Circadian repressors CRY1 and CRY2 broadly interact with nuclear receptors and modulate transcriptional activity

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Nuclear hormone receptors (NRs) regulate physiology by sensing lipophilic ligands and adapting cellular transcription appropriately. A growing understanding of the impact of circadian clocks on mammalian transcription has sparked interest in the interregulation of transcriptional programs. Mammalian clocks are based on a transcriptional feedback loop featuring the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1), and transcriptional repressors cryptochrome (CRY) and period (PER). CRY1 and CRY2 bind independently of other core clock factors to many genomic sites, which are enriched for NR recognition motifs. Here we report that CRY1/2 serve as corepressors for many NRs, indicating a new facet of circadian control of NR-mediated regulation of metabolism and physiology, and specifically contribute to diurnal modulation of drug metabolism.

nuclear hormone receptor | cryptochrome | circadian | corepressor | xenobiotic metabolism

Nuclear receptors (NRs) regulate transcription in response to lipophilic ligands (1). Most NRs contain four domains: The N-terminal activation function 1 (AF1) is followed by a highly conserved DNA binding domain (DBD), which is connected to the ligand binding domain (LBD) via a flexible hinge region. The LBD contains the hydrophobic ligand binding pocket and activation function 2 (AF2) located in helix 12 (H12). Structural rearrangement of H12 is crucial for ligand-dependent transactivation. Regulation of transcription by NRs depends on interaction with corepressors, such as nuclear receptor corepressor (NCoR) or silencing mediator for retinoid or thyroid-hormone receptors (SMRT), and coactivators, such as peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) or steroid receptor coactivators. Steroid hormone receptors are enriched in the cytoplasm in the absence of ligand. Upon agonist ligand binding, they translocate to the nucleus and bind DNA. Recruitment of coactivators then allows transcription to occur. Conversely, nonsteroid-binding NRs are bound to DNA and corepressors in the absence of ligand. Binding of agonist ligand displaces corepressors in favor of coactivators, allowing transcription initiation.

Mammalian circadian clocks synchronize physiology and metabolism with daily environmental changes. The mammalian clock is based on a transcription–translation feedback loop featuring the transcription factors circadian locomotor output cycles kaput (CLOCK), brain and muscle ARNT-like 1 (BMAL1), cryptochrome (CRY), and period (PER) (2). Forming a heterodimer, CLOCK and BMAL1 drive transcription of target genes, including those encoding their own repressors period (PER1, PER2, PER3) and cryptochrome (CRY1, CRY2). PER and CRY dimerize and repress CLOCK and BMAL1, allowing the cycle to repeat. This core clock directly or indirectly drives oscillating transcription of ≈43% of all protein coding genes (3).

NRs are intimately connected to the circadian clock and its function in adapting daily metabolic outputs to the 24-h day/night cycle. The NRs REV-ERBα/β and RORα/γ are key regulators of core clock function (4), and many NRs are rhythmically expressed (5). PER, CRY, and CLOCK can regulate NRs by diverse mechanisms (6–9). These and indirect effects, like rhythmic abundance of endogenous ligands and the rhythmic transcription of coactivators and corepressors, convey time-of-day information to NR-regulated pathways, such as lipid, glucose, and xenobiotic metabolism (4).

Upon binding of a xenobiotic ligand, the NRs pregnane X receptor (PXR) and constitutive androstane receptor (CAR) induce expression of proteins required for xenobiotic detoxification. Xenobiotic metabolism is subject to time-of-day–dependent regulation: in humans, the half-life of CYP3A substrates is shortest in the afternoon (10); in rodents, the lethal toxicity of a fixed dose of a drug depends on the time of administration (11). The circadian transcriptome (6, 7) and proteome (8) are enriched for components of xenobiotic detoxification pathways. Here we report a comprehensive survey of CRY–NR interactions and enhanced metabolism of the anesthetic ketamine in CRY-deficient mice.

