Dysregulation of the serotonergic system and abnormalities of the hypothalamic-pituitary-adrenal axis function have been implicated to be involved in neuropsychiatric disorders. Serotonin-1A receptors have been shown to be suppressed by corticosteroid hormones in a variety of animal studies. This effect may play a central role in the pathophysiology of depression. However, little is known about the molecular mechanism underlying this suppressive effect of corticosteroids. Here, we show by functional analysis of the promoter region of the rat serotonin-1A receptor gene that two NF-κB elements in the promoter contribute to induced transcription of the rat serotonin-1A receptor gene. Furthermore, we show that corticosteroids repress this NF-κB-mediated induction of transcription. Remarkably, we observed that only the glucocorticoid receptor and not the mineralocorticoid receptor was able to mediate this repressive effect of corticosteroids. We argue that negative cross-talk between the glucocorticoid receptor and NF-κB may provide a basis for the molecular mechanism underlying the negative action of corticosteroids on serotonin signaling in the brain.

The brain serotonin (5-hydroxytryptamine; 5-HT) system has been strongly implicated in the control of behavioral processes, including feeding, aggression, and response to stress (1). Extensive evidence also supports the involvement of serotonin function in neuropsychiatric disorders, such as depression and anxiety (2, 3). In addition, selective serotonergic compounds have been shown to be clinically effective antidepressants and anxiolytics (4, 5).

The complex physiological actions of serotonin are mediated by a family of related receptors of which particular attention has focused on the 5-HT1A receptor (6). The 5-HT1A receptor belongs to the family of G protein-coupled receptors, and it negatively regulates adenylate cyclase (7). In the midbrain raphe neurons, the receptor is thought to play a role in the feedback regulation of the 5-HT system. mRNA transcripts were found to be most abundant in areas of the limbic system (i.e. hippocampus, septum, and thalamus), cerebral cortex, and raphe nucleus (8–10).

In addition to dysregulation of the serotonin system, abnormalities of hypothalamic-pituitary-adrenal axis function also have been described to be involved in neuropsychiatric disorders. The hypothalamic-pituitary-adrenal axis regulates the synthesis and secretion of the adrenal corticosteroids, which influence numerous processes in the central nervous system modulating mood, behavior, and neuroendocrine function (11–13). Corticosteroids exert their action by binding to two types of intracellular corticosteroid receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (14, 15). Upon hormone binding, the receptor translocates to the nucleus and can activate transcription by binding to specific glucocorticoid response elements (GREs) in the regulatory region of target genes. Because of the high degree of homology in the DNA binding domain, MR and GR can interact with the same target DNA sequence (16). Of the two receptors, MR exhibits the highest affinity for corticosteroids, whereas GR mediates a higher stimulation of transcription (17). Besides activation of transcription, GR has been described to repress transcription of genes either via negative GREs or via protein-protein interactions with transcription factors such as AP-1 and NF-κB (18–20).

Both autoradiographic and immunohistochemical studies indicate that the hippocampus contains particularly high concentrations of both MR and GR compared with other brain regions (21, 22). Several studies with adrenalectomized rats have shown a suppressive effect of exogenous corticosteroids on 5-HT1A receptor mRNA expression in the hippocampus (23, 24). However, little is known about the molecular mechanism underlying this effect.

To investigate the mechanism by which corticosteroids repress rat 5-HT1A receptor expression, we functionally analyzed the promoter region of the rat 5-HT1A receptor gene in COS-1 cells and P19 embryonal carcinoma (EC) cells. Evidence is provided that two NF-κB elements in the rat 5-HT1A receptor promoter contribute to induced transcription of the rat 5-HT1A receptor gene. Furthermore, corticosteroids are shown to repress this NF-κB-mediated induction of transcription. Remarkably, we observed that only GR and not MR is able to mediate this repressive effect of corticosteroids. Our results suggest that repression of NF-κB activity by GR may provide a mechanism underlying the negative action of corticosteroids on 5-HT1A receptor activity.

**EXPERIMENTAL PROCEDURES**

*Special Reagents—Dexamethasone, D-aldosterone, and hydrocortisone were obtained from Sigma.

