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DAXX, FLASH, and FAF-1 Modulate Mineralocorticoid and Glucocorticoid Receptor-Mediated Transcription in Hippocampal Cells—Toward a Basis for the Opposite Actions Elicited by Two Nuclear Receptors?

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ABSTRACT

Mineralocorticoid (MR) and glucocorticoid (GR) receptors are two closely-related members of the steroid nuclear receptor family of transcription factors that bind common ligands in the brain (corticosterone and cortisol) and supposedly have identical hormone response elements. This raises the important question of how they can elicit differential biological actions in neurons in which they are often colocalized. One plausible explanation is that they differentially recruit proteins (coregulators or other receptor-interacting factors) through cell-specific interactions with regions that diverge between MR and GR to modulate target gene transcription in a receptor-specific manner. We therefore performed a yeast-two-hybrid screening of a human brain cDNA library with an AF1-containing region of the human MR as bait. This screening revealed several potential MR-interacting partners; among them were several clones bearing homology to DAXX, FLASH, and FAF-1, all previously implicated in apoptosis. Coexpression of candidate clones in a mouse hippocampal cell line confirmed these interactions in a mammalian neural cell environment as well. In transient transactivation assays, DAXX and FLASH influenced MR- and GR-driven transcription of the MMTV-Luc reporter similarly; in contrast, although FAF-1 did not transactivate GR, it did selectively stimulate MR-mediated transcription. Thus, the present findings, that 1) DAXX, FLASH, and FAF-1 modulate the transcriptional activities of MR and GR and that 2) FAF-1 selectively coactivates only MR, provide possible clues for how these closely related receptors might differentially influence neuronal function.

Mineralocorticoid (MR) and glucocorticoid (GR) receptors, members of the steroid family of nuclear receptors, are transcription factors that have a predominantly cytoplasmic localization when unliganded but translocate to the nucleus after ligand binding. GR are widely expressed throughout the brain, whereas MR are mainly restricted to limbic areas such as the hippocampus (Reul et al., 2000). Cortisol and corticosterone are the endogenous agonists for both receptors; MR has an approximately 10-fold greater affinity for these ligands (Reul et al., 2000). Corticosteroid actions in the brain include endocrine regulation, cognition, mood, and sleep (Holsboer, 2000). The manifestation of these functions has been proposed to depend on the relative occupation of MR and GR (de Kloet et al., 1998). Experiments delineating the specific roles of MR and GR in learning and memory support this view, as do studies demonstrating that GR and MR mediate opposing actions on the survival of hippocampal granule cells (Almeida et al., 2000; Sousa and Almeida, 2002).

Given that MR and GR bind the same agonist, have high structural homology (Laudet and Gronemeyer, 2002), may be coexpressed in the same cells (van Steensel et al., 1996), and apparently bind to the same DNA response elements (Yudt and Cidlowski, 2000), questions concerning receptor-specific signaling mechanisms and biological outcomes become a central issue. One current view holds that the differential transcriptional effects of MR and GR may result from the recruitment of specific factors to each receptor. Work over the last decade has demonstrated an essential role for coregulator

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ABBREVIATIONS: MR, mineralocorticoid receptor; GR, glucocorticoid receptor; YTH, yeast two-hybrid; DAXX, death-associated protein; FLASH, FLICE-associated huge; FAF-1, fas-associated factor 1; MTH, mammalian two-hybrid; hMR, human mineralocorticoid receptor; AF-1, activation function 1; AD, activation domain; HA, hemagglutinin; MMTV, mouse mammary tumor virus; FBS, fetal bovine serum; hGR, human glucocorticoid receptor; GFP, green fluorescent protein; ADX, adrenalectomy; PML, promyelocytic leukemia protein; RT, reverse transcription; PCR, polymerase chain reaction; POD, PML oncogenic domains; SV40, simian virus 40; ANOVA, analysis of variance.

(coactivator and corepressor) molecules in mediating nuclear receptor effects on transcription; the coregulators serve to bridge nuclear receptors with the basal transcriptional machinery (Glass and Rosenfeld, 2000). Coregulator proteins, present in limiting amounts in the cell, associate with nuclear receptors, modify the chromatin structure of target genes by decondensation or compaction, and direct the assembly of productive or abortive transcriptional preinitiation complexes at target promoters (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). To date, a number of bona fide coregulators have been identified using genetic screens such as the yeast two-hybrid (YTH) system. Whereas many coregulators bind to the activation function 2 located within the ligand-binding domain at the C terminus of steroid receptors, evidence is emerging that coactivators can also interact with the N-terminal AF-1 region of several nuclear receptors (e.g., Benecke et al., 2000; Watanabe et al., 2001; Wansa et al., 2002; Wardell et al., 2002). Studies in animals have shown that coactivators are expressed in specific neural cell types (with identical or differing nuclear receptor compositions) according to strict temporal patterns (Meijer et al., 2000; Mitev et al., 2003), indicating that transcriptional activity of a given receptor can occur in a cell-specific manner.

Most known coactivators interact with the activation function 2 region of GR (Necela and Cidlowski, 2003) but only a few have addressed interactions with MR (Fuse et al., 2000; Kitagawa et al., 2002). Because sequence homology of the AF-1 region of MR and GR is relatively weak (~15%), we reasoned that this region may be important for conferring specificity to the two receptors. Therefore, we first sought to identify proteins interacting with the AF-1 region of the MR using a classic YTH approach. Some of the clones emerging from this analysis showed sequence homology to DAXX (death-associated protein), FLASH (FLICE-associated huge), and FAF-1 (Fas-associated factor 1), all of which have been known, until now, mostly for their involvement in the transduction of apoptotic stimuli (see Peter and Krammer, 2003). In confirmation of the YTH results, clones corresponding to DAXX, FLASH, and FAF-1 were found to interact with the AF-1 domains of MR (amino acid residues 170–433) and GR (amino acid residues 1–450) in mammalian two-hybrid assays (MTH). Based on reporter gene assays, we provide evidence that these molecules can differentially enhance or repress MR- and GR-mediated gene transactivation in a neural cell type-specific fashion. Finally, the relevance of our findings to the better understanding of the differential actions of corticosteroids is indicated by our detection of DAXX, FLASH, and FAF-1 mRNA transcripts in the rat hippocampus and our demonstration that the expression of *DAXX*, but not of *FLASH* and *FAF-1*, is influenced by the corticosteroid environment in vivo.