Results

CRY1 Interacts with Many NRs. We examined the interaction of all mouse NRs with mouse CRY1 using coimmunoprecipitation (co-IP) (Fig. 1A). Approximately one-third of mouse NRs consistently interact with CRY1, whereas another third is weakly or variably associated with CRY1 (Fig. 1B). The strongest interactors include steroid hormone receptors, lipid-sensing peroxisome
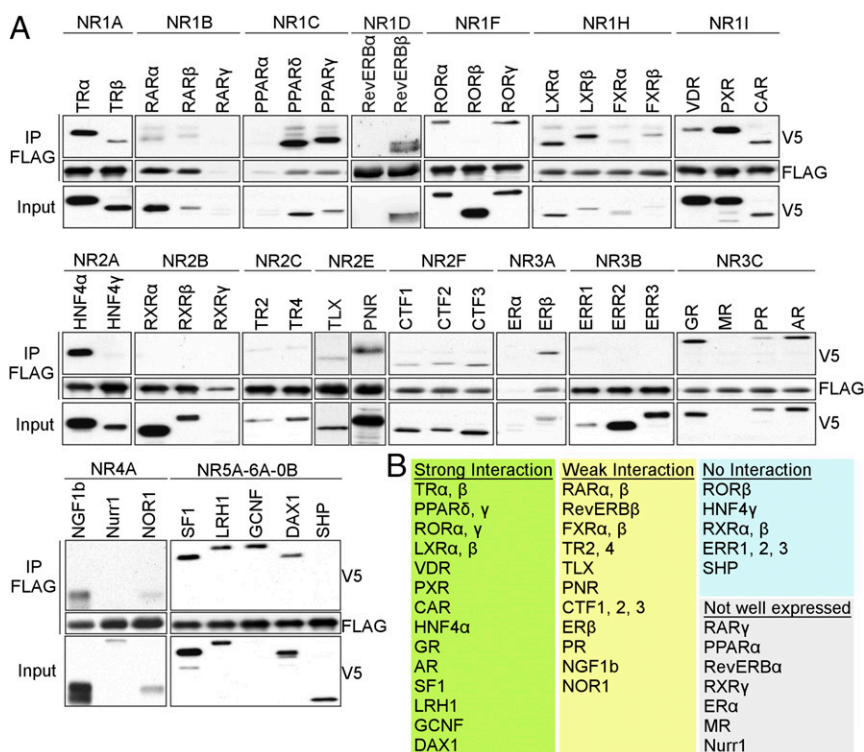
Fig. 1. CRY1 interacts with a subset of nuclear receptors. (*A*) Co-IP of FLAG-CRY1 with V5-NRs transiently expressed in HEK293T cells. (*B*) Table listing strongly or weakly interacting NRs, and NRs that were not or only poorly expressed.

proliferator-activated receptors (PPARs), vitamin D receptor (VDR), and the xenobiotic receptors PXR and CAR.

CRY1 and CRY2 bind many genomic sites independent of other clock proteins, and these unique sites were enriched for NR-binding motifs (9). Using these previously reported datasets to perform motif analysis for CRY1 and CRY2 genomic binding sites, we detected consensus sites of liver-expressed NRs, including hepatocyte nuclear factor 4-α (HNF4α), PPAR, Rev-ERB, farnesoid X receptor (FXR), and retinoic acid receptor (RAR) (Datasets S1 and S2). Comparing CRY1 and CRY2 genomic binding sites with those published for glucocorticoid receptor (GR) (12), Rev-ERBα, Rev-ERBβ (13), and PPARα (14) (Fig. 2*A*), we detected overlap of CRY1 and CRY2 binding with up to 37% of NR binding sites (Fig. 2*B*). These sites include *Pck1* (GR) (Datasets S3 and S4), confirming previous reports (15), *Pdk4* (PPARα) (Dataset S5), consistent with our finding that CRYs regulate PPARδ and *Pdk4* in muscle (16), and *Bmal1* (Rev-ERBα, Rev-ERBβ) (Datasets S6 and S7), suggesting that CRYs could contribute to *Bmal1* transcriptional regulation.

Many NRs display increased affinity for CRY2 compared with CRY1, allowing us to use CRY1/2 hybrid constructs (17) (Fig. 3*A*) to identify the domains required for preferential interaction. Co-IP of CRY hybrids with PXR and CAR revealed that the A and B domains, which correspond to the photolyase homology region (PHR), as well as the D domain, which mostly consists of the divergent C-terminal tail, contribute to the interactions (Fig. 3 *B* and *C*). We identified a helix on the surface of CRY2 in which three exposed amino acids differ from CRY1. Serine 394, valine 396, and arginine 397 are located near the rim of the secondary pocket of CRY2 (Fig. 3*E*). CRY2$^{S394E, V396M, R397K}$ (amino acids as in CRY1, hereafter denoted CRY2*) represses BMAL1:CLOCK-driven luciferase expression (Fig. 3*D*), indicating that these mutations did not prevent proper protein folding. Each of these mutations decreases the interaction of CRY2 with PXR or CAR, and CRY2* interacts with them like CRY1 (Fig. 3*E*), suggesting that this region is important for interaction.