*Cell Culture—Monkey COS-1 cells were obtained from American
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Type Culture Collection (Manassas, VA). Mouse EC cells, P19S1801A1 were cultured as described before (25). Both cell lines were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium (Life Technologies, Inc.), buffered with bicarbonate, and supplemented with 7.5% FCS. CVI-b cells were grown in DMEM (Life Technologies, Inc.), buffered with bicarbonate, and supplemented with 5% FCS. Dextran-coated charcoal-FCS was prepared by treatment of FCS with dextran-coated charcoal to remove steroids, as described previously (26).

Plasmids—The construct −1588luc containing 1588 base pairs of the rat 5-HT1A receptor 5′-flanking region was described previously (38). This −1588 base pairs Xba/I/BgII was cloned into pGL3 digested with NheI/BgII. Subsequent deletion constructs were obtained using restriction digests at specific sites in the promoter; −1388luc was created by digestion of −1588luc with HpaII/BgII and ligation into pGL3 digested with Smal/BgII; −901luc was created by partial digestion of −1588luc with StyI, filling-in and ligation into pGL3 digested with SalI, redigestion with HindIII, and religation; −880luc was created by digestion of −1588luc with KpnI/SalII, filling-in, and religation; −122luc was created by digestion of −1588luc with BamHI/BgII and ligation into pGL3 digested with SalI; −81luc was created by digestion of −1588luc with StyI, filling-in, and digestion with BgII and ligation into pGL3 digested with Smal/BgII; and −1588luc<sup>901−1322</sup> was created by partial digestion of −1588luc with StyI and religation. −901 365Mluc and −901 64Mluc were constructed by introducing point mutations into the original promoter constructs by site-directed mutagenesis using the oligonucleotides 5′-gcagggagttctgcaaggt-3′ and 5′-agcttcttggaaattccggagc-3′, respectively. 901 365/64Mluc was created by digestion of −901 64Mluc with SalCI/HindIII and ligation into −901 365Mluc digested with SalCI/HindIII. The reporter plasmid 2×GREKluc was described elsewhere (28). The CMV4 expression vectors containing full-length cDNAs encoding human p65 (RelA), p50 (NF-κB1), and GR have been described before (29). The CMV4 expression vector containing full-length cDNA encoding human MR was made by digestion of a kind gift from Evans (San Diego CA), with AvaI, filling-in, digesting with AclI, ligated into AclI/EcoRI-digested pBluescript SK−. Additionally, SK hMR was digested with KpnI/SmalI, and the fragment was ligated into CMV4 digested with KpnI/SmalI.

Transient Transfections—For transient transfections, COS-1 cells and P19 cells were cultured in 24-well plates in DMEM and Ham’s F-12 medium. DMEM and Ham’s F-12 medium supplemented with 5% dextran-coated charcoal-FCS for COS-1 cells and 7.5% dextran-coated charcoal-FCS for P19 cells was used when MR function was studied. Cells were transfected using calcium-phosphate coprecipitation with transfections, including two potential NF-κB sites from the 5HT1AR promoter (48). However, these proximal promoter fragments showed a 1588luc containing 1588 base pairs of the 5-HT1A receptor 5′-flanking region, was used with mRNA from unstimulated CV1-b cells or a 1:1 mixture of DMEM and Ham’s F-12 medium (Life Technologies, Inc.), buffered with bicarbonate, and supplemented with 10% FCS. Dextran-coated charcoal-FCS was prepared by treatment of FCS with dextran-coated charcoal to remove steroids, as described previously (26).