Materials and Methods

Yeast Two-Hybrid Screening. A region of the human mineralocorticoid receptor (hMR) encoding the AF-1-containing region (amino acids 170–433) was expressed as a fusion with the lexA DNA-binding domain of a DUAL-Hybrid vector, and used for screening of a human brain cDNA library (BD Biosciences Clontech, Heidelberg, Germany). Transformation of *Saccharomyces cerevisiae* was carried out on SD-Trp-Leu-His selection plates. Of 4.8×10^6 colonies screened, 96 X-Gal-positive clones were obtained. Library plasmids were isolated, amplified in *Escherichia coli* XL1 blue and subjected to restriction digest analysis with Bgl II to release the insert. Selected library clones were subsequently re-transformed into the yeast reporter strain carrying either the hMR bait construct or a control plasmid, and transformants were tested for β-galactosidase activity as a measure of bait dependence. pACT2 plasmids from bait-dependent clones were isolated and the cDNA inserts determined by automated sequencing using a forward primer located 5′ to the lexA/insert fusion site. A database search revealed several of the bait-dependent clones to be homologous to either DAXX, FLASH, or FAF-1 proteins.

Plasmids. The AF-1 bait used in mammalian two-hybrid assays was designed to correspond to that used in the YTH screening (see above). Briefly, Xma I and Xba I restriction sites were inserted, respectively, at the 5′ and 3′ ends of the primers, and the sequence amplified using pRShMR as template. This product was inserted into the pM vector (BD Biosciences Clontech) and fused with its GAL4 DNA-binding domain. Positive clones indicated by MTH experiments were excised from pACT2 yeast vector using BamHI/XhoI restriction sites and inserted into the pVP16 vector in frame with its activation domain (AD) (BD Biosciences Clontech). The full-length FAF-1 clone was directionally fused with HA tag of pcDNA vector (Invitrogen, Karlsruhe, Germany). All frames were confirmed by sequencing. The EagIxAflII fragment of the human full-length MR was inserted into pEGFP-C3 vector (BD Biosciences Clontech). Full-length hDAXX (in pcDNA) was a kind gift from Dr. Gerd Maul (Philadelphia, PA); full-length FLASH-SPORT expression vector was purchased from the Resource Center and Primary Database (RZPD, Berlin, Germany); pRShMR, pRShGR, and pMMTV-Luc were kindly provided by R. M. Evans (La Jolla, CA).

Cells. The HN9.10 cell line, a fusion of embryonic mouse hippocampal and human neuroblastoma cells, was a gift from Dr. Marsha Rosner (Chicago, IL); the SHY-SY5Y neuroblastoma cell line (established from a metastatic bone tumor) was purchased from the European Tissue Culture Collection (Salisbury, UK). All cell culture reagents were obtained from Invitrogen. Both cell lines were maintained in Dulbecco's minimum essential medium containing 10% (HN9.10) and 15% (SHY-SY5Y) fetal bovine serum (FBS) and kanamycin. Cells were regularly split after reaching 80 to 90% confluence. After transfection (see below), cells were maintained in 1% charcoal-stripped FBS to eliminate the interfering effects of steroids in normal FBS.

Transfections and Reporter Assays. Transfections were performed using the cationic polymer Jet-PEI, according to the manufacturer's instructions (Polytransfection, Illkirch, France). Before transfection, 100×10^3 cells/well were plated in 24-well plates; transfection was carried out 14 to 24 h later when cells had reached 50 to 80% confluence. One hundred twenty-five nanograms of each DNA construct was used, and the total amount of DNA was adjusted with empty pcDNA vector. Twenty-four hours after transfection, medium was changed to 1% charcoal-stripped FBS medium, and cells were treated with specific MR (aldosterone) or GR (dexamethasone) ligands. In common with the majority of published studies, these ligands were applied at a final concentration of 10^{-6} M to ensure robust responses from endogenous and exogenous GR. In some cases, the MR antagonist spironolactone was used at 10^{-5} M. All steroid receptor ligands were purchased from Sigma Chemicals (Deisenhofen, Germany). After 48 h, cells were lysed (with 75 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM Triton-X, and 2 mM ATP), washed with Tris-buffered saline, and centrifuged to remove cell debris before measurement of luciferase activity in the supernatant. For this, Luc assay buffer (75 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM ATP) and 10 mM luciferin (Roche, Mannheim, Germany) were added and light emission of each supernatant was measured. Raw Luc values varied according to cell number and transfection efficiency; in a typical experiment, basal Luc activity was ~50,000 light units, increasing to ~2 to 3 $\times 10^6$ units after induction by ligand. To correct for variation between independent experiments and to allow comparison of results, values were normalized to (the corresponding) β-galactosidase activity. The latter was measured after incubating

cell extracts with buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, and 50 mM β-mercaptoethanol) and *O*-nitrophenyl-D-galactopyranoside (Sigma) and measuring light absorption at 405 nm.

Western Blotting. A standard procedure was followed, using primary antibodies raised against hGR (Santa Cruz, Heidelberg, Germany), HA (BabCO, Richmond, CA), and GFP (BD Biosciences Clontech).

Coimmunoprecipitation. HN9.10 cells were transiently transfected with plasmids expressing HA-FAF1 and GFP-MR. Twelve hours after transfection, cells were treated with aldosterone (10^{-6} M), lysed 24 h later using ice-cold radioimmunoprecipitation assay-phosphate-buffered saline buffer (1× phosphate-buffered saline, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 15 mM *N*-ethyl-maleimide, and protease inhibitor cocktail). Lysates were sonicated and centrifuged, and the supernatants were precleared with 40 μl of protein A/G agarose beads (3 h). Beads were pelleted and proteins precipitated overnight (4°C) with either monoclonal anti-HA or rabbit polyclonal anti-GFP. Precipitated proteins were collected by incubation with fresh agarose beads (3 h, with rotation), spun down, washed three times with buffer I (150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 15 mM *N*-ethyl-maleimide, and protease inhibitors) and once with buffer II (without salt). Finally, beads were re-suspended in 50 μl of 2× Laemli buffer, boiled, and analyzed using SDS-PAGE; signals were detected with enhanced chemiluminescence reagents (Amersham, Braunschweig, Germany).

