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Selective allosteric ligand activation of the retinoid X receptor heterodimers of NGFI-B and Nurr1

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Abbreviations:
AF-2, activation function-2
9CRA, 9-*cis* retinoic acid
DR, direct repeat
FXR, farnesoid X receptor
H, helix
HEK, human embryonic kidney
IR, inverted repeat
LBD, ligand-binding domain
LXR, liver X receptor
NBRE, NGFI-B response element
PPAR, peroxisome proliferator-activated receptor
RAR, retinoic acid receptor
RXR, retinoid X receptor
TR, thyroid hormone receptor

ABSTRACT

NGFI-B, an orphan member of the NR4A subfamily of the nuclear receptors, recognizes specific sequences in the promoters of neuronal target genes as a monomer. Although NGFI-B also forms a heterodimer with the retinoid X receptor (RXR), a receptor for 9-*cis* retinoic acid (9CRA), endogenous targets of the heterodimer have not been identified. We investigated the role of RXR ligand binding in NGFI-B/RXR activation and found that dibenzodiazepine-derived ligands, such as the weak RXR agonist HX600, selectively activate NGFI-B/RXR heterodimers. HX600 also activated the heterodimer formed by RXR and Nurr1, another NR4A subfamily receptor. In an assembly assay that detects ligand-dependent reconstruction of the ligand-binding domain, HX600 and not 9CRA induced an allosteric ligand effect on NGFI-B through RXRα binding. The data indicate that the RXR heterodimers of NGFI-B and Nurr1 are selectively activated by the RXR ligand HX600, and that compounds such as HX600 will be valuable tools in investigating NGFI-B and Nurr1 function.

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1. Introduction

Nuclear receptors are ligand-dependent transcription factors that regulate many biological processes including cell growth and differentiation, embryonic development, and metabolic homeostasis [1,2]. The transcriptional activity of nuclear receptors is modulated by ligands such as steroids, retinoids, and other lipid-soluble compounds. Although 48 human nuclear receptors have been identified, one-third have no characterized ligands and are referred to as orphan nuclear receptors. Retinoid X receptor α (RXRα; NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3), which are receptors for 9-*cis* retinoic acid (9CRA), mediate retinoid signaling as both homodimers and as heterodimers with other nuclear receptors, including Vitamin D receptor (NR1I1), thyroid hormone receptor (TR; NR1A), retinoic acid receptor (RAR; NR1B), peroxisome proliferator-activated receptor (PPAR; NR1C), liver X receptors (LXRα; NR1H3 and LXRβ; NR1H2), and farnesoid X receptor (FXR; NR1H4), as well as the orphan receptor NGFI-B (NR4A1; also known as NGFI-Bα, Nur77, TR3, and NAK-1) [3]. Although several lines of evidence indicate that RXRs play an important role in the regulation of multiple nuclear receptor signaling pathways [4], an understanding of allosteric mechanisms which enable RXR ligand signaling in RXR heterodimers is only recently emerging [5].

NGFI-B belongs to the NR4A subgroup of orphan nuclear receptors which also includes Nurr1 (NR4A2; also known as NGFI-Bβ, RNR-1, TINUR, and HZF-3), NOR-1 (NR4A3; also known as NGFI-Bγ and MINOR), and the *Drosophila* ortholog DHR38 (NR4A4). NGFI-B was originally identified as an immediate-early gene that is rapidly induced by serum and growth factors such as nerve growth factor [6,7]. NGFI-B and its related family members are highly expressed in the adult nervous system. NGFI-B is also expressed in the adrenal, thyroid, muscle, lung, and prostate [8] and is upregulated in T cells undergoing apoptosis [9]. Nurr1 is expressed in the adult liver, pituitary gland, thymus, and osteoblasts, while NOR-1 is expressed at high levels in the pituitary gland and at low levels in the thymus, kidney, heart, skeletal muscle, and adrenal glands [8]. NGFI-B subfamily members have been shown to bind to a *cis*-acting sequence the NGFI-B response element (NBRE), AAAGGTCA, as monomers [10]. In addition, NGFI-B and Nurr1, but not NOR-1, can form heterodimers with RXR on an AGGTCA consensus sequence direct repeat spaced by five nucleotides (DR-5) and can activate transcription in promoter assays in a 9CRA-dependent manner [11,12]. NR4A subfamily receptors have been shown to regulate the expression of genes in the hypothalamic-pituitary-adrenal axis [8]. NBREs are identified in the promoter regions of the pituitary proopiomelanocortin gene [13], adrenal steroid 17-hydroxylase, steroid 21-hydroxylase, 20α-hydroxysteroid dehydrogenase, aldosterone synthase, and 3β-hydroxysteroid dehydrogenase type 2 genes [14–18], as well as neuronal tyrosine hydroxylse and the dopamine transporter gene [19,20]. Despite this progress, endogenous target genes of the NGFI-B/RXR and Nurr1/RXR heterodimers have not been identified. In addition to its genomic actions, NGFI-B was reported to regulate apoptosis in a transcription-independent manner [21]. Since NGFI-B null mice have no discernible phenotype and display no abnormalities in hypothalamic regulation, pituitary function, adrenal steroidogenesis [22], and in thymic and peripheral T cell death [23], functional redundancy appears to exist among NR4A nuclear receptors. On the other hand, mice lacking Nurr1 fail to generate midbrain dopaminergic neurons [24]. NOR1-deficient mice were reported to exhibit abnormal ear and hippocampus development and have a defect in gastrulation [25,26].

In this study, we show that dibenzodiazepine-derived RXR ligands are selective activators of the NGFI-B/RXR and Nurr1/RXR heterodimers and identify inter-dimer allosteric signaling as a key molecular mechanism of action. Interestingly, the dibenzodiazepine agonist HX600 activates NGFI-B/RXR through allosteric properties distinct from that of 9CRA. The data indicate that the RXR ligands engage allosteric signals in the NGFI-B/RXR and Nurr1/RXR heterodimers and that dibenzodiazepine-derived RXR ligand may be useful in revealing previously unknown physiological roles of the NGFI-B/RXR and Nurr1/RXR heterodimers.