Electrophoretic Mobility Shift Assay—COS-1 cells were grown in 10-cm dishes and transfected as described above with 20 μg of expression plasmid and 10 μg of both plasmids when combinations were used. Whole cell extracts were prepared in Dignam C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and incubated for 30 min at 4 °C, and membranes were pelleted. Protein concentration of the supernatant was determined by the Bio-Rad protein assay according to the manufacturer’s protocol. Double-stranded oligonucleotides containing the NF-κB sites from the HIV long terminal repeat (5′-agcttaggaggggtaattggccg-3′), the intercellular adhesion molecule-1 promoter (5′-agctttgcgaattgcccggagc-3′), the 5HT1AR promoter (−365, 5′-agcttaggaggggtaattggccg-3′), and the mutated NF-κB sites from the 5HT1AR promoter (−365M, 5′-agcttaggaggggtaattggccg-3′; −65, 5′-agcttaggaggggtaattggccg-3′) were labeled with [α<sup>32P</sup>]dCTP using the Klenow fragment of DNA polymerase I. Whole cell extracts (5 μg/assay) were incubated with 15,000 cpm of probe (0.1–0.5 ng) and 2 μg of poly(dI-dC) for 30 min at room temperature in a total reaction mixture of 20 μl containing 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 μg/μl bovine serum albumin. Samples were loaded on a 5% polyacrylamide (29:1) gel containing 0.25 × Tris borate, EDTA, pH 8.0, as running buffer, and the gel was run at room temperature at 150 V for 2–3.5 h. After electrophoresis, gels were dried and autoradiographed for 1 day at −80 °C.

RESULTS

Activation of the 5-HT1A Receptor Promoter by NF-κB—Although previous studies have shown that addition of corticosteroids to adrenocorticotized rats resulted in suppression of 5-HT1A receptor mRNA expression in the hippocampus (23, 24), the mechanism underlying this effect remained largely unknown. To elucidate the molecular mechanism by which corticosteroids repress 5-HT1A receptor expression, we functionally analyzed the promoter region of the rat 5-HT1A receptor gene. Sequence analysis of the 1.6-kb promoter did not reveal any GREs. However, sequence analysis did reveal potential response elements for other known transcription factors, including two potential NF-κB elements, located at −365 and −64 in the promoter. Activation of NF-κB in the brain, in response to a variety of stimuli, has been reported (31), and we examined whether NF-κB transcription factors were able to influence the transcriptional activity of the 5-HT1A receptor promoter.

To investigate the effect of NF-κB, we transiently transfected COS-1 cells and P19 EC cells with several reporter constructs, containing a variety of 5-HT1A promoter deletions (Fig. IA), in combination with expression vectors encoding the p50 and p65 subunits of NF-κB. As shown in Fig. 1 (B and C), NF-κB was clearly capable of inducing luciferase activity of the −1588 promoter construct in both cell lines. Furthermore, deletion of the region between −122 and −81 resulted in complete abolishment of the NF-κB effect (−81 luc and −1588<sup>901−1322</sup>luc). Deletion of the distal region of the promoter from −1588 to −122 resulted in promoter fragments, which possessed stronger NF-κB-induced activity when transfected into COS-1 cells (Fig. 1D). However, these proximal promoter fragments showed less induction by NF-κB when transfected into P19 EC cells (Fig. 1C). This result suggests that the upstream region contains cis-acting DNA sequences that specifically repress transcription depending on the cell line used.

The 5-HT1A receptor promoter is a TATA-less promoter, and multiple transcription initiation sites have been found in the mouse and human 5-HT1A receptor promoter (32). To determine the transcription initiation sites in the rat promoter and to investigate whether increased reporter activity actually reflects increased 5-HT1A receptor promoter-directed transcription, primer extension analysis was carried out. A primer directed at the chloramphenicol acetyltransferase coding sequence, just downstream of the 5-HT1A receptor 5′-flanking region, was used with mRNA from unstimulated CVI-b cells or
mRNA from CV1-b cells stimulated with expression vector encoding p65. In the unstimulated condition, two bands of equal intensity were found representing major transcription initiation sites around position −90 and −105 (Fig. 2, lane 1). After stimulation with p65, the lower band (−90) significantly increased in intensity suggesting recruitment of the transcription machinery specifically at this site (Fig. 2, lane 2). This site is located just upstream of the nearby NF-κB binding site at position −64. Longer exposure of the film revealed multiple minor bands (not shown).