CRYs Exhibit Many Characteristics of NR Corepressors. CRY1 and CRY2 are transcriptional repressors within the core molecular clock. Our results and those of others (9, 15) suggest that CRYs may function independently of other core clock proteins to regulate NR-driven transcription. NR corepressors are recruited to the LBD, through a conserved hydrophobic motif, the corepressor NR box (CoRNR box), comprising I/L-X-X-I/V-I sequences (18, 19). Corepressors dissociate following a conformational change of H12 caused by agonist ligand binding (20). PXR, because of its crucial role in drug metabolism, has been extensively studied structurally. Potent and specific synthetic agonist ligands are available, making PXR a prime candidate to explore the biochemical features of the interaction with CRY.

To determine whether the LBD of PXR is sufficient for the interaction with CRYs, we performed co-IP of full-length CRY1 and CRY2 with full-length (FL) PXR or the PXR LBD, and observed that the LBD is sufficient for interaction (Fig. 4*A*). Furthermore, recombinant CRY2 (amino acids 1–512) interacts directly with recombinant PXR LBD (Fig. 4*B*). However, the affinity of the interaction is weak, possibly as a result of CRY2 lacking the C-terminal tail, which we and others have thus far been unable to express and purify (21, 22) (Fig. 4*C*). CRYs contain an amino acid sequence that resembles a CoRNR box, in the PHR domain. However, mutating this motif does not disrupt interaction with PXR as similar mutations in NCOR and SMRT do (18, 19, 23), suggesting that it is not required for interaction of CRY with NRs (Fig. 4*D*). This finding is further supported by the 3D structures of CRY1 and CRY2 (21, 24) in which the LXXII helix is unusually short and hydrophobic residues face the core of the protein.

NR corepressors interact with unliganded or antagonist-bound receptors. Upon binding of agonist ligand, they dissociate, enabling recruitment of coactivators. Consistent with our hypothesis that CRYs are corepressors for PXR, the interaction between CRY1 or CRY2 and PXR is decreased in the presence of the PXR agonist ligand pregnenolone-16α-carbonitrile (PCN), but not CAR agonist ligand TCPOBOP (Fig. 4*E*). This occurs in a dose-dependent manner (Fig. 4*F*). Extensive structural characterization of NR LBDs suggests dynamic conformational changes upon ligand binding (25). In the apo form, the C-terminal H12 is extended away from the body of the LBD allowing corepressor binding. In the ligand bound (holo) form, H12 folds back onto the
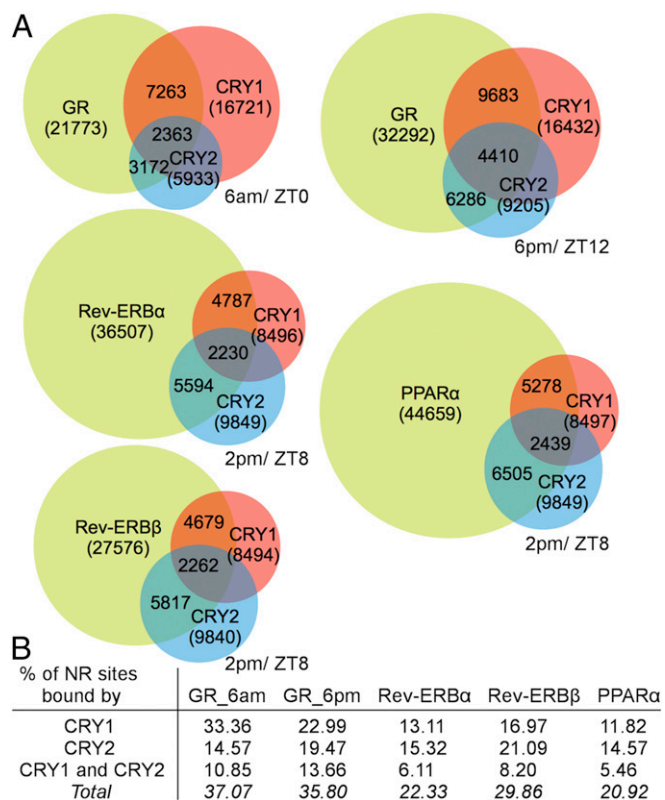
Fig. 2. CRYs and NRs co-occupy many sites in the genome. (*A*) Overlap of DNA binding sites for indicated proteins in mouse liver at matched ZT. Numbers in parentheses represent total number of binding sites for each protein. Numbers in overlapping areas indicate: (*Top*) total overlap between NR and CRY1; (*Middle*) total overlap between CRY1, CRY2, and NR; (*Bottom*) total overlap between CRY2 and NR. (*B*) Table depicting percent NR sites bound by CRY1 and CRY2.