2. Materials and methods

2.1. Chemical compounds

Dibenzodiazepine derivatives shown in Fig. 1 were synthesized as reported previously [27] and 9CRA was purchased from Wako (Osaka, Japan).

2.2. Plasmids

Fragments of rat NGFI-B (UniProt Accession no. P22829), human RXRα (GenBank Accession no. NM_002957), RXRβ (GenBank Accession no. NM_021976), RXRγ (GenBank Accession no. NM_006917), LXRα (GenBank Accession no. NM_005693), LXRβ (GenBank Accession no. NM_007121), FXR (GenBank Accession no. NM_005123), and mouse Nurr1 (GenBank Accession no. S53744) were inserted into pCMX vector to make pCMX-NGFI-B, pCMX-RXRα, pCMX-RXRβ, pCMX-RXRγ, pCMX-LXRα, pCMX-LXRβ, pCMX-FXR, and pCMX-Nurr1, respectively, as reported previously [28]. The ligand-binding domains (LBDs) of human RXRα, RXRβ, RXRγ, NOR1 (GenBank Accession no. NM_006981), FXR, TRβ (GenBank Accession no. NM_000461), RARα (GenBank Accession no. NM_000964), rat NGFI-B, mouse Nurr1, PPARγ (GenBank Accession no. NM_011146), and *Drosophila* DHR38 (GenBank Accession no. X89246) were inserted into pCMX-GAL4 vector to make pCMX-GAL4-RXRα, pCMX-GAL4-RXRβ, pCMX-GAL4-RXRγ, pCMX-GAL4-NOR1, pCMX-GAL4-FXR, pCMX-GAL4-TRβ, pCMX-GAL4-RARα, pCMX-GAL4-NGFI-B, pCMX-GAL4-Nurr1, pCMX-GAL4-PPARγ, and pCMX-GAL4-DHR38, respectively. A full-length RXRα cDNA was inserted into the pCMX-VP16 vector to make pCMX-VP16-RXRα. GAL4-responsive MH100(UAS)x4-tk-LUC, NGFI-B- and Nurr1-responsive NX3′x3-tk-LUC, and LXR- and FXR-responsive IR1x3-tk-LUC reporters were utilized [12,28]. The NGFI-B amino acid 1–574 fragment, 353–398 fragment, and the RXRα amino acid 1–448 fragment, and 222–249 fragment were inserted into pCMX-GAL4 to generate pCMX-GAL4-NGFI-B-dAF-2, pCMX-GAL4-NGFI-B (H1), pCMX-GAL4-RXRα-dAF-2, and pCMX-GAL4-RXRα (H1), respectively. The NGFI-B amino acid 399–597 fragment and the RXRα amino acid 250–462 fragment were inserted into pCMX-VP16 to generate pCMX-VP16-NGFI-B (H3–H12) and pCMX-VP16-RXR (H3–H12), respectively.

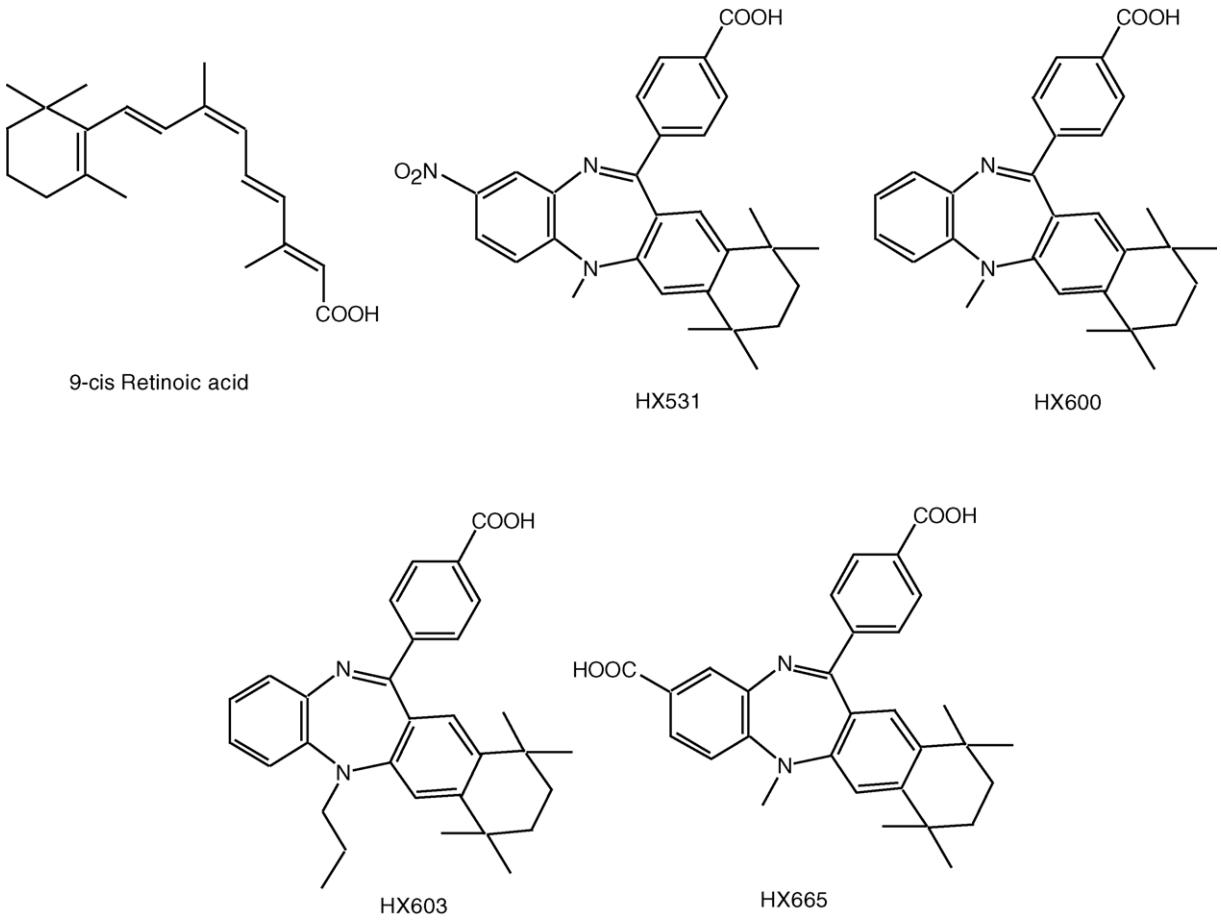

Fig. 1 – Chemical structure of 9-*cis* retinoic acid and the dibenzodiazepines (HX531, HX600, HX603, and HX665).