Animals. Adult male Wistar rats (Charles River, Sulzfeld, Germany), maintained under standard laboratory conditions, were used. A subgroup of animals were surgically adrenalectomized (ADX) under halothane anesthesia (see Patchev and Almeida, 1996); all ADX animals were maintained on 0.9% saline drinking solution. Some of the ADX animals were given daily injections of either aldosterone (50 mg/kg) or dexamethasone (100 μg/kg) from the time of surgery; all other ADX and adrenal-intact animals received vehicle (sesame oil) injections. Animals were sacrificed after 4 days; RNA was extracted from their hippocampi, reverse-transcribed, and assayed for DAXX, FLASH, and FAF-1 mRNA levels by RT-PCR. The local animal welfare regulatory authorities approved all procedures on live animals; National Institutes of Health and Society of Neuroscience codes of practice were followed.

Semiquantitative RT-PCR. RNA was extracted from rat hippocampi (30 mg) using the RNeasy-kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Next, 1 μg of RNA was reverse-transcribed using QIAGEN's Omniscript reverse transcriptase kit and oligo(dT) primers from Promega (Mannheim, Germany). Hippocampus-derived cDNA was amplified using primers that spanned intron-exon boundaries to control for contamination by genomic DNA. DAXX: forward, gaa gat gaa gca gt gct cag; reverse, gat cca ac cct ttt cct gca g; FLASH: forward, ctg aat agt cca gtg aga c; reverse, tca tct aca aac ttc cac a; FAF-1: forward, gca gct atc aat ggt gta ata c; reverse, gtg taa gga cat aaa gac tgt tg (primers were synthesized by MWG-Biotech, Ebersberg, Germany). The annealing temperatures/cycling parameters were as follows: DAXX, 57°C/23 cycles; FLASH, 56°C/26 cycles; FAF-1, 56°C/25 cycles. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The amplified products were separated on 1.5% agarose gel, and optical densities (O.D.) quantified by using standard imaging software.

Statistics. All data are presented as means ± S.D. Numerical data were subjected to statistical analysis (ANOVA, followed by post hoc tests). Significant differences were accepted when $P \leq 0.05$.

Results

Selection of Potential MR-Interacting Partners. A total of 96 positive clones that interacted with a bait consisting of amino acids 170 to 433 of the AF-1 region of hMR (Fig. 1A) were identified using a YTH screen. None of these clones corresponded to any of the known coregulators, but several showed full sequence homology to proteins implicated in cell death: five clones were homologous to DAXX, three clones were homologous to FLASH, and one clone was homologous to the full-length sequence of human FAF-1 (Fig. 1B). These candidates were selected for further analysis in the light of the previously described differential effects of MR and GR activation in neural cells (Almeida et al., 2000; Sousa and Almeida, 2002). In a next step, we sought to confirm the interactions obtained in the YTH in a mammalian cell environment, using mammalian two-hybrid (MTH) assays. For this, DAXX, FLASH, and FAF-1 cDNA clones were fused to the AD of pVP16, and the AF-1 bait was fused to the Gal DNA binding domain of the pM vector. These constructs were transfected into either a mouse hippocampus-derived cell line (HN9.10) or a human neuroblastoma cell line (SHY-5Y). The degree of interaction is expressed as the level of transactivation of Luciferase reporter (pFR-Luc) relative to values obtained in controls (empty pM, pVP16 cotransfected with either pMGal-AF1, or clones fused to pVP16AD-clones). The ability of Gal-hMR(170–433) and Gal-hGR(1–450) to independently transactivate the pFR-Luc reporter was controlled in a separate experiment (data not shown).

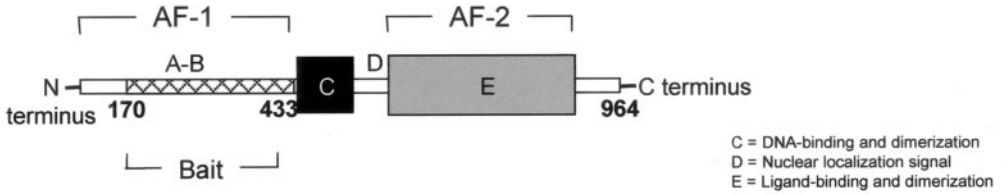
The transfection assays indicated weak, but reproducible, interactions between the DAXX-, FLASH-, and FAF1-clones and the AF-1–containing region of MR in HN9.10 cells (Fig. 1C). When candidate partners were coexpressed with full-length GAL4-MR, interaction levels remained comparable with those found with GAL4-AF1, except for the case of the DAXX clone, suggesting that regions other than the AF-1 are required for the interaction between MR and DAXX; in addition, this interaction depends on the cellular environment (see below). Using a bait comprising residues 1 to 450 of the human GR [Gal-GR(1–450)] revealed that DAXX, FLASH, and FAF-1 have the potential to interact also with GR (Fig. 1C), suggesting that both receptors possess surfaces for docking all three factors in their N-terminal domains.

MTH assays conducted in neuroblastoma SHY-5Y cells yielded interaction profiles that were, with one exception, similar, albeit weaker, to those obtained in HN9.10 cells (Fig. 1C, inset), indicating that post-translational modifications may contribute to these interactions. The strong induction of pFR-Luciferase reporter when DAXX was coexpressed with full-length MR in HN9.10 cells was not observed in SHY-5Y cells, suggesting the importance of the cellular context for MR-DAXX interaction.

Modulation of MR- and GR-Mediated Transcription by DAXX, FLASH, and FAF-1. In extension of the MTH assay, we next conducted transcriptional assays with full-length MR and GR to obtain more functional information on the effects of candidates of interest on MR- and GR-mediated transactivation. For this, HN9.10 cells were transfected with either MR or GR, full-length DAXX, FLASH, or FAF-1, and MMTV-Luc. Reporter activity was measured after treatment of cells with the prototypic MR and GR agonists, aldosterone and dexamethasone, respectively; both ligands were applied at a dose of 10^{-6} M.

Controls. As demonstrated recently, cotransfection of different expression vectors can influence the transactivation of the reporter gene through squelching phenomena and alteration of the concentrations of individual components; i.e., the

A.



B.

Sequence homology to	Times isolated in YTH	Clone length (bp)*	Interactions in Mammalian 2- hybrid (MTH) assay: AF-1 of MR	Interactions in Mammalian 2- hybrid (MTH) assay: Full-length MR
DAXX	5	2353 (2461)	YES	YES
FLASH	3	1200 (6781)	YES	YES
FAF-1	1	Full length	YES	YES

* Figures in brackets indicate base pairs in full-length sequences

C.

