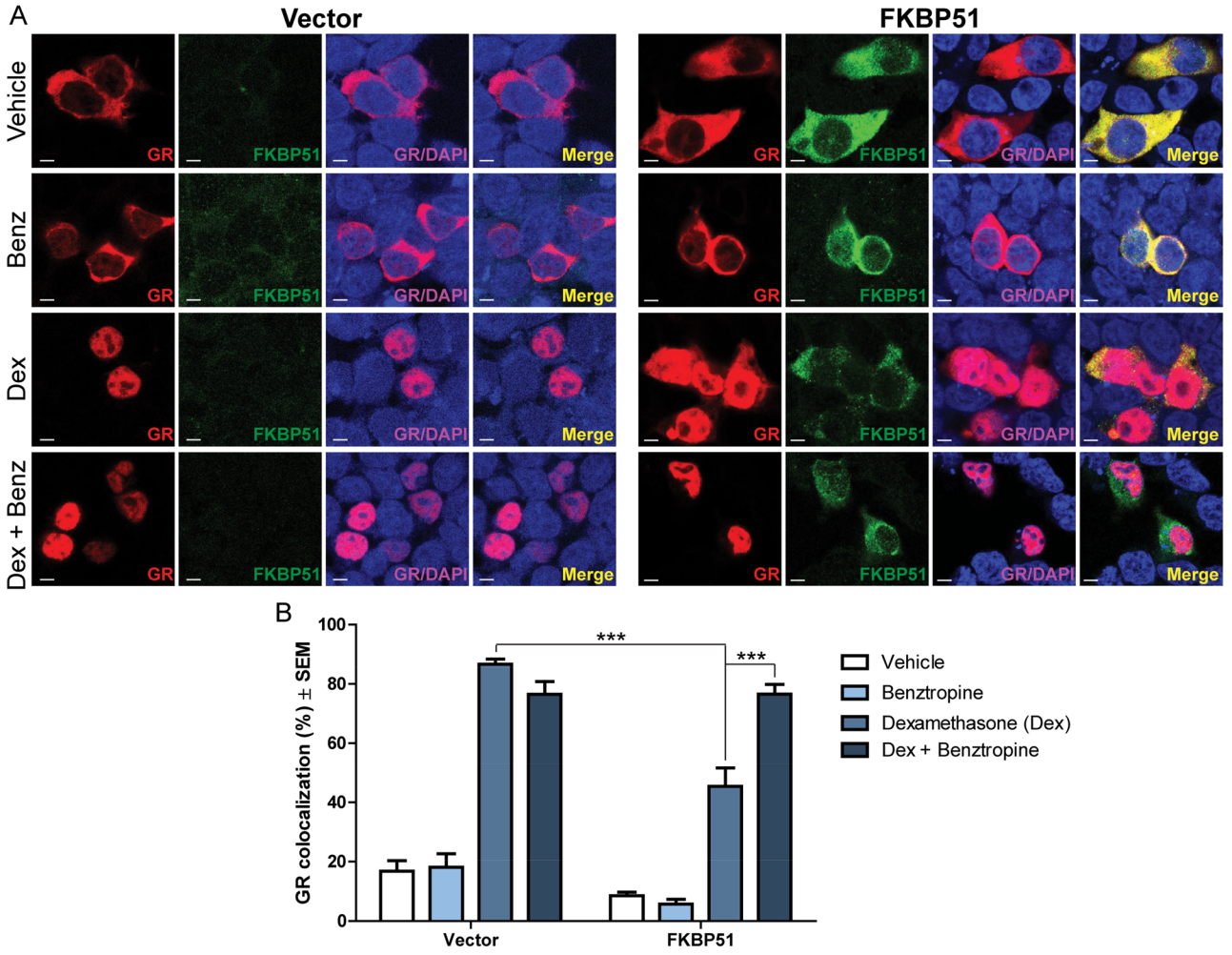


**Figure 6.** Benztropine rescued FKBP51-mediated GR inhibition. (A,B) Confocal microscopy images of HEK293T cells transfected with RFP-GR (A) or RFP-GR and FKBP51 and stained with FKBP51 and DAPI. Cells were treated as indicated for 90 min. Scale bar = 5 $\mu$m. (B) Quantification of z-stack images of percent GR nuclear colocalization. ***$p < 0.001$ by two-way ANOVA.