2.3. *Cell culture and cotransfection assay*

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and antibiotic–antimycotic (Nacalai, Kyoto) at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were performed by the calcium phosphate coprecipitation assay as described previously [28]. Eight hours after transfection, test compounds were added. Cells were harvested after 16–24 h for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Cotransfection experiments used 50 ng of reporter plasmid, 20 ng of pCMX-β-galactosidase, 15 ng of each receptor expression plasmid, and pGEM carrier DNA to give 150 ng of DNA per well of a 96-well plate. Luciferase data were normalized to an internal β-galactosidase control and represent the mean ± S.D. of triplicate assays.

3. Results

3.1. *Selective activation of NGFI-B/RXR heterodimers by dibenzodiazepines*

The effects of dibenzodiazepines (Fig. 1) on RXRα, RXRβ, and RXRγ transactivation were examined using the GAL4-chimeric receptor assay. Because the GAL4-responsive element luciferase reporter is activated only by a GAL4-chimera receptor, the potentially confounding effects of endogenous receptors are eliminated. While 9CRA effectively activated RXRα, RXRβ, and RXRγ, HX600 was a weak RXR agonist and HX531, HX603, and HX665 did not induce RXR transactivation (Fig. 2A). Transactivation of GAL4-RXRγ by 9CRA was weaker than that of GAL4-RXRα and GAL4-RXRβ as reported previously [29], although the mechanism remains unknown. The ability of the dibenzodiazepines to inhibit GAL4-RXRα response to effective dose of 9CRA was examined. Addition of 1 μM HX603 inhibited the RXRα activity induced by 100 nM 9CRA, while treatment with HX531, HX600, and HX665 had no effect (Fig. 2B). These findings indicate that HX600 and HX603 are a weak agonist and an antagonist for RXR, respectively. Next, the possibility that the dibenzodiazepines might affect the activity of NGFI-B/RXR heterodimers was tested using a GAL4-NGFI-B chimera receptor. The GAL4-NGFI-B chimera receptor was not activated by 9CRA or the benzodiazepines (Fig. 2C). Since NGFI-B forms a heterodimer with RXR and can be activated by rexinoids [12], we cotransfected an RXRα, RXRβ, or RXRγ expression vector with GAL4-NGFI-B. As expected, 9CRA effectively activated the GAL-NGFI-B/RXRα heterodimer. Interestingly, although HX600 was a very weak RXRα agonist, this compound effectively activated the GAL4-NGFI-B/RXRα heterodimer (Fig. 2C). HX600 (1 μM) and 9CRA (1 μM), which