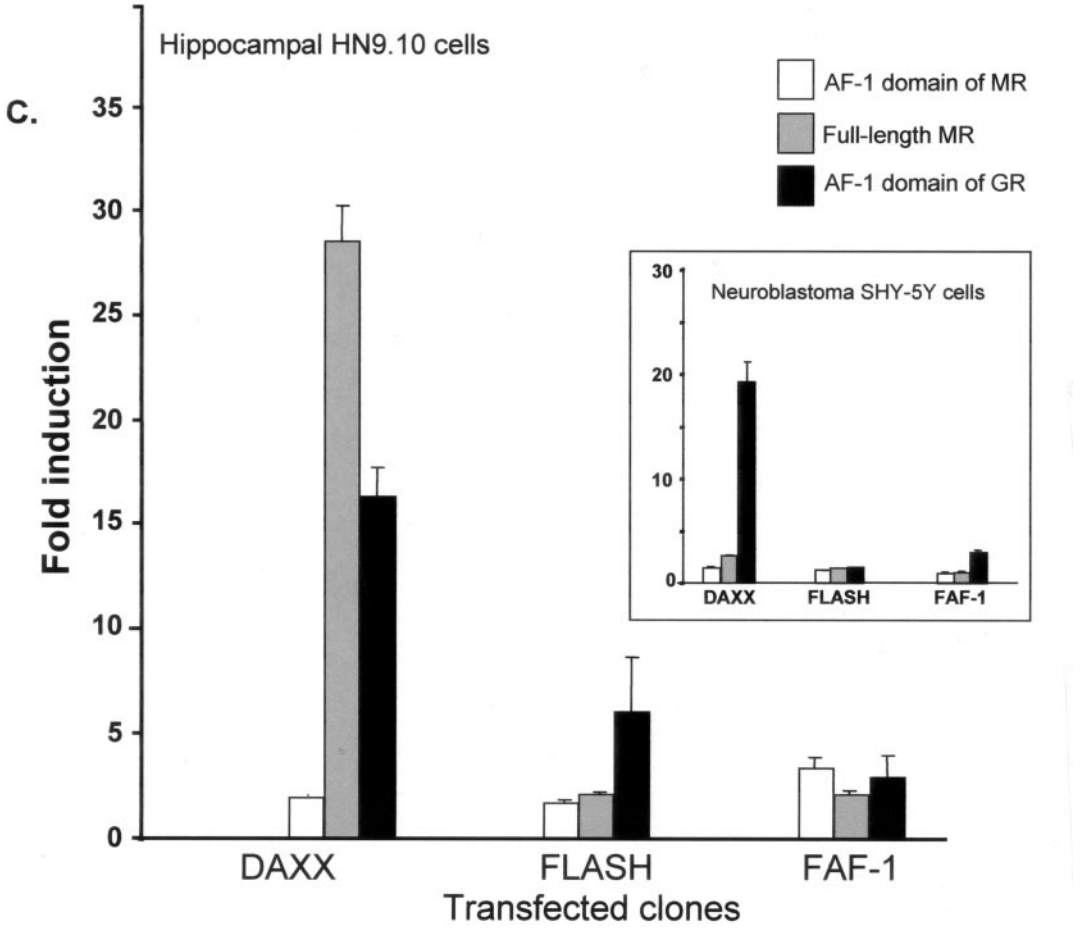


Fig. 1. Interaction of DAXX, FLASH, and FAF-1 clones with the AF-1 domain of the MR in a mammalian-two-hybrid assay. A, schematic representation of the domain structure of the human MR, indicating its AF-1 region used as bait in yeast two-hybrid screening. B, summary of results of mammalian two-hybrid assays performed in hippocampal HN9.10 cells. C, HN9.10 cells were cotransfected with plasmids containing either 1) Gal-hMR(170–433) (□), full-length Gal-MR (▨), or Gal-hGR(1–450) (■); 2) DAXX-, FLASH-, and FAF1-clones, or 3) pFR-Luc (reporter) and pSV40βGal (to normalize for transfection efficiency). Same amounts of all constructs were used (125 ng) and reporter activity assayed after 48 h. Results are depicted as fold induction [i.e., ratio between normalized luciferase activity and corresponding control (VP16-clone or pMGal-bait alone) values]. Each bar represents mean ± S.D. from five independent experiments performed in duplicate. The *inset* shows the results obtained in comparable assays in neuroblastoma SHY-5Y cells.

resulting data may not necessarily reflect the influence of the factor being tested on receptor-mediated transcription (Hofman et al., 2000). Therefore, the expression levels of MR and GR were monitored by immunoblotting in the presence of transfected DAXX, FLASH, and FAF-1 cDNAs. As shown in Fig. 2, protein levels of both receptors remained constant, thus excluding the possibility that altered receptor levels contributed to the changes in reporter transactivation. Also, to exclude the possibility that DAXX, FLASH, and FAF1 were themselves acting on the reporter, they were transfected in a broad concentration range in the absence of MR or GR, with either MMTV-Luc or the glucocorticoid response element-less cytomegalovirus-Luc. In no case was reporter activation observed (not shown), suggesting that the transcriptional effects of DAXX, FLASH, and FAF-1 reported below are mediated by MR and/or GR.

DAXX Represses Transactivation by MR and GR. Extending the MTH assays, which demonstrated physical interaction of DAXX with MR and GR baits (Fig. 1C), studies were undertaken to examine whether DAXX might have the potential to influence the transcriptional activity of full-length MR and GR. For this, HN9.10 cells were transiently transfected with DAXX, MR, or GR and MMTV-Luc reporter and treated with either aldosterone or dexamethasone for 24 h. As shown in Fig. 3, A and B, DAXX significantly repressed MR- and GR-driven transactivation by ~45% and 65%, respectively. To our knowledge, this is the first report of the repressive actions of DAXX on MR transactivation. The repressive effects of DAXX were found to be dose-dependent in a concentration range of 1 to 125 ng (Fig. 3C).

FLASH Enhances MR- and GR-Mediated Transactivation. In contrast to DAXX, coexpression of FLASH with either MR or GR consistently led to increased transactivation of the MMTV-Luc reporter by the individual receptors (Fig. 4, A and B); the transactivation potential of both receptors increased with the amount of FLASH cDNA transfected, as shown in Fig. 4C. Interestingly, FLASH was recently shown to have an inhibitory effect on GR-mediated transactivation in human colon carcinoma-derived cells (Kino and Chrousos, 2003), suggesting a cell-specific action of FLASH on GR-mediated transcription activation.