sone-induced GR nuclear translocation (Figure 7A,B), suggesting that benztropine restored FKBP51-mediated GR suppression in these slices. These results were further confirmed in primary neurons. To do this, primary neurons were transduced with eGFP or FKBP51 adeno-associated virus serotype 9 (AAV9) for 10 days and then treated with dexamethasone and/or benztropine for 3 h. The neurons were lysed, and nuclear and cytosolic fractions were collected. Western blot data revealed a decrease in the GR translocation ratio following FKBP51 overexpression, which was rescued by benztropine (Figure 7C,D). Taken together, these data confirm that benztropine restores GR translocation in the presence of high FKBP51.

## DISCUSSION

Here, we have identified benztropine mesylate as a novel FKBP51-specific inhibitor. Treatment with benztropine in models of high FKBP51 expression rescued FKBP51 suppression of GRE activation and GR nuclear translocation, likely by inhibiting the FKBP51-GR interaction. Using recombinant protein studies, we found that benztropine can bind directly to FKBP51 and that disruption of the FKBP51/GR/Hsp90 heterocomplex was increased in the presence of hormones. Together, these findings, in addition to identifying benztropine as a FKBP51 inhibitor, highlight the feasibility of high-throughput screening for modulators of steroid hormone receptor activity.

The identification of FKBP51-specific inhibitors is of great interest to both provide tools to further study the effects of FKBP51 inhibition and as potential therapeutics for FKBP51-related disorders. However, the direct targeting of FKBP51 has been challenging due to the high sequence similarity among the FKBP family members. Target specificity is critical for inhibitors of FKBP51, since other isomerases, like FKBP52, have different, but essential, functions. Mice lacking FKBP51 showed an antidepressive behavior without cognitive impairment, while mice lacking FKBP52 experienced detrimental effects on developmental, stress sensitivity, and neuroendocrine processes.[5,41]

Recently, some compounds have been developed that are selective for FKBP51 over other family members, including FKBP52.[49] These molecules have been shown to reduce anxiety-like behavior in mice,[50] indicating that targeting FKBP51 may be a viable approach to treat anxiety disorders and other stress-related diseases. These molecules were designed to target the hydrophobic FK506-binding pocket of FKBP51, which disrupts PPIase activity. However, PPIase activity has been shown to be dispensable for GR activity,[10,51]


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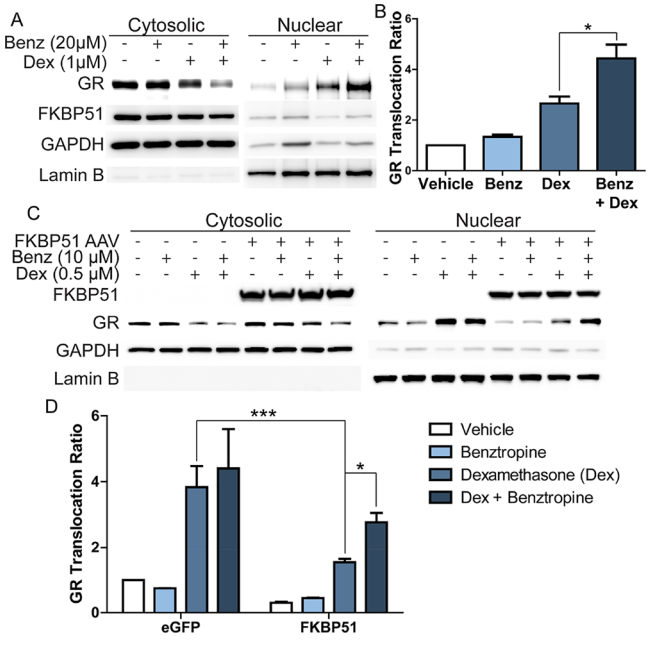