% of NR sites bound by	GR_6am	GR_6pm	Rev-ERBα	Rev-ERBβ	PPARα
CRY1	33.36	22.99	13.11	16.97	11.82
CRY2	14.57	19.47	15.32	21.09	14.57
CRY1 and CRY2	10.85	13.66	6.11	8.20	5.46
Total	*37.07*	*35.80*	*22.33*	*29.86*	*20.92*

LBD facilitating coactivator binding. Truncation of H12 leads to constitutive corepressor binding in the presence of ligand (26). Truncation of PXR H12 prevents ligand-induced dissociation of CRY (Fig. 4*G*). Based on this characterization, CRYs strongly resemble established NR corepressors.

The Features of the PXR Interaction with CRY Are Reproduced in Those of Many NRs. We tested the impact of agonist ligands, truncation of H12, and the CRY2* mutations on the interaction of CRY2 with a subset of the most strongly interacting NRs from our screen (Fig. 1*A*). VDR, PPARδ, and HNF4α each interact preferentially with CRY2 compared with CRY1, and the CRY2* mutant recapitulates their reduced binding to CRY1 (Fig. 5*A*). GR and androgen receptor (AR), which show a lesser preference for binding CRY1 or CRY2, are less affected by the CRY2* mutation. Together, these data indicate that this region near the secondary pocket of CRYs plays an important role in the interaction between CRY1/2 and most NRs. Truncation of H12 of CAR, PPARδ, GR, and AR abolishes ligand-dependent dissociation of CRY (Fig. 5*B*). Dissociation of the interaction between VDR and CRY2 is attenuated in the absence of H12. We quantified ligand-dependent dissociation of CRY2 from PXR, CAR, VDR, PPARδ, GR, and AR for a range of doses (Fig. S1*A*). At the highest dose, the signal intensity is decreased by at least half (44% of V5-AR remains bound to CRY2). The maximum disruption is almost 90% (13% of V5-tagged VDR was detected in precipitated complexes) (Fig. S1*B*). In previous studies, we observed that the presence of the agonist GR ligand dexamethasone increased the amount of GR pulled down by CRY1 (15). Because steroid hormone receptors (like GR and

AR) translocate into the nucleus upon ligand binding, altered spatial proximity confounds interpretation of the effect of ligand on their interactions with CRYs.

CRYs Repress PXR-Driven Transcriptional Activity. We used a luciferase reporter under the control of a PXR LBD–GAL4 DBD fusion protein. CRY2 significantly represses PXR-driven luciferase expression in a dose-dependent manner (Fig. 6*A*), whereas CRY1 and CRY2* show a trend toward dose-dependent repression, consistent with the observed preferential interaction of PXR with CRY2 compared with CRY1 and CRY2*. The CRY2 mutants G351D and G354D do not repress BMAL1:CLOCK-driven transcription (27) (Fig. 6*B*). Interestingly, these mutants retain the ability to repress PXR (Fig. 6*C*). These data suggest that CRY1, and more potently CRY2, repress NR-mediated transcription through interaction with the LBD by a distinct mechanism from that underlying repression in the core circadian clock.

To study the effect of CRY1/2 on endogenous PXR and CAR, we used HepaRG cells (28) that, unlike most liver-derived cell lines, express crucial xenobiotic metabolism genes. Although we were not able to manipulate CRY expression in a manner that allowed us to determine the effects on PXR- or CAR-mediated gene transcription in HepaRG cells (Fig. S2), these cells exhibit rhythmic expression of xenobiotic genes and could be used to study the impact of circadian rhythm on drug pharmacokinetics in vitro (Fig. S3).