To further examine the importance of the NF-κB elements in the regulation of the 5-HT1A receptor promoter, we specifically mutated both NF-κB elements in the promoter. To investigate the effect, we transfected COS-1 cells and P19 EC cells with promoter constructs, containing either one or two mutated NF-κB elements (Fig. 3A) in combination with empty expression vectors encoding the p50 and p65 subunits of NF-κB. Deletion of the induction of luciferase activity evoked by NF-κB over cells transfected with empty expression vector. Bars represent the means of at least three independent experiments ± S.D. C, P19 EC cells were transfected as under B.

**Fig. 1.** Transcriptional activation of the 5-HT1A receptor promoter by NF-κB. A, schematic representation of luciferase (luc) reporter constructs used, containing rat 5-HT1A receptor (5-HT1AR) promoter deletions. The open circles represent potential NF-κB binding sites. B, COS-1 cells were transiently transfected with the different reporter constructs as indicated in combination with empty expression vector or expression vectors encoding the p50 and p65 subunits of NF-κB. Depicted is the induction of luciferase activity evoked by NF-κB over cells transfected with empty expression vector. Bars represent the means of at least three independent experiments ± S.D. C, P19 EC cells were transfected as under B.

**Fig. 2.** NF-κB selectively induces transcription initiation of the 5-HT1A receptor around position −90. CV1-b cells were transiently transfected with the −1588 reporter in combination with empty expression vector (lane 1) or expression vectors encoding the p65 subunits of NF-κB (lane 2). Total RNA was isolated, and primer extension was performed with a primer directed at base pairs 52–17 downstream of the 5-HT1A receptor 5′-flanking region. Two major start sites are indicated that represent initiation around position −90 and −105. M, size markers.

**Fig. 3.** Importance of κB elements in 5-HT1A receptor promoter regulation. A, schematic representation of luciferase (luc) reporter constructs used in this experiment, containing wild type or mutated κB elements. The open circles represent potential NF-κB binding sites. The open circles with × represent mutated κB elements. B, COS-1 cells were transiently transfected with the different reporter constructs as indicated in combination with empty expression vector or expression vectors encoding the p50 and p65 subunits of NF-κB. Depicted is the induction of luciferase activity evoked by NF-κB over cells transfected with empty expression vector. Bars represent the means of at least three independent experiments ± S.D. C, P19 EC cells were transfected as under B. wt, wild type.
κB elements from the HIV long terminal repeat and the intercellular adhesion molecule-1 promoter (Fig. 4B). As described before (33), the κB element in the HIV long terminal repeat preferentially bound p50 homodimers (I) and p50/p65 heterodimers (II) and more weakly p65 homodimers (III), whereas the κB element in the intercellular adhesion molecule-1 promoter preferentially bound p65 homodimers (III) and more weakly p50 homodimers (I) and p50/p65 heterodimers (II). Together these data indicate that specific NF-κB complexes are able to bind to the κB elements in the 5-HT1A receptor promoter.

Repression of NF-κB-induced 5-HT1A Receptor Promoter Activity by Corticosteroids—Previous studies have shown that GR is able to repress transcription of target genes via protein-protein interactions with other transcription factors, including NF-κB. To examine the effect of corticosteroids on NF-κB-induced activity of the 5-HT1A receptor promoter, we transfected COS-1 cells and P19 EC cells with three different reporter constructs, containing 5-HT1A promoter deletions (−1588 luc, −901 luc, and −208 luc; see Fig. 1A) in combination with expression vectors encoding the p50 and p65 subunits of NF-κB and GR. As shown in Fig. 5A, the NF-κB-induced activity of all three promoter constructs could be repressed by GR after treatment of COS-1 cells with dexamethasone. Comparison of the promoter constructs showed that the repression varied from 40% when −1588 luc and −901 luc were used to 65% when −208 luc was used. In P19 EC cells (Fig. 5B), the NF-κB-induced activity of both −1588 luc and −901 luc was also repressed hormone-dependently by GR to approximately 55%, whereas the activity of the −208 luc construct was not affected by GR. This was due to a low level of NF-κB activity in the absence of GR.