**Figure 7.** Benztropine enhanced GR translocation in the presence of high FKBP51. (A) Western blots of acute *ex vivo* slices from aged wild-type mice ($N$ = 3) treated as indicated for 4 h. (B) Quantification of the GR translocation ratio. *$p$ < 0.05 by one-way ANOVA. (C) Western blots from wild-type primary neurons ($N$ = 3) transduced with GFP or FKBP51 AAV for 10 days and treated with dexamethasone (0.5 $\mu$M) and/or benztropine (10 $\mu$M) for 3 h. (D) Quantification of the GR translocation ratio. *$p$ < 0.05, ***$p$ < 0.001 by two-way ANOVA.

so the link between the physiological effects and mechanism of action of these compounds is still uncertain. Therefore, it is unclear if these compounds will achieve the desired outcome on GR activity and stress regulation even though they are highly selective for this domain of FKBP51. Perhaps compounds such as benztropine that target FKBP51-independently of regulating PPIase activity and that appear to have increased activity in the presence of hormones will have better clinical utility.

Benztropine, labeled as Cogentin, is a blood−brain barrier permeable compound that has been widely used to reduce extrapyramidal side effects of antipsychotic treatments and as a second line drug treatment for Parkinson's disease.[52] It works through decreasing the imbalance between neurotransmitters acetylcholine and dopamine to reduce tremors and rigidity.[53] It is possible that some of these effects may be in part related to this newly identified mechanism of altering in GR signaling through FKBP51. For example, benztropine increases dopamine levels by blocking reuptake and storage[53] as well as improves motility through reduced rigidity.[53] Studies have shown that CORT levels play an important role in controlling dopamine balance[54,55] and locomotor activity.[56,57] One of the known side effects of benztropine is impaired long-term memory.[58] It has been well-documented that CORT affects learning and memory.[59−61] Interestingly, benztropine was recently shown to be beneficial in reversing deficits in myelination caused by $\alpha$-synuclein;[62] similar white matter deficits have been previously measured in PTSD for patients with *FKBP5* risk SNPs.[32] While there are many parallels in the effects of benztropine and CORT regulation, additional studies are needed to determine if each of these described effects or any of the other side effects, including coma, mania, anticholinergic toxicity, and delirium,[52,63−65] are mediated through an FKBP51-dependent mechanism.

Since benztropine is BBB permeable, it could serve as a solid platform for a medicinal chemistry campaign in the hopes of refining its specificity and improving its potency for the FKBP51/GR/Hsp90 interaction while abrogating the negative effects. In fact, a number of *N*-substituted benztropine analogues have been developed, which retain high affinity for dopamine transporters with minimal off-target effects.[66,67] An even greater number of benztropine derivatives have been developed with the goal of discovering novel monoamine transporter inhibitors.[68] Similar future studies may be used to improve specificity against the FKBP51/GR/Hsp90 complex.

In conclusion, our cell-based screen identified a very promising molecule that can selectively inhibit FKBP51-mediated GR suppression. Attenuation of FKBP51 activity, or expression, is desirable for neuropsychiatric disorders, Alzheimer's disease, and cancer. Moreover, the unique design of our screen could be used to identify inhibitors of other steroid hormone receptors, other cochaperones, or components tertiary to steroid signaling pathways. Therefore, these studies have broad implications and utility in the development of novel therapeutics targeting not only FKBP51 complexes but also other dynamic complexes in the cell.