CRY-Deficient Mice Exhibit Reduced Anesthesia Sleep Time. Our findings suggest that CRYs could limit activation of xenobiotic receptors, thus contributing to rhythmicity of xenobiotic metabolism. To investigate whether CRY1/2 regulate PXR and CAR in vivo, we measured xenobiotic gene expression in livers of WT and $Cry1^{-/-};Cry2^{-/-}$ (dKO) mice at two times during the day. The PXR and CAR target genes *Cyp3a11*, *Cyp3a13*, and *Cyp2b10* encode drug-metabolizing enzymes (homologs of human *CYP3A4* and *CYP2B6*). Each of these transcripts was elevated in dKO mouse livers; the effect of daytime on their expression was more variable (Fig. 7*A*). *Tnfα* and *Il-6* were unaffected by the CRY genotype (Fig. S4), suggesting that increased inflammation (29) cannot explain the elevated Cyp expression. Taken together, these data suggest that the time of day, as well as CRY expression, could influence drug metabolism. Ketamine is a widely used anesthetic that is metabolized in human liver by CYP3A4, CYP2B6, and CYP2B9 (30). In mice, increased PXR and CAR activity is expected to increase expression of the *CYP3A4* and *CYP2B6* orthologs *Cyp3a11*, *Cyp3a13*, and *Cyp2b10*, leading to enhanced ketamine metabolism and reduced ketamine-induced sleep. Female dKO mice anesthetized with ketamine sleep less than their WT littermates (Fig. 7*B*). Both WT and dKO female mice wake up more quickly at zeitgeber time (ZT; hours after lights on) 16 when *Cyp* expression was higher. In male mice, we observed a significant effect of genotype but not of time. Taken together, these data support the idea that CRYs limit PXR and CAR activity to suppress xenobiotic metabolism in vivo.

Discussion

It is increasingly clear that mammalian circadian clocks coordinate metabolic physiology with predictable daily fluctuations in metabolic demand because of rhythms in the external environment (31). NRs regulate large gene networks that are critical to adjusting metabolic physiology in response to hormones, vitamins, and other lipophilic ligands (1). The notion that clock proteins function as NR coregulators is supported by the finding that CLOCK, PER2, and CRYs modify or physically interact with some NRs to regulate NR-mediated transcription (32–34). Here we demonstrate that CRY1 interacts with more than half of all mouse NRs, suggesting a widespread mechanism of circadian NR regulation. Analysis of genome-wide DNA binding revealed that CRYs and NRs co-occupy many genomic sites.
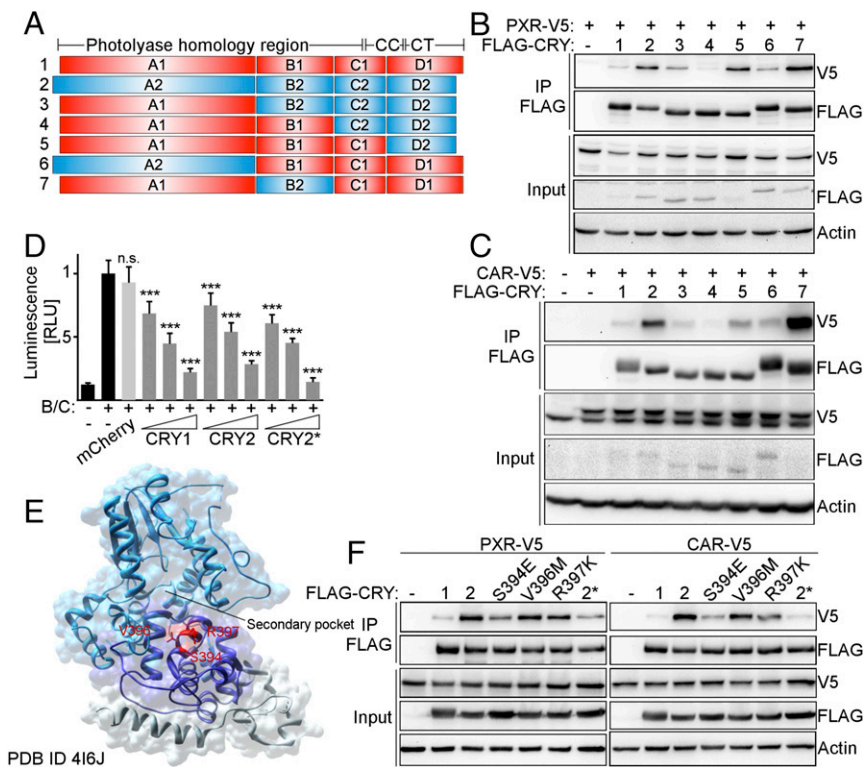
Fig. 3. The CRY2-NR interaction is disrupted by mutations near the secondary pocket of CRY2. (*A*) CRY1/2 hybrid constructs (red: CRY1; blue: CRY2). CC, coiled coil; CT, C-terminal tail. (*B* and *C*) Co-IP of FLAG-CRY hybrids with V5-PXR (*B*) and V5-CAR (*C*) from transfected cells. (*D*) Repression of BMAL1:CLOCK by CRY1, CRY2, and CRY2*. U2OS cells transiently expressed *Per2-luciferase*, *Bmal1*, *Clock*, and *Cry1*, *Cry2*, or *Cry2*. *mCherry* was used as a negative control. Luminescence was normalized to β-Galactosidase activity. (*E*) CRY2 (PDB ID code 4I6J). A, B, and C domains are light blue, dark blue, and gray, respectively. Amino acids mutated in CRY2* are red. (*F*) Co-IP of FLAG-CRY1, -CRY2, and -CRY2* with V5-PXR and -CAR from transfected cells. In *D*, data represent the mean + SD for five to six replicates per condition from one of three experiments. n.s., not significant; ***P < 0.005 vs. BMAL1:CLOCK by *t* test.