Because corticosteroids are known to exert their action not only via GR but also via MR, we examined whether MR was also able to mediate this repressive effect of corticosteroids. We transfected COS-1 cells and P19 EC cells with the 5-HT1A promoter construct in combination with expression vectors encoding the p50 and p65 subunits of NF-κB and GR or MR. Although treatment of the cells with dexamethasone or hydrocortisone in the case of GR resulted in repression of NF-κB-

Fig. 4. Differential binding of NF-κB subunits to the κB elements in the 5-HT1A receptor promoter. NF-κB subunits, p50 and p65, were overexpressed alone or in combinations in COS-1 cells. A, subsequently, whole cell extracts (WCE) were analyzed by EMSA with 32P-labeled probes containing the κB elements form the 5-HT1A receptor promoter (−365 and −64). Specific complexes (I, II, and III) are indicated. B, whole cell extracts were analyzed by EMSA with 32P-labeled probes containing the κB elements form the HIV long terminal repeat (LTR) or the intercellular adhesion molecule-1 promoter (ICAM-1). Specific complexes (I, II, and III) are indicated.

Fig. 5. Corticosteroids repress NF-κB-induced 5-HT1A receptor promoter activity. A, COS-1 cells were transiently transfected with the different reporter constructs as indicated in combination with empty expression vector or expression vectors encoding the p50 and p65 subunits of NF-κB and expression vector encoding GR. Cells were either untreated (black bars) or treated with 0.1 μM dexamethasone (dex; hatched bars) for 24 h. Depicted is the induction of luciferase activity evoked by NF-κB over cells transfected with empty expression vector. Bars represent the means of at least three independent experiments ± S.D. B, P19 EC cells were transfected as under A.

κB complexes, EMSA was also performed with probes containing

II, seen as an additional complex with intermediate mobility, could also be observed. In contrast, the −64 κB element showed only efficient binding of p50 homodimers (I), whereas weak binding of p50/p65 heterodimers (II) could be observed when cell extract containing p65 was used. This specific p50/p65 heterodimer is probably formed with endogenous p50 from COS-1 cells. No binding of p65 homodimers could be observed with this −64 κB element. Both mutated κB elements (−365M and −64M) as used earlier in the promoter studies were unable to bind any p50- or p65-containing complex (results not shown).

As a control for binding and mobility of the specific κB complexes, EMSA was also performed with probes containing
induced promoter activity, treatment of the cells with aldosterone or hydrocortisone in the case of MR had no effect on the promoter activity in both cell lines (Fig. 6, A and B). In a control experiment, MR was able to activate transcription from a GRE-containing reporter construct after treatment of the cells with ligand, although the transcriptional activity of MR was found to be less strong than that of GR (results not shown), a phenomenon that has been described earlier (17). Taken together, our results indicate that repression of NF-κB activity by GR could be the mechanism by which corticosteroids suppress rat 5-HT1A receptor expression in the brain.

DISCUSSION

To unravel the mechanism underlying the negative action of corticosteroids on serotonin signaling, we investigated the promoter region of the rat 5-HT1A receptor gene. Previous studies in rats showed that the 5-HT1A receptor gene is negatively regulated by corticosteroids (23, 24). Because the gene does not contain obvious negative GREs, this negative regulation may be the result of protein-protein interactions of the corticosteroid receptors with other transcription factors. This negative action of GR via direct protein-protein interaction with other transcription factors, such as NF-κB and AP-1, is thought to play a pivotal role in the anti-inflammatory action of glucocorticoids (18–20).

In the present study, we show that NF-κB binding sites in the rat 5-HT1A receptor promoter region contribute to induced transcription of the gene, whereas corticosteroids can repress this NF-κB-mediated induction of transcription via GR. Mutation analysis showed the importance of two NF-κB binding sites in activation of the 5-HT1A receptor promoter. Mutation of either one of the two elements resulted in a 50% decrease in NF-κB-induced promoter activity. These data indicate that there is no synergism between the two NF-κB elements but just an additive effect. The fact that the κB element at −64 is conserved between mouse and rat and the element at −365 is conserved between mouse, rat, and human (32) already suggests the importance of these elements in the regulation of the 5-HT1A receptor gene. Functional binding of NF-κB subunits was determined by EMSA, and it was shown that the −64 κB element preferentially bound p50 dimers, whereas p50/p65 heterodimers preferentially bound to the −365 κB element. These differences in affinity for NF-κB subunits were consistent with binding characteristics as predicted for these sequences, based on binding of NF-κB subunits to artificial κB oligonucleotides (34).