## MATERIALS AND METHODS

**Plasmids.** FKBP51 and RFP-GR constructs were generated in our laboratory and inserted into pCMV6 vectors. The mouse mammary tumor virus (MMTV) glucocorticoid response element (GRE) luciferase reporter construct was a gift from Dr. Marc Cox (University of Texas, El Paso).

**Cell Culture.** HeLa, M17, and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) as previously described;[69,70] for all hormone treatments, charcoal-stripped FBS was used instead for at least 16 h before treatment started. For the high-throughput screen, HeLa cells were transfected in suspension with 200 ng of the GRE luciferase reporter and FKBP51 or 6/TR (six repeats of the tetracycline repressor sequence) as a control using 3 $\mu$L/$\mu$g of Mirus TransIT2020 transfection reagent (Mirus Bio, Madison, WI) in serum-free media and plated in 96-well plates. Cells were treated 24 h later with hydrocortisone (50 nm; Sigma-Aldrich) and compounds from the LOPAC 1280 drug library (Sigma-Aldich, St. Louis, MO) in triplicate wells at 10 $\mu$M. Cells were harvested 16 h later in Cell Culture Lysis Reagent (Promega, Madison, WI) and transferred to opaque, white polystyrene 96-well plates. Luciferase assays were performed with the Luciferase Assay System (Promega) according to the manufacturer's protocols, and luminescence was immediately read on a multimodal plate reader (Biotek Synergy H1, Biotek, Winooski, VT).

For RFP-GR experiments, HEK293T cells were plated on poly-L-lysine coated coverslips and transfected with 1 $\mu$g of RFP-GR and 1 $\mu$g of FKBP51 or 6/TR using 2.5 $\mu$L/$\mu$g of Lipofectamine 2000 (Life Technologies) in Opti-Mem. After 4 h, media were replaced with DMEM supplemented with 10% charcoal stripped FBS, Glutamax, and Pen/Strep. Cells were treated 24 h later with the synthetic glucocorticoid, dexamethasone (100 nm), and benztropine (10 $\mu$M) for 90 min and then fixed in 4% PFA for 30 min. Cells were permeabilized with 0.1% Triton-X-100 and stained for FKBP51, and DAPI was applied for 5 min to stain nuclei. All conditions were run in duplicate, and confocal microscopy images were captured by a blinded experimenter.

**Microscopy.** Slides were imaged on an Olympus FV1200 confocal microscope as previously described for analysis.[71] Fifteen confocal z-stack images were captured per treatment at 60× magnification. To examine the extent of GR nuclear colocalization, ImageJ software (National Institutes of Health) was utilized. Briefly, DAPI-positive


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nuclei were masked, and the percent of total GR-positive signal to DAPI-overlapping was calculated as previously described.[34,71] A Zeiss LSM 880 AxioObserver laser scanning confocal microscope was used for representative images. A 63×/1.40 PLAN APO oil objective was 1-$\mu$m Z-stacked images with Argon (for FKBP51-positive signal in green) and Red HeNe (for GR-positive signal in red).

**Expression, Purification, and Labeling of Recombinant Proteins.** The GR-LBD protein, a stabilized variant of the GR ligand binding domain, was purified as previously reported.[45] FKBP51, FKBP52, and Hsp90$\alpha$ proteins were generated as described.[72] Briefly, full-length wild-type human FKBP51, FKBP52, or Hsp90$\alpha$ was cloned into a pET28 vector, expressed in *E. coli*, and purified *via* Ni-NTA-agarose chromatography followed by size exclusion chromatography. Protein purity was verified by Coomassie blue SDS-PAGE. Purified proteins were labeled for flow cytometric protein−protein interaction evaluation as previously described.[73,74] Recombinant GR-LBD was incubated with biotin for 1 h followed by washes to remove excess biotin. The biotin-labeled protein was then immobilized with streptavidin coated polystyrene particles (Spherotech, Lake Forest, IL) for 1 h followed by washes to remove any unbound protein. For the experiment using biotinylated benztropine, we used the Dynabeads M-280 Streptavidin particles (Thermo Fisher Scientific, #11205D) that have a smaller diameter and a strong binding affinity for biotin allowing better drug−protein interaction. FKBP51, FKBP52, and BSA were chemically labeled with Alexafluor 488 carboxylic acid succinimidyl ester (Life Technologies) following the manufacturer's protocol.