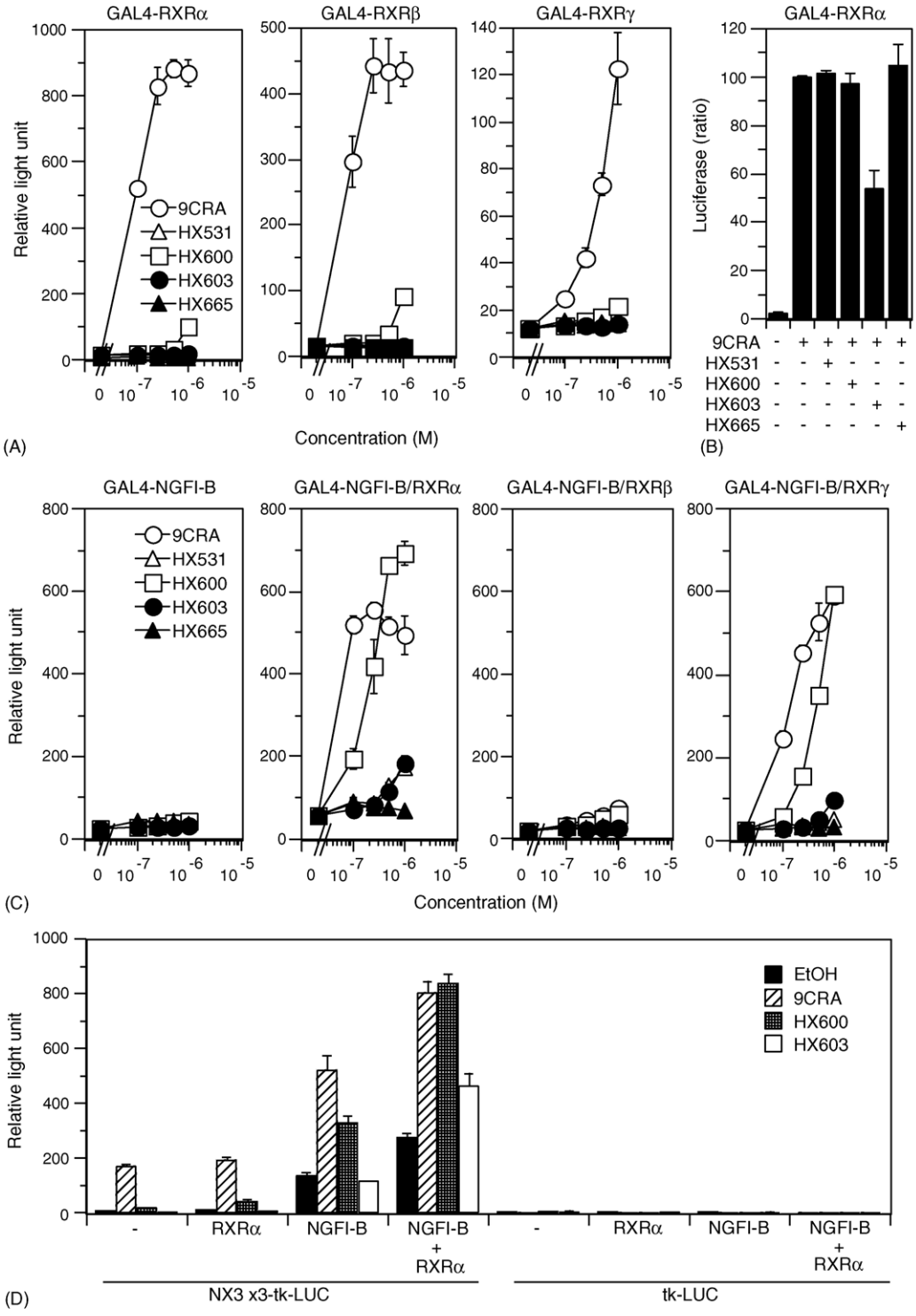

Fig. 2 – Dibenzodiazepines activate NGFI-B/RXR heterodimers. (A) Effect of dibenzodiazepine treatment on RXR activity. HEK293 cells were cotransfected with CMX-GAL4-RXRα, GAL4-RXRβ, or GAL4-RXRγ and the MH100(UAS)x4-tk-LUC reporter, and were treated with 9CRA or dibenzodiazepines at a range of concentrations. (B) Inhibition of 9CRA-induced RXRα activity by dibenzodiazepines. HEK293 cells were cotransfected with CMX-GAL4-RXRα and MH100(UAS)x4-tk-LUC and were treated with 100 nM 9CRA in combination with 1 μM HX531, HX600, HX603, or HX665. (C) Activation of NGFI-B/RXR heterodimers by dibenzodiazepines. HEK293 cells were transfected with CMX-GAL4-NGFI-B, in combination with control CMX vector, CMX-RXRα, CMX-RXRβ, or CMX-RXRγ, and MH100(UAS)x4-tk-LUC and were treated with a dose range of test compounds. (D) Effect of 9CRA, HX600, and HX603 treatment on full-length NGFI-B/RXRα heterodimer activation of a DR-5 element-containing reporter. HEK293 cells were cotransfected with CMX-NGFI-B, CMX-RXRα, and NX3′x3-tk-LUC or control reporter tk-LUC, and were treated with ethanol (EtOH), 1 μM 9CRA, HX600, or HX603.

activated GAL4-RXRα 8.1- and 71-fold, respectively (Fig. 2A), increased the GAL4-NGFI-B/RXRα activity 12- and 8.5-fold, respectively (Fig. 2C). HX531, which had no effect on RXRα, and HX603, a RXRα antagonist, activated the NGFI-B/RXRα heterodimer to a lesser extent than HX600. While the effect of HX600 on GAL4-NGFI-B/RXRβ is very weak, HX600 effectively induced the GAL4-NGFI-B/RXRγ activation. The weak NGFI-B/RXRβ activation by the ligands may be due to the weak responsiveness of RXRβ isotype [30]. The N-terminal region of RXRβ was reported to be involved in this limited transactivation activity [31]. This is consistent with the finding that GAL4-RXRβ chimera receptor, which DNA binding domain was replaced with that of GAL4, was effectively activated by 9CRA (Fig. 2A). Although the activity of HX600 on GAL4-NGFI-B/RXRα, GAL4-NGFI-B/RXRβ, and GAL4-NGFI-B/RXRγ was weaker than that of 9CRA, HX600 (1 μM), which produced only partial agonistic activity on GAL4-RXRs (Fig. 2A), activated the GAL4-NGFI-B/RXRs as effectively as 9CRA (1 μM) (Fig. 2C). These data indicate the dibenzodiazepines, especially HX600, selectively induce the activation of NGFI-B/RXR heterodimers.

Although an endogenous target gene of NGFI-B/RXR has not been identified, the heterodimer was reported to bind to an artificial DR-5 element [12]. We cotransfected a full-length NGFI-B expression plasmid and/or an RXRα plasmid with a DR-5-containing luciferase reporter, NX3′x3-tk-LUC, and assayed the effects of treatment with 9CRA, HX600, and HX603. Reporter luciferase activity was slightly induced by 9CRA in the absence of cotransfection of RXR or NGFI-B (Fig. 2D). Since HEK293 cells express endogenous receptors, this effect is likely due to RAR/RXR heterodimer binding to the DR-5 element [12]. Cotransfection of RXR had little effect on luciferase activity. In the absence of NGFI-B cotransfection, the effect of HX600 was negligible. While cotransfection of NGFI-B increased the luciferase activity induced by both 9CRA and HX600, transcriptional activation by HX600 was weaker than that of 9CRA (Fig. 2D). With cotransfection of NGFI-B and RXRα, promoter activation by HX600 and 9CRA were comparable, and HX603 activation was detected. A control luciferase reporter lacking the DR-5 element was not activated in any of the conditions tested. Thus, dibenzodiazepines such as HX600 are potent activators of the full-length NGFI-B/RXRα heterodimer.

3.2. *Activation of Nurr1/RXRα heterodimer by HX600*

The effect of HX600 and HX603 on the Nurr1/RXRα heterodimer was examined. Closely related to NGFI-B, Nurr1 is another NR4A subgroup nuclear receptor that forms heterodimers with RXR. 9CRA, HX600, and HX603 were able to activate the GAL4-Nurr1 receptor even in the absence of cotransfected RXRα (Fig. 3A). The interaction of endogenous RXR with GAL4-Nurr1 may be sufficient to allow activation by these compounds. Cotransfection of RXRα augmented GAL4-Nurr1/RXR response to HX600 and HX603. Interestingly, at 1 μM concentration, HX600 and HX603 activated the GAL4-Nurr1/RXRα heterodimer more effectively than 9CRA. We examined the effects of 9CRA and HX600 at concentrations ranging from 100 nM to 1 μM on GAL4-Nurr1 activation in the absence or presence of RXRα cotransfection. The GAL4-Nurr1 was activated by HX600 more efficiently than 9CRA in the absence of RXRα cotransfection, and the RXRα addition augmented the ligand-inducible GAL4-Nurr1/RXRα transactivation (Fig. 3B). HX600 also induced the activation of full-length Nurr1 and RXRα on the NX3′x3-tk-LUC reporter (Fig. 3C). These findings indicate that HX600 is also potent on Nurr1/RXRα heterodimer.