Many mechanisms contribute to circadian regulation of NR target gene expression. For example, PAR bZip transcription factors promote the rhythmic expression of *Car* and CAR targets (35). Whereas DBP, HLF, and E4BP4 may contribute to rhythmic expression of *CYP3A4* in HepG2 cells (36) and of *Mdr1* in mouse intestine (37), mechanisms facilitating rhythmic

transcription of PXR targets generally are not well understood. Our data suggest that direct repression by CRY1/2 enables circadian modulation of nonrhythmic NRs like PXR, and pose an additional avenue for circadian clock regulation of rhythmically transcribed NRs, including CAR. The mechanisms by which CRYs regulate NRs remains largely undetermined. Interestingly,
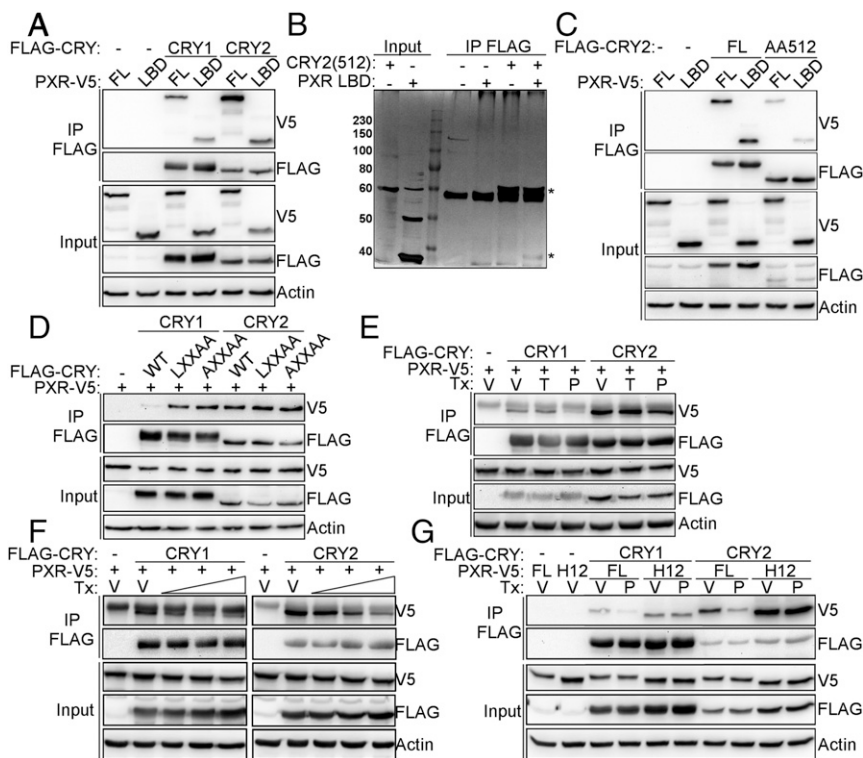