**Biotinylated Benztropine Synthetic Procedures.** All materials were obtained from commercial suppliers and used without further purification. Dry THF was obtained *via* distillation from sodium benzophenone ketyl. Preparative chromatography was carried out using Sorbtech silica gel (60 Å porosity, 40−63 $\mu$m particle size) in fritted MPLC cartridges and eluted with Thomson Instrument SINGLE StEP pumps. Thin layer chromatography analyses were conducted with 200 $\mu$m precoated Sorbtech fluorescent TLC plates. Plates were visualized by UV. LC/MS and LRMS data were obtained using an Agilent 1100 HPLC/MSD system equipped with a diode array detector running an acetonitrile/water gradient and 0.1% formic acid. High resolution mass spectral data were obtained using an Agilent 6540 QTOF mass spectrometer. Nuclear magnetic resonance spectrometry was run on a Varian Inova 500 MHz spectrometer, and chemical shifts are listed in parts per million correlated to the solvent used as an internal standard.

*Benztropine (**2**).*[39] Tropine (**1**, 1.052 g, 7.45 mmol) and benzhydryl chloride (1.4 mL, 7.9 mmol) were added to a 25 mL round-bottomed flask containing a stir bar to give a tan solution. The mixture was heated with stirring to 160 °C for 1 h, then allowed to cool to RT. The residue was dissolved in dichloromethane and then evaporated to obtain a brown oil. The oil was triturated in ether to yield a tan solid as the HCl salt of the crude product. Saturated sodium carbonate was added to this suspension; then the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried with sodium sulfate and evaporated under reduced pressure to yield **2** (2.12 g, 93%) as a light brown oil. $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 7.36−7.27 (m, 8 H), 7.24−7.16 (m, 2 H), 5.40 (s, 1 H), 3.74−3.53 (m, 1 H), 3.17 (br s, 2 H), 2.32 (s, 3 H), 2.21 (br d, $J$ = 7.5 Hz, 2 H), 2.07−1.86 (m, 6 H) ppm. LRMS $m/z$: $[M + H]^+$ = 308.

*Methyl (3-Endo)-3-benzhydryl-8-azabicyclo[3.2.1]octane-8-carboxylate (**3**).* A solution of **2** (4.07 g, 13.2 mmol) in 1,2-dichloroethane (65 mL) was stirred in a 250 mL round bottomed flask, and sodium carbonate (5.64 g, 53.2 mmol) and 1-chloroethyl chloroformate (5.78 mL, 53.0 mmol) were added. The mixture was heated with stirring to 85 °C for 3 h, then cooled and filtered. The filtrate was concentrated on a rotary evaporator, then dissolved in methanol and stirred at RT overnight. This mixture was concentrated on a rotary evaporator to give an off-white solid that was dissolved in dichloromethane and then washed with 5.5 M ammonium hydroxide and water. The organic layer was then dried with magnesium sulfate and concentrated on a rotary evaporator to give **3** as a brown oil (4.34 g, 93%) that was used without further purification. $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 7.37−7.27 (m, 8 H), 7.25−7.17 (m, 2 H), 5.42 (s, 1 H), 4.24 (br s, 2 H), 3.70−3.65 (m, 4 H), 2.23 (q, $J$ = 6.3 Hz, 2 H), 2.07−1.86 (m, 6 H) ppm. LRMS $m/z$: $[M + H]^+$ = 352.