We next examined the effects of HX600 on other nuclear receptors, including NR4A subfamily receptors NOR1 (NR4A3) and DHR38 (NR4A4), by transfecting GAL4-chimeric receptors with RXRα. 9CRA and HX600 did not activate NOR1 or DHR38 (Fig. 3D). This may be due to inability of NOR1 to form RXR heterodimer [11] and requirement of cell type-specific cofactor complex for *Drosophila*-derived DHR38 [32]. While 9CRA (1 μM) activated FXR/RXRα 3.5-fold and PPARγ/RXRα 114-fold, HX600 did not induce the transactivation of these heterodimers. Because TRβ/RXRα is not permissive to RXR ligand stimulation [5], this heterodimer did not respond to 9CRA and HX600. RARα/RXRα heterodimer was activated by 9CRA, because this compound can bind to RARα as well as RXRα [33]. HX600 had no activity on the RARα/RXRα heterodimer. LXRα and LXRβ form permissive RXR heterodimers which can be activated by RXR ligands as well as FXR [5]. We examined the effect of RXR ligands on GAL4-LXRα/RXRα and GAL4-LXRβ/RXRα, but these heterodimers were not activated by the RXR ligands (data not shown). The RXR ligand stimulation may induce RXR homodimerization rather than heterodimer activation in this system. We next examined the effect of 9CRA and HX600 on full-length LXRα, LXRβ, and FXR in RXR heterodimer. While the natural RXR ligand 9CRA activated LXRα/RXRα, LXRβ/RXRα, and FXR/RXRα heterodimers on a luciferase reporter containing an inverted repeat-1 (IR-1) element, HX600 did not induce their activation (Fig. 3E). These findings indicate that the dibenzodiazepine HX600 preferentially activates RXR heterodimers formed by the NR4A receptors NGFI-B and Nurr1.

3.3. *The allosteric network in the NGFI-B/RXR heterodimer is activated by HX600*

Since high-resolution crystal structures reveal that the NGFI-B family of nuclear receptors lack a ligand-binding cavity [32,34], ligand-inducible activation of NGFI-B/RXR is mediated by ligand binding to RXR. We examined whether the effect of HX600 on the NGFI-B/RXRα heterodimer is mediated by binding to RXRα with a competition assay using the RXR antagonist PA452 [35]. Addition of PA452 inhibited GAL4-RXRα transactivation induced by 9CRA and HX600 (Fig. 4A). This is consistent with the previous finding that HX600 directly binds to RXRα [36]. PA452 also repressed the activity of GAL4-NGFI-B/RXRα induced by 9CRA, HX600, and HX603 in a concentration-dependent manner (Fig. 4B). These data suggest that activation of the NGFI-B/RXRα heterodimer by dibenzodiazepines is mediated by direct RXR interaction. Inhibition of 9CRA-induced GAL4-NGFI-B/RXRα activity by PA452 was weaker than that of GAL4-RXRα and PA452 efficiently repressed GAL4-NGFI-B/RXRα activation by HX600 and HX603, suggesting that ligands exert different allosteric effects in RXRα homodimers and NGFI-B/RXRα heterodimers. Next, we examined the combined effect of 9CRA and HX600 at low concentrations on GAL4-NGFI-B/RXRα activation. HX600 at 10 and 100 nM had no and only slight activity on the GAL4-NGFI-B/RXRα,