Differential Effects of FAF-1 on MR- and GR-Driven Transactivation. FAF-1 was identified as an MR-interacting partner in a YTH screen in which the AF-1 region of MR served as bait. As opposed to DAXX and FLASH, which respectively repressed and enhanced transcription mediated by MR and GR, FAF-1 was found to differentially affect the transactivation properties of these two receptors: introduction of FAF-1 at doses ranging from 1 to 125 ng potentiated MR-mediated transactivation and had no effect on the GR-driven response from the reporter (Fig. 5, A and B). Thus, FAF-1 can differentially modulate the transactivation potential of MR versus GR. As shown in Fig. 5C, FAF-1 influenced MR-mediated transactivation even in the presence of a competitive MR antagonist (spironolactone). These observations suggest that 1) FAF-1 interacts with surfaces of MR that are not affected by conformational changes induced by agonists or antagonists, 2) FAF-1 binding is compatible with agonist-induced coactivator binding, and 3) possible recruitment of corepressor (complexes) by the antagonist does not impair FAF-1 association. Together, this is in keeping with the observation that MR AF-1 mediates FAF-1 binding. Indeed, this interaction was confirmed by coimmunoprecipitation and was observable in both presence and absence of ligand. GR did not interact with FAF-1 (Fig. 5D), explaining the lack of responses in the transcription assays (Fig. 5B).

Cell Context-Dependence. To examine whether the herein-reported influences of DAXX, FLASH, and FAF-1 on the transactivational properties of MR and GR in hippocampal HN9.10 cells also occur in other neural cell types, we performed similar cotransfection experiments in neuroblastoma SHY-5Y cells. We found that FLASH and FAF-1 had either slightly repressing, or no effects on MR and GR transactivation in SHY-5S cells (Fig. 6). In addition, in keeping with a previous report that DAXX is not exclusively a transcriptional repressor (Michaelson et al., 1999), we observed that DAXX respectively potentiated and repressed MR- and GR-mediated transcription. Thus, our comparison of MR and GR transactivation in HN9.10 and SHY-5S shows that the effects of DAXX, FLASH, and FAF-1 vary from one cell type to another, indicating the importance of the cellular environment.

DAXX, FLASH, and FAF-1 mRNA Expression in Rat Hippocampus. mRNA transcripts of DAXX, FLASH, and FAF-1 were detected in rat hippocampus, supporting a possible physiological role for these molecules. In light of previous observations showing that the corticosteroid milieu plays a crucial role in determining the survival of hippocampal neurons (Sousa and Almeida, 2002), it was of interest to analyze whether manipulation of circulating corticosteroid levels alters DAXX, FLASH, and FAF-1 mRNA expression. Extinction of endogenous corticosteroids by adrenalectomy did not affect FLASH and FAF1 but significantly down-regulated DAXX mRNA expression (Fig. 7). No significant changes were found in the expression of FLASH and FAF-1 mRNA after treatment of ADX rats with aldosterone or dexamethasone; however, this treatment paradigm resulted in a partial restoration of DAXX mRNA levels to those found under control (adrenal-intact) conditions (Fig. 7).

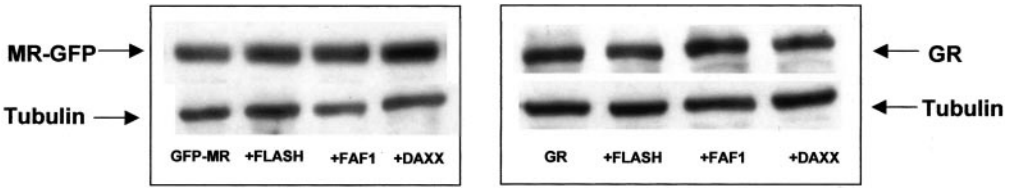


Fig. 2. MR and GR levels are not influenced by transfection of cells with DAXX, FLASH, or FAF-1 cDNA. To control for possible interfering effects on MR and GR expression, HN9.10 cells were transfected with plasmids expressing 1) full-length DAXX, FLASH, or FAF-1, 2) GR or GFP-MR, and 3) pMMTV-Luc (575 ng each). Expression of MR and GR in cell extracts (30 μg/lane) was monitored by immunoblotting and normalized to tubulin expression. One representative Western blot showing that levels of MR and GR were not affected by coexpression of DAXX, FLASH, or FAF-1 is presented.

Discussion

To address the question of how the highly homologous MR and GR can elicit opposite effects in hippocampal cells (de Kloet et al., 1998; Almeida et al., 2000; Sousa and Almeida, 2002), we here considered the possibility that recruitment of receptor-specific proteins results in the activation or repression of relevant downstream genes. Several GR coactivators have been described previously (Necela and Cidlowski, 2003), but the data on MR-interacting partners is sparse (Fuse et al., 2000; Kitagawa et al., 2002). MR and GR show greatest sequence divergence in their AF-1 domains. Reasoning that this domain might therefore provide unique protein interaction surfaces, we used MR AF-1 (amino acids 170–433) as bait in a yeast 2-hybrid (YTH) screen to identify potential MR-specific interacting partners. Some of the clones selected for the analysis in the present study, were homologous to DAXX, FLASH, and FAF-1; these proteins were previously shown to be also involved in Fas receptor-mediated apoptosis (Chu et al., 1995; Yang et al., 1997; Chang et al., 1998; Imai et al., 1999).

MTH assays in hippocampal (HN9.10) and neuroblastoma (SHY-5Y) cell lines demonstrated interactions between parts of DAXX, FLASH, and FAF-1 (see Fig. 1B) and the AF-1 region of MR as well as with full-length MR; the interactions were more pronounced in HN9.10 cells, indicating the importance of cellular context. Furthermore, using GR (1–450) as a bait showed that GR can dock these same molecules in its N-terminal; more robust interactions with the latter probably reflect the fact that it was longer than the MR bait, although the involvement of other facilitative adapter molecules should also be considered. In light of the YTH and MTH data, we proceeded to conduct a comparative analysis of the influence of full-length DAXX, FLASH, and FAF-1 on MR- and GR-mediated transcription.