*(3-Endo)-3-benzhydryl-8-propargyl-8-azabicyclo[3.2.1]octane (**4**).* A stirred mixture of **3** (1.334 g, 3.79 mmol) in 2 M potassium hydroxide (37 mL, 74 mmol) and ethanol (35 mL) in a 100 mL round bottomed flask was heated to 100 °C for 14 h, then concentrated on a rotary evaporator and extracted with ether. The combined organic extracts were washed with water and concentrated on a rotary evaporator to give the crude secondary amine (1.22 g), which was used without further purification. This residue was taken up in dry THF (5 mL) and added dropwise to a suspension of 60% sodium hydride (183 mg, 4.57 mmol) in dry THF (5 mL) at 0 °C under argon. Upon completion of the addition, the mixture was allowed to warm to RT for 10 min, and then propargyl bromide (500 $\mu$L, 4.64 mmol) was added *via* syringe, and the reaction was allowed to stir at RT overnight. The reaction was quenched with brine, and the mixture was extracted with ethyl acetate. The combined organic extracts were dried with brine, then concentrated on a rotary evaporator to give a brown oil. Purification by flash column chromatography provided unreacted **3** (364.3 mg) as well as pure **4** (65.7 mg, 7%). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 7.36−7.26 (m, 8 H), 7.25−7.07 (m, 2 H), 5.40 (s, 1 H), 3.59 (br s, 1 H), 3.35 (br s, 2 H), 3.26−3.07 (m, 2 H), 2.28−2.15 (m, 3 H), 2.03−1.86 (m, 6 H) ppm. $^{13}$C NMR (126 MHz, $CDCl_3$): $\delta$ 143.0, 128.4, 127.2, 126.8, 80.7, 71.6, 69.3, 58.3, 41.6, 37.6, 36.5, 25.7 ppm. LRMS $m/z$: $[M + H]^+$ = 332. HRMS $m/z$, $[M + H]^+$ Calcd for $C_{23}H_{26}NO$: 332.2014. Found: 332,2012.

*Final Compound (**5**).* A solution of **4** (49.0 mg, 148 $\mu$mol) and azide PEG3 biotin conjugate (85 mg, 190 $\mu$mol, used as obtained from Sigma-Aldrich) in water (500 $\mu$L) and *t*-butanol (500 $\mu$L) was stirred in a 10 mL round bottomed flask. Sodium ascorbate (6.90 mg, 35 $\mu$mol) and copper(II) sulfate pentahydrate (4.20 mg, 17 $\mu$mol) were added (causing the mixture to turn blue), and the reaction was stirred at RT overnight. The mixture was concentrated on a rotary evaporator, then taken up in water and extracted with dichloromethane. The combined organic extracts were dried over sodium sulfate, then concentrated on a rotary evaporator. The residue was purified by flash column chromatography to give **5** (92 mg, 80%) as a white solid. $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 8.08 (s, 1 H), 7.37 (d, $J$ = 7.3 Hz, 4 H), 7.33−7.27 (m, 4 H), 7.26−7.19 (m, 2 H), 5.53 (s, 1 H), 5.04−4.90 (m, 1 H), 4.60 (t, $J$ = 5.0 Hz, 2 H), 4.47 (dd, $J$ = 7.8 Hz, 4.8 Hz, 1 H), 4.28 (dd, $J$ = 7.8 Hz, 4.5 Hz, 1 H), 3.99 (s, 2 H), 3.90 (t, $J$ = 5.0 Hz, 2 H), 3.67−3.55 (m, 10 H), 3.51 (t, $J$ = 5.5 Hz, 2 H), 3.36−3.33 (m, 3 H), 3.22−3.14 (m, 1 H), 2.90 (dd, $J$ = 12.7 Hz, 5.0 Hz, 1 H), 2.68 (d, $J$ = 12.8 Hz, 1 H), 2.45−2.35 (m, 2 H), 2.19 (br t, $J$ = 7.3 Hz, 4 H), 2.14−2.00 (m, 4 H), 1.76−1.53 (m, 5 H), 1.48−1.37 (m, 2 H) ppm. $^{13}$C NMR (126 MHz, $CDCl_3$, mixture of amide rotamers): $\delta$ 175.0, 164.6, 142.6, 140.9, 128.0, 127.0, 126.5, 125.5, 80.8, 70.10, 70.08, 69.98, 69.8, 69.1, 68.0, 61.9, 60.2, 59.5, 55.6, 50.1, 48.4, 45.6, 39.6, 38.9, 35.3, 34.5, 31.8, 28.3, 28.1, 25.4, 24.7, 20.6 ppm. LRMS $m/z$: $[M + H]^+$ = 776. HRMS $m/z$, $[M + H]^+$ Calcd for $C_{41}H_{58}N_7O_6S$: 776.4169. Found: 776.4180.