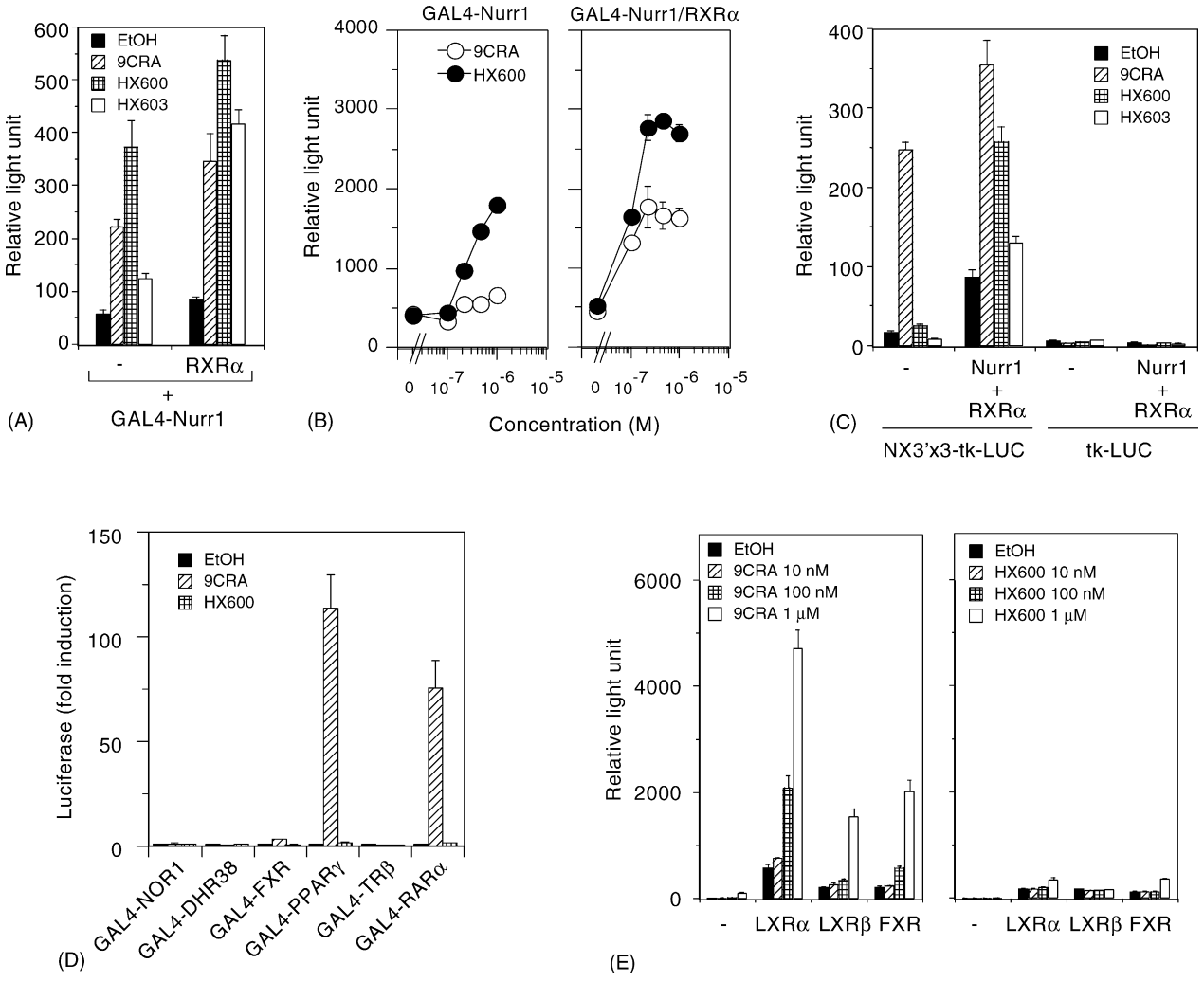

Fig. 3 – HX600 and HX603 effectively activate the Nurr1/RXR heterodimer but not other RXR heterodimers. (A) Activation of GAL4-Nurr1/RXRα by HX600 and HX603. HEK293 cells were cotransfected with CMX-GAL4-Nurr1 with or without CMX-RXRα and MH100(UAS)x4-tk-LUC and were treated with ethanol (EtOH), 1 μM 9CRA, HX600, or HX603. (B) Concentration-dependent activation of GAL4-Nurr1/RXRα by HX600. HEK293 cells were cotransfected as in (A) and treated with several concentrations of 9CRA and HX600. (C) Activation of full-length Nurr1/RXRα heterodimer by HX600 and HX603. HEK293 cells were cotransfected with CMX-Nurr1 or CMX vector, CMX-RXRα and NX3′x3-tk-LUC or control reporter tk-LUC and were treated with ethanol (EtOH), 1 μM 9CRA, HX600, or HX603. (D) Effect of 9CRA and HX600 on NOR1, DHR38, FXR, PPARγ, TRβ, and RARα. HEK293 cells were cotransfected with GAL4-NOR1, GAL4-DHR38, GAL4-FXR, GAL4-PPARγ, GAL4-TRβ, or GAL4-RARα, CMX-RXRα and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), 1 μM 9CRA, or HX600. (E) Effects of 9CRA and HX600 on full-length LXRα, LXRβ, and FXR. HEK293 cells were cotransfected with control CMX vector, CMX-LXRα, CMX-LXRβ or CMX-FXR, and IR1x3-tk-LUC and were treated with several concentrations of 9CRA or HX600.

respectively (Fig. 4C). Interestingly, HX600 at these low concentrations enhanced the effect of 9CRA at 0.1 and 1 nM on GAL4-NGFI-B/RXRα, which was little activated by 9CRA at these low concentrations. The effect of 9CRA and HX600 on NGFI-B/RXRα heterodimerization was examined by using a mammalian two-hybrid assay as reported previously [37]. While VP16-RXRα did not interact with the control GAL4 protein, it formed heterodimer with GAL4-NGFI-B (Fig. 4D). 9CRA decreased the heterodimerization, because this compound may induce RXR homodimerization although it can activate NGFI-B/RXRα heterodimer. On the other hand, HX600 slightly increased the interaction between NGFI-B and RXRα. These findings also suggest a different allosteric effect of HX600 from 9CRA. Upon ligand binding, nuclear receptors undergo a conformational change that induces C-terminal activation function-2 (AF-2)-dependent recruitment of coactivators [38]. X-ray crystallographic analyses reveal that NGFI-B family receptors have an atypical AF-2 helix and lack a conventional coactivator-binding site [32]. We examined whether the AF-2 domains of both RXRα and NGFI-B are required for NGFI-B/RXRα activation by 9CRA and the dibenzodiazepines. Deletion of the RXRα AF-2 domain resulted in a complete loss of ligand activation (Fig. 4E), as reported previously [39]. The effect of RXR ligands on the heterodimer formed by NGFI-B and the RXRα AF-2 deletion mutant was examined. 9CRA, HX600, and HX603 activated this