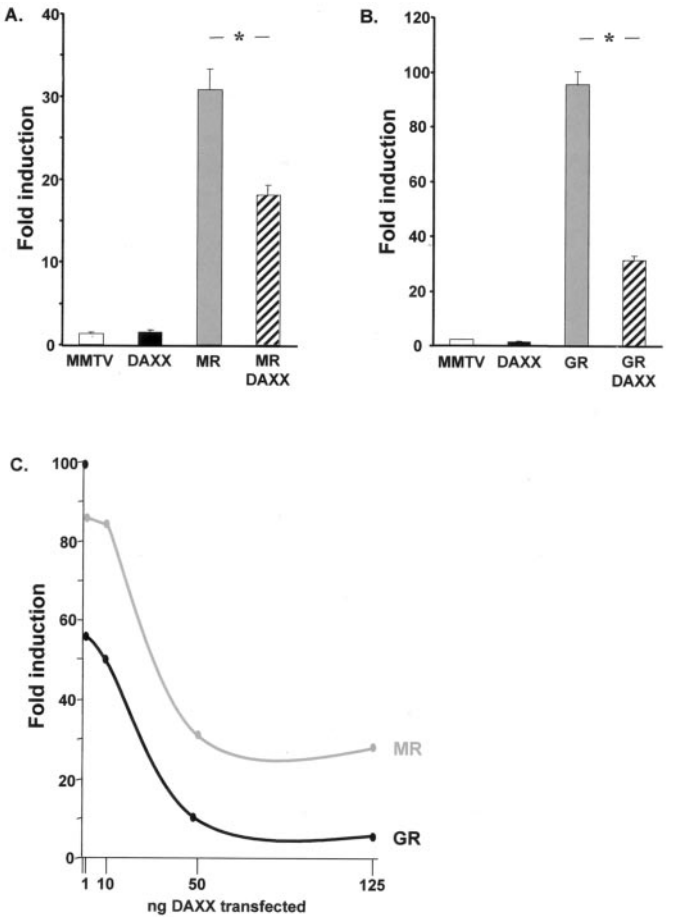


Fig. 3. DAXX represses MR- and GR-mediated transactivation in hippocampal cells in a dose-dependent manner. A and B, HN9.10 cells were transfected with a combination of 1) full-length DAXX-expressing vector, 2) MR or GR, 3) pMMTV-Luc, and 4) pSV40βGal. Total amounts of transfected DNA were kept constant (125 ng of construct/well), controls were filled with empty pcDNA plasmid where necessary. Cells were treated with 10^{-6} M of either aldosterone (A) or dexamethasone (B) 12 h after transfection, and reporter gene activity was measured after a further 24 h. Results are given as induction fold (ratio of luciferase activity normalized to β-Gal activity in the absence and presence of the cognate ligand). Bars represent mean ± S.D. of data from at least three independent experiments. *, $P \leq 0.05$ (one-way ANOVA followed by post hoc analyses). C, dose-dependence of the repressive effects of DAXX on MR- and GR-mediated transcription was demonstrated by cotransfecting HN9.10 cells with increasing concentrations of a DAXX-expressing plasmid (0–125 ng) together with the reporter plasmids pMMTV-Luc and pSV40βGal. Results are depicted as fold induction (ratio of luciferase activity normalized to β-Gal activity in the presence and absence of cognate ligand); the arbitrary value of 100 was assigned to the level of induction obtained when empty plasmid was transfected. Mean values of data obtained when MR or GR were coexpressed in at least three different experiments are shown.

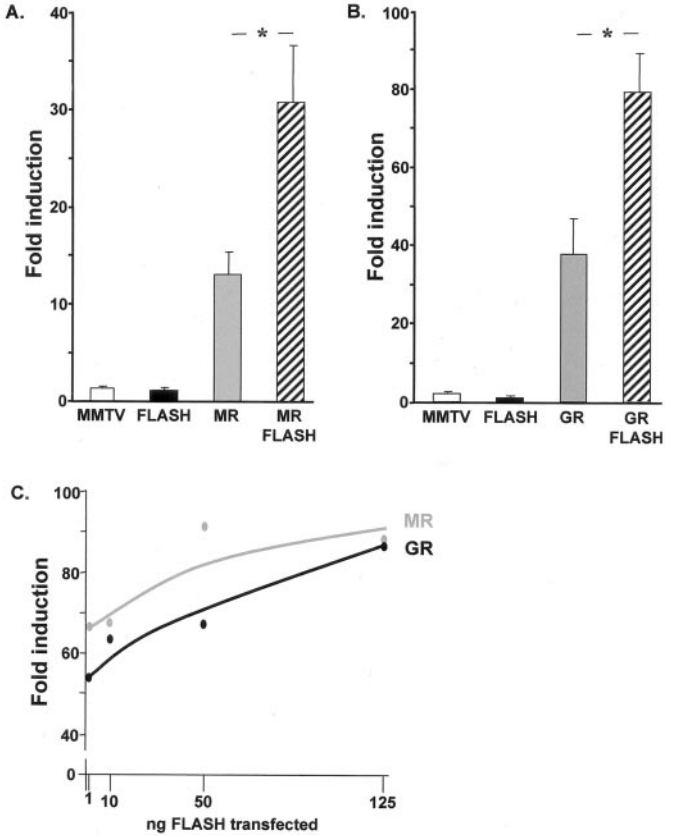


Fig. 4. MR- and GR-mediated transactivation of the MMTV reporter is enhanced by FLASH. A and B, the effects of FLASH on MR- and GR-driven transactivation of the MMTV reporter (see experimental details in legend to Fig. 3, A and B) are shown. Fold induction of the reporter gene was calculated as described in the legend to Fig. 3; *, $P \leq 0.05$ (one-way ANOVA followed by post hoc analyses). C, using conditions similar to those described in the legend to Fig. 3 (C), cotransfection with a FLASH-expressing plasmid (0–125 ng DNA) revealed that enhancement of the transcriptional activity of MR- and GR-driven reporter tends to be dose-related. Mean levels of MR- and GR-mediated transactivation in three independent assays are shown.