**Flow Cytometry Protein Interaction Assay.** The assay was conducted as previously described.[73,74] For the drug binding and complex interaction experiment, biotinylated GR-LBD and Alexafluor 488 conjugated FKBP51 were incubated together, with Hsp90$\alpha$ to facilitate the interaction, in equimolar ratios for 30 min with varying concentrations of drug. For the benztropine specificity assay, Dynabeads Streptavidin particles were washed three times with BSA/PBS (0.2 mg $mL^{-1}$). The streptavidin particles were then incubated for 30 min with recombinant fluorescently labeled FKBP51, FKBP52, or BSA in the presence or absence of a biotinylated-benztropine. All conditions were run in triplicate on separate days. Binding was evaluated using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) to quantify median bead-associated fluorescence.[74] Background scatter and fluorescence were subtracted from the signal.



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**Co-Immunoprecipitation.** Co-immunoprecipitation (co-IP) was performed as previously described.[48] Briefly, HeLa cells were transfected with 3 $\mu$g of FKBP51 and treated overnight with hydrocortisone (50 nm) and benztropine (10 $\mu$M). Cells were harvested in M-PER buffer, and lysates were incubated overnight in rabbit anti-GR antibody (Cell Signaling) at 4 °C. Magnetic protein A beads (Life Technologies) were incubated with samples for 4 h at 4 °C, followed by five washes and a denaturing elution. The resulting precipitates were subjected to SDS-PAGE.

**SDS/PAGE.** Western blot experiments were performed as previously described.[75] Membranes were incubated overnight with primary antibodies directed against rabbit GR (1:1000; Cell Signaling), mouse GAPDH (1:5000; Meridian Life Sciences, Mempthis, TN), rabbit histone H3 (1:1000; Abcam, Cambridge, MA), or goat Lamin B (1:500; Santa Cruz, Dallas, TX). Chemiluminescence was detected with an ImageQuant LAS 4000 imaging system (GE Life Sciences, Pittsburgh, PA), and band densitometry was analyzed using Scion Image. All samples were run in duplicate and normalized to GAPDH for cytosolic fractions or Histone H3 or Lamin B for nuclear fractions prior to analysis.

***Ex Vivo* Slice Culture and Subcellular Fractionation.** *Ex vivo* slices were processed as previously described.[76] Briefly, mice were decapitated, and whole brains were rapidly dissected. Horizontal 400 $\mu$m slices between 2.5 and 4.0 mm ventral to the surface of the skull were prepared on a vibratome and acclimated in artificial cerebrospinal fluid (ACSF). Slices from 9-month-old wild-type mice were treated with benztropine (Sigma-Aldrich) or dimethyl sulfoxide (DMSO) and/or the synthetic CORT, dexamethasone, or DMSO for 4 h prior to harvest. To examine GR translocation, slices underwent subcellular fractionation as described previously for isolation of specific tissue fractions.[77] Briefly, slices were homogenized in a buffer containing 10 mM HEPES, 10 mM NaCl, 1 mM $KH_2PO_4$, 5 mM $NaHCO_3$, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease/phosphatase inhibitors (Sigma-Aldrich) and centrifuged at 1000*g* for 10 min at 4 °C following a 20 min incubation on ice. Supernatant was collected and centrifuged again at 107 000*g* for 30 min to remove the membrane fraction. The resulting supernatant contained the cytosolic fraction. Pellets from the initial spin were resuspended in 1 mL of TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, and protease/phosphatase inhibitors) and centrifuged at 4000*g* for 5 min. The supernatant was discarded, and pellets were washed with 0.5 mL of TSE buffer until the supernatant was clear. The pellet was resuspended with 100 $\mu$L of TSE buffer and stored as the nuclear fraction.