Fig. 4. CRYs exhibit many characteristics of NR corepressors. (*A* and *C*–*G*) Proteins detected by Western blot in IPs from 293T cells expressing the indicated plasmids. (*B*) Silver stain of recombinant proteins after co-IP performed in vitro. An asterisk (*) indicates CRY2(512) and PXR LBD. In *D*, CRY1/2 CoRNR box-like sequences were mutated as indicated. In *E*–*G* cells were treated overnight with (*E*) DMSO (V, vehicle), 250 nM TCPOBOP (T, CAR agonist ligand) or 10 μM PCN (P, PXR agonist ligand), or (*F*) DMSO (V, vehicle), or 2, 10, and 50 μM PCN (P, PXR agonist ligand), or (*G*) DMSO (V, vehicle) or 10 μM PCN (P, PXR agonist ligand). Tx, treatment.
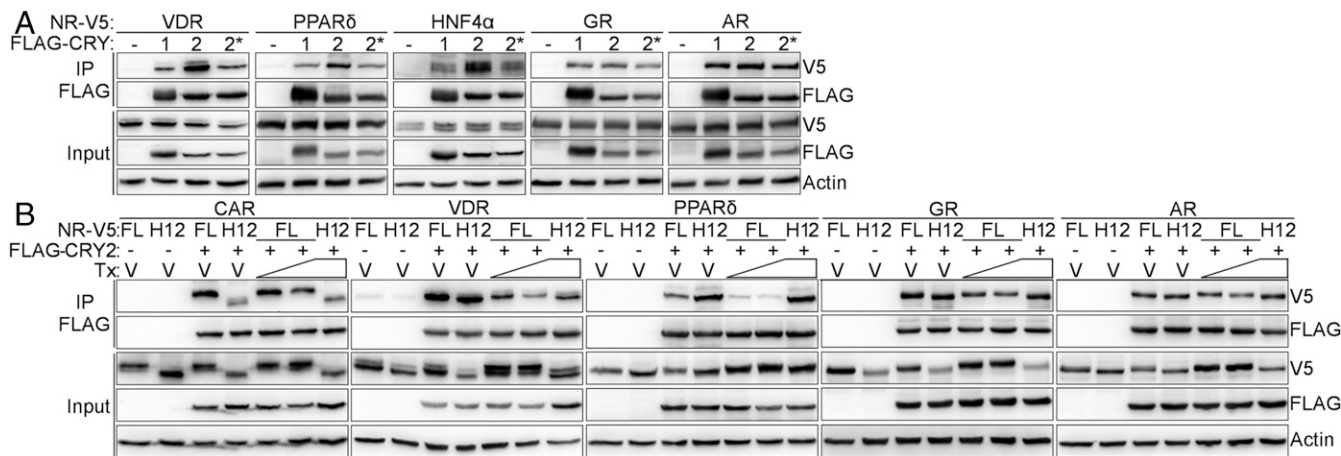
Fig. 5. The features of PXR–CRY interaction are reproduced with other NRs. (*A* and *B*) Proteins detected by Western blot in FLAG IPs from 293T cells expressing the indicated plasmids. In *B*, cells were treated with DMSO (V, vehicle) or 100/500 nM TCPOBOP (CAR agonist ligand), 10/100 nM calcipotrol (VDR agonist ligand), 0.1/1 μM GW1516 (PPARδ agonist ligand), 0.1/1 μM dexamethasone (GR agonist ligand), and 0.1/1 μM testosterone (AR agonist ligand) for 6 h in the presence of 10 μM MG132.

we found that CRYs share many characteristic biochemical features with the well-studied NR corepressors NCOR1 and SMRT, although they do not require a CoRNR box (18, 19, 23, 38) to interact with NRs.

Structurally, CRYs are completely distinct from NCOR1 and SMRT, which are large, disordered scaffolds that recruit histone-modifying enzymes like HDAC3 (39). CRYs are compact and well-ordered, with the exception of the C-terminal tail (21, 24). We identified an α-helix near the secondary pocket of CRY2 that is crucial for interaction with NRs. It was recently discovered that CLOCK interacts directly with the secondary pocket of CRY1 (40) and our results, together with that finding, suggest that this area of the CRY1/2 surface could be an important site of interaction with transcription factor targets of CRY-mediated repression more generally.

The mechanisms by which CRYs repress transcription are not fully understood (41–44). In our hands, CRY transcriptional repression of BMAL1:CLOCK is much more robust than CRY repression of PXR or GR (15), which could reflect technical limitations of this assay. Interestingly, CRY2 mutants G351D and G354D that cannot repress BMAL1:CLOCK (27) repress PXR-dependent transcription. This finding indicates that CRYs repress NRs by a distinct mechanism.