DAXX, which colocalizes with promyelocytic leukemia protein (PML) oncogenic domains (PODs) in the nucleus, has been shown previously to act as a transcriptional modulator (Torii et al., 1999; Li et al., 2000; Zhong et al., 2000). Recently, *DAXX* was shown to be up-regulated in the hippocampus of patients with Alzheimer's disease (Colangelo et al., 2002). Consistent with reports that DAXX can silence various promoters (Li et al., 2000), we found that DAXX dose-dependently repressed MR-mediated transcription from the MMTV-Luc reporter in hippocampal HN9.10 cells; also, in agreement with a recent report by Lin et al. (2003), we found that DAXX can repress GR-mediated transcription. On the other hand, DAXX was seen not to have any significant effect on either MR or GR in neuroblastoma SHY-5Y cells; this confirms the observations made by other authors (Michaelson et al., 1999; Michaelson and Leder, 2003), and shows that the modulatory effects of DAXX on gene transactivation can vary from one cell type to another. Although insights into the mechanisms of transcription modulation by DAXX are beginning to emerge—DAXX associates with histone deacetylase II (Hollenbach et al., 2002)—there are indications that histone deacetylation, which plays a critical role in transcriptional silencing, cannot alone explain all of the actions of DAXX. Indeed, our recent unpublished data (D. Obradović) confirm that transcriptional repression by DAXX cannot be relieved by the histone deacetylase inhibitor trichostatin A, confirming similar observations by others (Wilson et al., 2002).

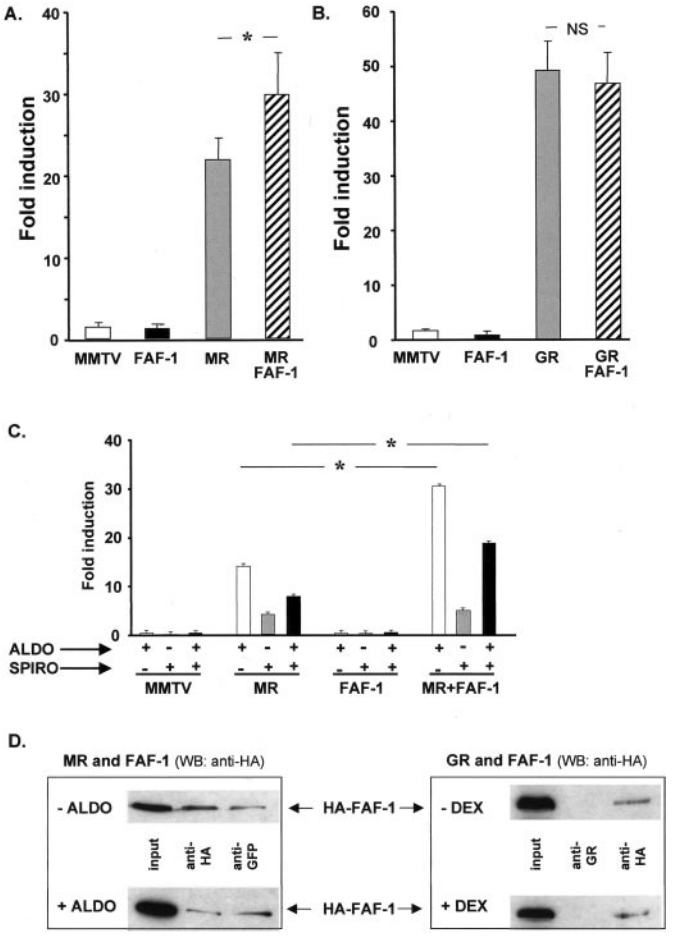


Fig. 5. FAF-1 differentially influences MR- versus GR-mediated transactivation of the MMTV-Luc reporter gene. A and B, expression of a FAF1-expressing plasmid in HN9.10 cells resulted in a significant ($P \leq 0.05$) enhancement of MR-driven transactivation of an MMTV-Luc reporter gene (A). In contrast, GR-mediated transactivation was unaltered when FAF-1 was coexpressed (B). Experimental details were identical to those described in the legend to Fig. 3, A and B. C, FAF-1 enhanced MR-mediated transactivation even in the presence of a competitive MR antagonist spironolactone (10^{-5} M). In these experiments, HN9.10 cells were cotransfected with MR, FAF-1, or a combination of both and exposed to the indicated ligands 24 h later; reporter activity was measured after a further 24 h. Results are presented as means ± S.D. and come from at least three independent experiments; *, $P \leq 0.05$. Fold induction was calculated as described previously. D, FAF-1 interacts with MR, but not GR, in the HN9.10 cell line. Cells were transiently transfected with vectors expressing 1) HA-FAF1 and 2) GR or GFP-MR and treated with aldosterone or dexamethasone, respectively. Lysates were immunoprecipitated with anti-GFP, anti-GR, and anti-HA antibodies, and Western blots were visualized with anti-HA.

Reporter gene assays revealed that FAF-1 specifically enhances transcription driven by MR, but not GR, in HN9.10 hippocampal cells. FAF-1, a molecule with a predominantly nuclear localization (Frohlich et al., 1998; Guerra et al., 2001; Jensen et al., 2001; Ryu et al., 2003), has a role in Fas receptor-mediated apoptosis (Chu et al., 1995). A role for FAF-1 in the brain seems likely in view of a recent report that FAF-1 expression is increased in the brains of patients with Alzheimer's disease (Wang et al., 2003). In the present experiments, MR signal enhancement by FAF-1 occurred in a ligand- and dose-dependent manner. Interestingly, we found FAF-1 to override the transcriptional blockade caused by exposure of cells to the MR-specific antagonist spironolactone, suggesting that FAF-1 probably interacts through a surface of the MR distinct from that to which the antagonist binds. Our studies revealed that FAF-1 selectively potentiates MR, but not GR, transactivation; in contrast, DAXX and FLASH exerted repressive and amplifying effects, respectively, on MR and GR transactivation in hippocampal HN9.10 cells. Together, the present findings suggest some novel mechanisms through which MR and GR activity can be differentially modulated (de Kloet et al., 1998; Almeida et al., 2000; Sousa and Almeida, 2002).