**Animal Studies.** All animal studies were approved by the University of South Florida Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Mice were group-housed under a 12-h light−dark cycle (lights on at 06:00) and permitted *ad libitum* access to food and water.

**Primary Neuronal Cultures.** Primary cortical neurons were isolated from E16.5 wild-type mouse brains using previous established protocols.[78] Briefly, brains were extracted, meninges were removed, and cortices were placed in ice-cold isotonic buffer (137 mM NaCl, 5 mM KCl, 0.2 mM $NaH_2PO_4$, 0.2 mM $KH_2PO_4$, 5.5 mM glucose, and 6 mM sucrose, pH 7.4). Following washes, cortices were minced, digested in trypsin, triturated, and resuspended in DMEM supplemented with 10% FBS, penicillin/streptomycin, and Amphotericin B. The DMEM was exchanged 24 h later for Neurobasal medium supplemented with Glutamax and B27 supplement (Life Technologies). Primary neurons were transduced at DIV4 with 2 $\mu$L/well of eGFP or FKBP51 AAV in PBS at $10^{13}$ viral particles per microliter. At DIV14, neurons were treated with dexamethasone (0.5 $\mu$M) and/or benztropine (10 $\mu$M) for 3 h prior to harvesting using subcellular fractionation to isolate cytosolic and nuclear fractions as described above.

**Statistical Analyses.** Statistical significance for each analysis was determined with Student's *t*-tests or one- or two-way analysis of variance (ANOVA) with Tukey or Bonferroni post-tests to compare groups. All figures and statistics were generated using GraphPad Prism software; each graph represents the mean ± the standard error of the mean (SEM). For analysis of the compound library screen, luciferase values were normalized on each plate to two separate values: hormone-induced GR activity and FKBP51 + hormone GR activity. Using SPPS statistical software, standardized residuals were calculated from the normalized FKBP51-hormone values with hormone alone values used as a covariate. Z scores were generated. Significance was determined by SPPS with $p < 0.0001$ used as a criterion.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00454.

Additional details on methods, Supporting Figures 1 and 2, Supporting Table 1 (PDF)

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### Notes

The authors declare the following competing financial interest(s): C.A.D. and J.J.S. are co-inventors for the following patent: Inhibitors of the FKBP51 Protein from a High-Throughput Drug Screen and Methods of Use, U.S. Patent# US9399039 B1. The other authors have no conflicts of interest to disclose.

[#]Deceased.

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## ■ ABBREVIATIONS

Acetylcholine, Ach; Adeno-associated virus, AAV; Artificial cerebrospinal fluid, ACSF; Cornus Ammonis 1, CA1; Corticosterone/hydrocortisone/glucocorticoid, CORT; dopamine transporters, DATs; FKBP4/FKBP52, FK506-binding protein 4; FKBP5/FKBP51, FK506-binding protein 5; Flow cytometry protein interaction assay, FCPIA; Glucocorticoid receptor, GR; Glucocorticoid Response Element, GRE; 90 kDa Heat Shock Protein, Hsp90; Major Depressive Disorder, MDD; National Institute of Health, NIH; Paraventricular Nucleus, PVN; Peptidyl-prolyl isomerase, PPIase; Post-traumatic stress disorder, PTSD; single-nucleotide polymorphisms, SNPs.

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