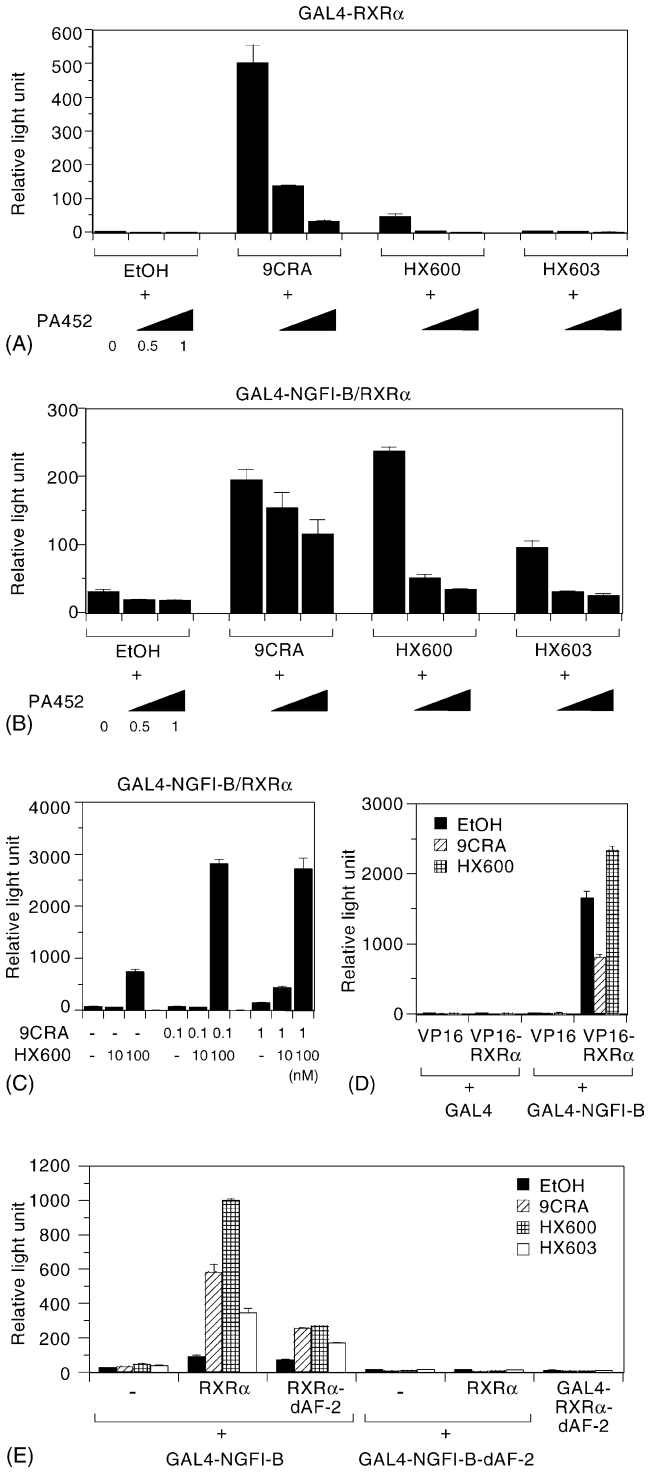

Fig. 4 – Effects of HX600 and HX603 are mediated by RXRα but do not require the RXRα AF-2 domain. Inhibition of GAL4-RXRα (A) and GAL4-NGFI-B/RXRα (B) transcription activated by 9CRA, HX600, or HX603 by the RXR antagonist PA452. HEK293 cells were cotransfected with CMX-GAL4-RXRα (A) or CMX-GAL4-NGFI-B/RXRα and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), 500 nM 9CRA, HX600, or 1 μM HX603 in combination with 0, 0.5, or 1 μM PA452. (C) Combined effect of low concentrations of 9CRA and HX600 on GAL4-NGFI-B/RXRα. HEK293 cells were cotransfected as in (B) and were treated with 9CRA (0, 0.1, or 1 nM) plus HX600 (0, 10, or 100 nM). (D) Effect of 9CRA and HX600 on NGFI-B/RXRα heterodimerization. HEK293 cells were cotransfected with control CMX-VP16 vector or CMX-VP16-RXRα, control CMX-GAL4 vector or CMX-GAL4-NGFI-B and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), 1 μM 9CRA, or HX600. (E) The activation of NGFI-B/RXR requires the NGFI-B AF-2 domain, but not the RXRα AF-2 domain. HEK293 cells were cotransfected with CMX-GAL4-NGFI-B in combination with control CMX vector, CMX-RXRα or CMX-RXRα-dAF-2, and CMX-GAL4-NGFI-B-dAF-2 in combination with control CMX vector, CMX-RXRα, or CMX-GAL4-RXRα-dAF-2 and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), 1 μM 9CRA, HX600, or HX603.

heterodimer, although induction was decreased compared to that of the wild-type heterodimer (Fig. 4E). These ligands had no effect on a NGFI-B AF-2 deletion mutant transfected alone or the presence of RXRα. We assayed the interaction of cofactors (steroid receptor coactivator-1, nuclear receptor corepressor, and silencing mediator for retinoid and thyroid hormone receptors) and NGFI-B/RXRα heterodimer in the mammalian two-hybrid assay using GAL4-cofactor chimeras having nuclear receptor interacting domains and VP16-NGFI-B chimeric receptor, and did not observe ligand-dependent association (data not shown). These findings indicate that the AF-2 domain of NGFI-B, and lesser that of RXRα, is required for activation of the NGFI-B/RXR heterodimer by RXR ligands.

In addition to an allosteric effect on the positioning of helix (H) 12, ligand binding is also known to induce a conformational change that results in stabilization of the LBD. This ligand-dependent structural rearrangement can be detected by assaying the interaction between the isolated H1 domain and an engineered receptor lacking H1 [40]. The H1 fragments of NGFI-B and RXRα were fused to GAL4 DNA-binding domain, and the LBDs lacking H1 were fused to the transactivation domain of the herpesvirus VP16 protein [34,40]. 9CRA strongly induced the assembly of the H1 and H3–H12 fragments of the RXRα LBD as reported previously [40]. The weak RXR agonist HX600 and the RXR antagonist HX603 also induced the association of H1 with the remainder of the LBD (Fig. 5), indicating that this assay can detect conformational changes induced by both agonists and antagonists. The H1 of NGFI-B interacted weakly with the H3–H12 fragment of NGFI-B, and addition of RXR ligands did not affect the association. Cotransfection of full-length RXRα increased the assembly of the NGFI-B LBD H1 and H3–H12 in the absence of ligand. Surprisingly, although the addition of 9CRA did not stabilize the interaction, the dibenzodiazepines HX600 and HX603 strongly induced reconstitution of the NGFI-B LBD (Fig. 5). These data reveal that HX600 and HX603, but not 9CRA, exhibit an allosteric effect on NGFI-B through RXRα binding.

4. Discussion

In this study, we identified the dibenzodiazepine HX600 as a selective activator of the NGFI-B/RXR and Nurr1/RXR heterodimers. Although the RXR antagonist PA452 effectively