As mentioned, DAXX, FLASH, and FAF-1 are best known for their participation in the transduction of Fas receptor-

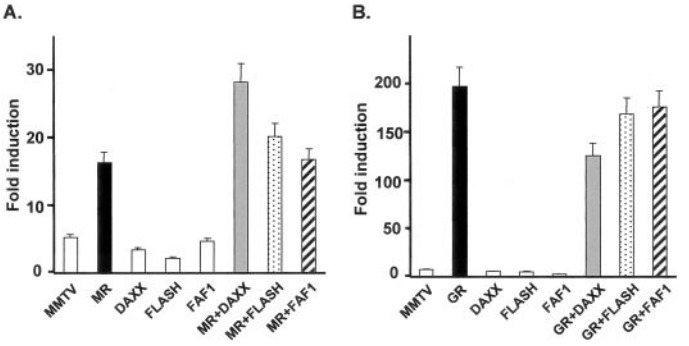


Fig. 6. DAXX, FLASH, and FAF-1 do not equally influence MR- and GR-mediated transcription in all neural cell environments. DAXX, FLASH, and FAF-1 had slightly repressing or no effects on MR- and GR-mediated transactivation in SHY-5Y cells, contrasting with observations in HN9.10 cells and emphasizing the importance of the cellular environment (see Figure 3). For these experiments, cells were transfected with a combination of 1) full-length DAXX, FLASH, or FAF-1, 2) MR or GR, 3) pMMTV-Luc, and 4) pSV40βGal. The amounts of transfected DNA were kept constant and equal (125 ng of construct/well), filling in with empty pcDNA plasmid where necessary. Cells were treated with 10^{-6} M of aldosterone or dexamethasone, and activity of the reporter measured after 24 h. Data shown represent mean ± S.D. from two experiments performed in triplicates.

mediated apoptosis (Medema et al., 1999; Peter and Krammer, 2003). Many studies have now shown the crucial role of corticosteroids in the regulation of hippocampal cell survival (Almeida et al., 2000). In this study, we observed that *DAXX*, *FLASH,* and *FAF-1* mRNAs are expressed in the rat hippocampus, raising the question of their likely involvement in mediating corticosteroid actions on neuronal survival. In a first attempt to address this issue, we measured the expression of the mRNAs coding for these molecules in the hippocampi of animals exposed to either of two triggers of apoptosis (ADX and dexamethasone treatment), or to a treatment previously shown to have pro-survival effects (stimulation of MR with aldosterone (for a review, see Sousa and Almeida, 2002). None of these treatments altered the expression of FLASH and FAF-1. However, DAXX mRNA levels were significantly down-regulated by both the pro-apoptotic as well as the pro-survival stimuli. These findings clearly indicate that the physiological role(s) of DAXX is not exclusively related to cell survival, a suggestion made previously by other authors (Michaelson et al., 1999; Michaelson and Leder, 2003). It should be noted in support of this view that the overexpression of DAXX, FLASH, or FAF-1 in either HN9.10 or SHY-5Y cells did not decrease cell viability. Furthermore, the results obtained in a recent study in a Fas ligand-sensitive human breast (T47D) cell line suggest that DAXX, FLASH, and FAF-1 are unlikely to be general mediators of corticosteroid-induced apoptosis (D. Obradović and H. Gronemeyer, unpublished observations).

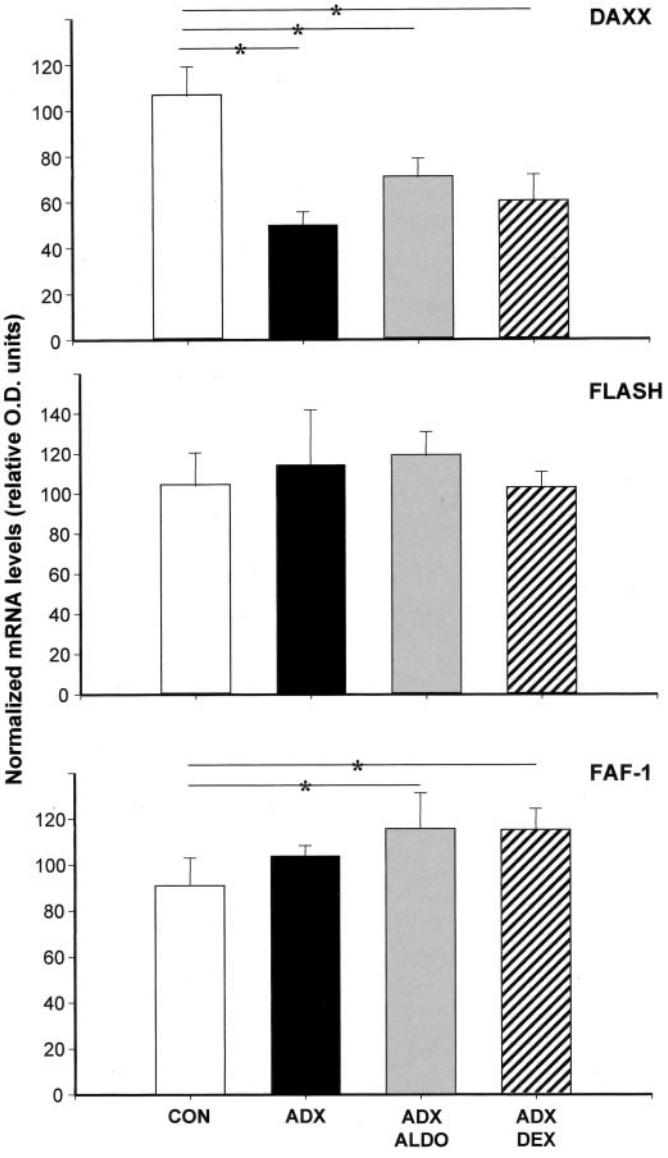


Fig. 7. Influence of the corticosteroid milieu on expression of DAXX, FLASH, and FAF-1 mRNA levels in the rat hippocampus. Semiquantitative RT-PCR was used to measure steady-state mRNA levels of DAXX, FLASH, and FAF-1 in the hippocampi of control (CON), adrenalectomized (ADX), aldosterone (ALDO)-treated ADX, and dexamethasone (DEX)-treated ADX adult male rats. Hippocampi were obtained 4 days after commencement of treatments; control animals were sham-adrenalectomized and received vehicle (oil) injections. Levels of DAXX, FLASH, and FAF-1 mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels, and are expressed in relative optical density (O.D.) units. Means ± S.D. originating from four to eight animals per treatment group are depicted. *, $P \leq 0.05$; significant differences detected after one-way ANOVA and post hoc testing.

In summary, we have identified DAXX, FLASH, and FAF-1 as MR- and GR-interacting partners that can modulate the transactivation potential of MR and GR in neural cells. In addition, our results demonstrate that DAXX, FLASH, and FAF-1 can have signaling roles that may be unrelated to their apoptotic effects in other cellular or treatment environments. Besides confirming the recently described ability of DAXX to repress GR-mediated transcription (Lin et al., 2003), we now show that DAXX can also repress transactivation by the MR. Last, we provide evidence that FAF-1 preferentially potentiates the transcriptional activity of the MR without having any effect on GR-mediated transactivation; this suggests a plausible mechanism to explain some of the differential effects mediated by MR and GR in neural cells.

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