Promoter deletion analysis revealed the presence of several important regulatory regions in the rat 5-HT1A receptor promoter. The region between −122 and −81 was shown to be essential for NF-κB-induced activation of transcription. Although the −81 luc construct still contained an NF-κB binding site, transcriptional activity was completely abolished. In both the human and mouse 5′-flanking sequence, RNA 5′ end mapping experiments demonstrated the existence of numerous transcription start sites (32). Primer extension analysis of the rat 5-HT1A receptor promoter showed the presence of two major start sites that represent initiation around −90 and −105. Both sites were equally used for basal transcription. Stimulation with p65 specifically induced initiation around position −90. This site is located just upstream of the NF-κB binding site located at −64 and absent from the inactive −81 luc reporter construct, suggesting recruitment of the transcription machinery selectively at this site located around −90.

Because this arrangement of start sites is conserved between mouse and human, it is also likely to have some functional significance. The presence of several transcription initiation sites may provide a mechanism for differential control of 5-HT1A receptor transcription in different cell types and brain regions. Furthermore, deletion of the distal part of the promoter from −1588 to −122 resulted in promoter constructs that were more active in COS-1 cells and less active in P19 EC cells. This could be due to the presence of cis-acting DNA sequences in the upstream region that repress transcription specifically in COS-1 cells, suggesting that a different complement of transcription factors is present in P19 EC cells as compared with COS-1 cells.

In both cell lines, GR was shown to repress NF-κB-induced transcription of the 5-HT1A receptor after addition of hormone. Both the synthetic glucocorticoid, dexamethasone, and a naturally occurring corticosteroid, hydrocortisone, were able to mediate this effect of GR. In contrast, MR, the other corticosteroid receptor, important for activity in the brain, was unable to repress this NF-κB-induced transcription after addition of the mineralocorticoid, aldosterone, or hydrocortisone. In previous studies, similar results have been obtained on repression of AP-1 activity by both receptors. Under conditions in which GR represses AP-1-stimulated transcription, MR was inactive (35, 36). Although both receptors can bind to the same target DNA sequence (GRE), only GR has been found to negatively regulate transcription via protein-protein interactions with other transcription factors. These data suggest that negative cross-talk...
between GR and NF-κB may provide a basis for the molecular mechanism underlying the negative action of corticosteroids in the brain.

Besides the 5-HT1A receptor and the corticosteroid receptors, GR and MR, NF-κB has also been found to be expressed in the brain, particularly in cortex and hippocampus (37). As in the periphery, the target genes for NF-κB in brain also encode proteins involved in inflammation. However, new evidence is accumulating that NF-κB also plays a specific role in the brain in processes such as neuronal plasticity and neurodegeneration (31). In addition, it has been shown that expression of the NF-κB target gene, interleukin-1β, is increased during long-term potentiation of synaptic transmission, a process thought to underlie certain forms of learning and memory (27). Obviously, the exact role of NF-κB in brain function related to serotonin needs to be further examined.

Because removal of circulating corticosteroids by adenalec- tomy causes up-regulation of 5-HT1A receptor expression and addition of exogenous corticosteroids suppresses 5-HT1A receptor expression, it seems clear that changes in hypothalamic-pituitary-adrenal axis function can result in dysregulation of the serotonergic system. Controlling hippocampal 5-HT1A recep- tor expression is one way by which corticosteroids may act to decrease sensitivity to 5-HT in the brain. Based on the role of 5-HT1A receptors in the feedback regulation of the 5-HT system, we hypothesize that 5-HT1A receptors may be down-regulated in depression and therefore display an inadequate ability to convert binding of 5-HT to its receptors to an adequate physiological response.

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