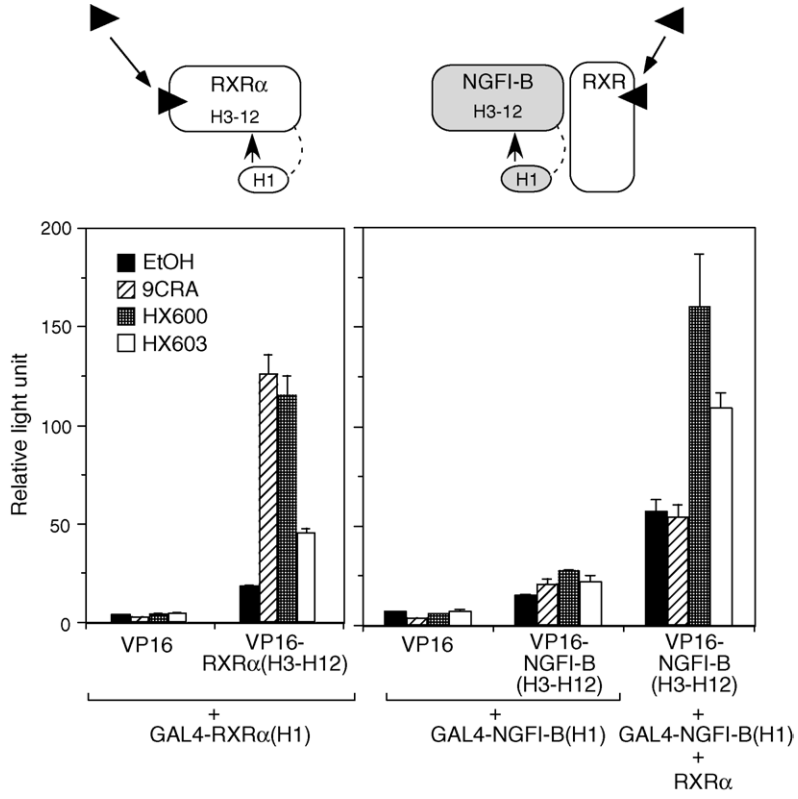

Fig. 5 – HX600 and HX603 allosterically modulate NGFI-B through RXRα. To investigate allosteric ligand effect in RXR heterodimers, we utilized the LBD H1/H3–H12 assembly assay. HEK293 cells were cotransfected with CMX-GAL4-RXRα (H1) in combination with CMX-VP16 or CMX-VP16-RXRα (H3–H12), or CMX-NGFI-B (H1) in combination with CMX-VP16 or CMX-VP16-NGFI-B (H3–H12), CMX-GAL4-NGFI-B (H1) in combination with CMX-VP16-NGFI-B (H3–H12) plus CMX-RXRα, and were treated with ethanol (EtOH), 1 μM 9CRA, HX600, or HX603.

inhibited the GAL4-RXRα activity induced by 9CRA, its effect on 9CRA-induced GAL4-NGFI-B/RXRα transactivation was weak (Fig. 4A and B). Interestingly, PA452 still had effective inhibitory activity on HX600-induced GAL4-NGFI-B/RXRα. Combination of low concentrations of 9CRA and HX600 exhibited a synergistic effect on NGFI-B/RXRα activation as shown in Fig. 4C. Fig. 4D shows that the effect of 9CRA and HX600 on NGFI-B/RXRα heterodimerization was different. These findings suggest that HX600 interacts with RXRα in a different allosteric manner from 9CRA. The assembly assay shown in Fig. 5 reveals that, unlike 9CRA, HX600 and HX603 increased the association of the isolated H1 fragment with the remainder of the NGFI-B LBD, indicating that these dibenzodiazepine RXR ligands induce inter-dimer allosteric communication from RXRα to NGFI-B. Recently, the anticancer drug 6-mercaptopurine was reported be an activator of Nurr1 and NOR1 [41,42]. Activation by 6-mercaptopurine is not mediated by RXR, since NOR1 does not heterodimerize with RXR and 6-mercaptopurine action is dependent on the N-terminal region of NOR1. X-ray crystal structures of the LBDs of NGFI-B, Nurr1, and its *Drosophila* homolog DHR38 reveal that NR4A subfamily receptors lack a cavity for ligand binding and a conventional coactivator-binding site, indicating that their transactivation activity is not directly regulated by ligands [32,34,43]. HX600 directly binds to RXRα, RXRβ, and RXRγ with K_i values of 1.9, 0.64, and 1.0 μM, respectively [36]. These findings indicate that the effect of HX600 on the transactivation of NGFI-B/RXRα heterodimer is mediated by RXRα stimulation.

The dibenzodiazepines, including HX600, were reported to enhance the activity of RAR/RXR heterodimer activated by RAR ligands, although these compound alone did not activate RARs [36]. HX600 and a RARα-selective agonist Am80 synergistically induced the differentiation of leukemia HL-60 cells. The dibenzodiazepines also enhanced the PPARγ/RXR activity stimulated by a PPARγ ligand [44]. Fig. 3D shows that HX600 alone did not induce the activation of RARα/RXRα and PPARγ/RXRα heterodimers, indicating that the activation of these heterodimers by HX600 requires ligand-stimulated partner receptors. NGFI-B/RXR and Nurr1/RXR heterodimers are activated by the dibenzodiazepine RXR ligands alone. The unique co-regulator surfaces of NGFI-B and Nurr1 may contribute to increased sensitivity of the NGFI-B/RXR and Nurr1/RXR heterodimers to the dibenzodiazepines [43,45]. The dibenzodiazepine HX531 was reported to be an RXR antagonist selectively inhibiting PPARγ/RXR heterodimer [46]. HX531 weakly activated NGFI-B/RXRα heterodimer (Fig. 2C). Thus, the dibenzodiazepine RXR ligands affect RXR heterodimers differently from the natural ligand 9CRA. HX600, HX531, HX603, and HX665 have a common dibenzodiazepine skeleton (Fig. 1). HX600 is a potent activator of NGFI-B/RXR, HX531 and HX603 are weak, and HX665 is not effective. The

structure–activity relationship of the benzodiazepines should be further examined.

We examined the effect of 9CRA and HX600 on the expression of tyrosine hydroxylase gene, a Nurr1 target, in neuronal PC12 cells, but these RXR ligands did not increase its expression (data not shown). Nurr1 was reported to bind a NBRE in the tyrosine hydroxylase promoter in an RXR-independent manner [19]. Several lines of evidence suggest that NGFI-B and Nurr1 function as heterodimers with RXR in vivo, although endogenous target genes for these heterodimers remain unknown. DHR38 forms heterodimers with USP, the *Drosophila* RXR homolog, and responds to ecdysteroids in the epidermis and fat body through an unknown molecular mechanisms [32]. RXR ligands increase the number of dopaminergic cells and other neurons in the rodent embryonic central nervous system in a process mediated by Nurr1-RXR heterodimers [47]. Suppression of haloperidol-induced dyskinesia in mice treated with RXR ligands, including HX531, required the expression of NGFI-B [48]. As shown in Fig. 2, although HX600 is a more potent agonist, HX531 can activate NGFI-B/RXR heterodimers. Recently, mutations in the Nurr1 gene were found to be associated with familial Parkinson's disease [49]. These findings suggest that NGFI-B and Nurr1 function are regulated by RXR ligand signaling in vivo and that selective ligands such as HX600 will be useful tools in investigating the pathophysiological roles of NFGI-B family nuclear receptors and in the development of novel therapeutics.

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