We observed a striking sex difference in ketamine-induced sleep duration. Sex differences in the metabolism of drugs have been described for many species, including humans and mice. Increased expression of drug-metabolizing enzymes (45) and increased sensitivity of PXR and CAR (46–49) in female mice, could contribute to the more pronounced time-of-day–dependent variations in ketamine clearance of female mice.
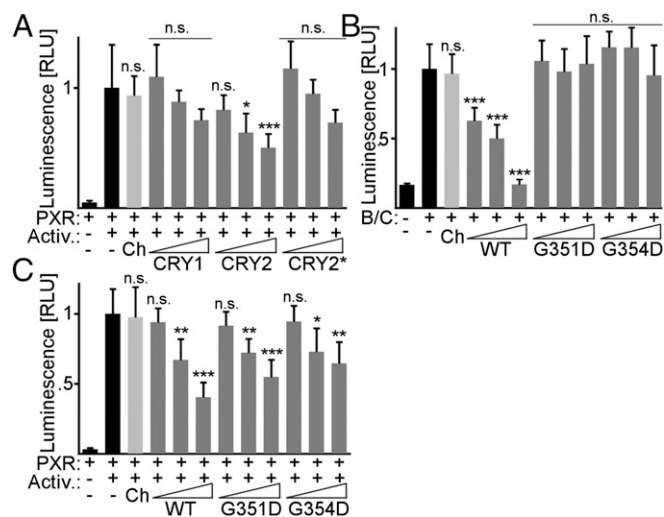

Fig. 6. CRYs repress PXR-driven transcriptional activity. (*A*) Luciferase activity in HepG2 cells expressing *Gal4-luciferase*, GAL4 DBD–PXR LBD, and 0.5, 1, or 2 ng of *CRY1*, *CRY2*, or *CRY2**. PXR was activated by *PGC1α* expression and 1 μM PCN overnight (Activ.). (*B*) Luciferase activity in U2OS cells expressing *Per2-luciferase*, *Bmal1*, *Clock*, and 10–50 pg of *Cry2* (WT), *Cry2^{G351D}* (G351D), or *Cry2^{G354D}* (G354D). (*C*) Luciferase activity as in *A* with 0.5, 1, or 2 ng of *Cry2* (WT), *Cry2^{G351D}* (G351D), or *Cry2^{G354D}* (G354D). In *A–C* mCherry (Ch) was used as a negative control and luminescence was normalized to β-galactosidase activity. Data represent the mean + SD for five to six replicates per condition from a representative of at least three experiments. n.s., not significant; RLU, relative luminescence units; *P < 0.05, **P < 0.01, ***P < 0.005 vs. PXR+activators in *A*, *C*, or BMAL1:CLOCK in *B* by *t* test.
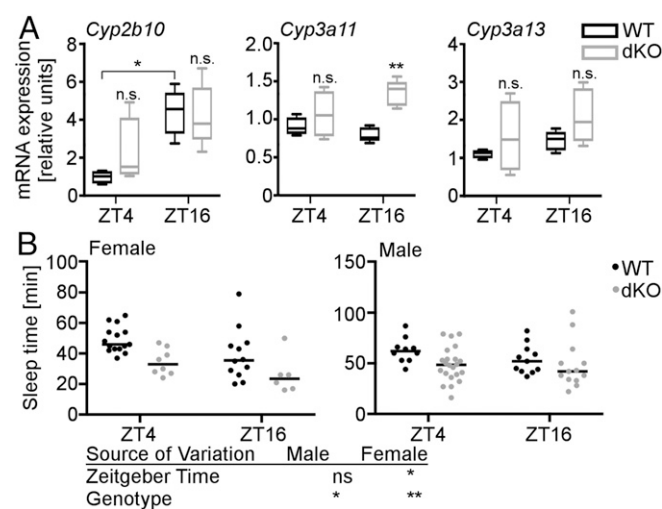

Fig. 7. CRY-deficient mice exhibit reduced anesthesia sleep time. (*A*) *Cyp2b10*, *Cyp3a11*, and *Cyp3a13* gene expression in livers from female dKO mice and WT littermates. *n* = 4–5 female animals per group. *P < 0.05, **P < 0.01, ***P < 0.005 by *t* test. (*B*) Duration of ketamine induced sleep in dKO mice and WT littermates. *n* (female) = 6–15 per genotype, *n* (male) = 10–22 per genotype. n.s., not significant; *P < 0.05, **P < 0.01 by two-way ANOVA.

Appreciation of the daily temporal regulation of physiology and metabolism is only recently emerging. Moving forward, this new dimension of CRY proteins corepressing NRs could be integrated into drug target discovery, as well as the timing and dosage regimen of existing drugs directed at NRs.

Materials and Methods

For details on co-IP, Western blotting, ChIP-seq data analysis, luciferase assays, qPCR, and ketamine sleep-time assay, please see SI Materials and Methods.

All animal care and treatments were in accordance with The Scripps Research Institute guidelines for the care and use of animals and were approved by the The Scripps Research Institute Institutional Animal Care and Use Committee under protocol #10–0019.

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