Title Page

Coordinated Regulation of Hepatic Phase-I and -II Drug Metabolizing Genes and Transporters using AhR-, CAR-, PXR-, PPARα-, and Nrf2-null mice

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Running Title Page

Running Title: Transcription Factor-Gene Interactions

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Non-Standard Abbreviations: aldehyde dehydrogenase (Aldh), aryl hydrocarbon receptor (AhR), ATP-binding cassette (Abc), constitutive androstane receptor (CAR), clofibrate (CFB), cytochrome P450 (Cyp), glutathione-*S*-transferase (Gst), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), multidrug and toxin extrusion (Mate), multidrug resistance-associated protein (Mrp), NAD(P)H:quinone oxidoreductase 1 (Nqo1), nuclear factor erythroid 2-related factor 2 (Nrf2), oltipraz (OPZ), organic anion transporting polypeptide (Oatp), peroxisome proliferator-activated receptor α (PPARα), pregnane X receptor (PXR), 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2), pregnenolone-16-alpha-carbonitrile (PCN), retinoid X receptor alpha (RXRα), solute carrier (Slc), sulfotransferase (Sult), 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP), 2',3',7',8'-tetrachlorodibenzo-*p*-dioxin (TCDD), UDP-glucuronysyl transferase (Ugt)

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Abstract

The transcription factors AhR, CAR, PXR, PPARα, and Nrf2 regulate genes encoding drug metabolizing enzymes and transporters in livers of mice following chemical activation. However, the specificity of their transcriptional regulation has not been determined systematically in vivo. The purpose of this study was to identify genes encoding drug metabolizing enzymes and transporters altered by chemical activators in a transcription factor-dependent manner using wild-type and transcription factor-null mice. Chemical activators were administered ip to mice once daily for 4 days. Livers were collected 24h after the final dose, and total RNA was isolated for mRNA quantification of cytochrome P450s (Cyp), NAD(P)H quinone oxidoreductase 1 (Nqo1), aldehyde dehydrogenases (Aldhs), glutathione-*S*-transferases (Gsts), sulfotransferases (Sults), UDP-glucuronosyltransferases (Ugts), organic anion transporting polypeptides (Oatps), and multidrug resistance-associated proteins (Mrps). Pharmacological activation of each transcription factor leads to mRNA induction of drug metabolic and transport genes in livers of male and female wild-type mice, but no change in null mice: AhR (Cyp1a2, Nqo1, Aldh7a1, Ugt1a1, 1a6, 1a9, 2b35, Sult5a1, Gstm3, Mrp4), CAR (Cyp2b10, Aldh1a1, 1a7, Ugt1a1, 2b34, Sult1e1, 3a1, 5a1, Papps2, Gstt1, a1, a4, m1-4, Mrp2-4), PXR (Cyp3a11, Ugt1a1, 1a5, 1a9, Gsta1, m1-m3, Oatp1a4, Mrp3), PPARα (Cyp4a14, Aldh1a1, mGst3, Gstm4, Mrp4), and Nrf2 (Nqo1, Aldh1a1, Gsta1, a4, m1-m4, mGst3, Mrp3-4). Collectively, these data reveal transcription factor specificity and overlap in regulating hepatic drug disposition genes by chemical activators. Coordinated regulation of phase-I, -II, and transport genes by activators of transcription factors can have implications in development of pharmaceuticals as well as risk assessment of environmental contaminants.

Introduction

Early work by our laboratory demonstrated that xenobiotics known to induce microsomal enzyme activity also altered the hepatic excretion of chemicals (Klaassen, 1970; 1974; 1976). Over the following years, differential expression of phase-I and -II drug metabolizing enzymes and hepatobiliary transporters after chemical treatment became recognized as an important pharmacological phenomenon that contributes to changes in drug disposition. Coordinated regulation of drug metabolizing enzymes and transporters is mediated by a number of hepatic transcription factors (Handschin and Meyer, 2003; Klaassen and Slitt, 2005; Xu et al., 2005). Transcription factor-mediated regulation of drug metabolizing and transport genes involves the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR, NR1I2), and peroxisome proliferator-activated receptor α (PPARα, NR1C1). These receptors (with the exception of AhR) function by heterodimerizing with the retinoid X receptor alpha (RXRα, NR2B1). Other transcription factors involved in hepatic gene regulation include the oxidative stress sensor, NF-E2-related factor 2 (Nrf2, NFE2L2).

AhR binds to xenobiotic responsive elements and mediates the induction of cytochrome P450 (Cyp) 1a1 by chemicals such as 2',3',7',8'-tetrachlorodibenzo-*p*-dioxin (TCDD) (Whitlock et al., 1989). Isoforms of the Cyp2B subfamily are associated with CAR activation (Wang and Negishi, 2003). PXR is a major chemical sensor known to induce the expression of Cyp3A enzymes. Foreign substances, such as pregnenolone-16 alpha-carbonitrile (PCN), trigger PXR heterodimerization with RXRα and binding to its response elements in the promoter regions of genes involved in detoxification and transport (Staudinger et al., 2001a). Similar to agonists of CAR and PXR, chemical activators such as clofibrate (CFB) cause PPARα to bind to specific response elements (Dreyer et al., 1993). The Cyp4A subfamily are most sensitive to PPARα signaling (Johnson et al., 1996). Nrf2 is a transcription factor that is activated in response to electrophiles and oxidative stress. Exposure to oxidative stress or chemicals such as oltipraz (OPZ) causes Nrf2 to bind to antioxidant response elements in the regulatory regions of target genes, such

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as NAD(P)H:quinone oxidoreductase 1 (Nqo1), and activates transcription (Venugopal and Jaiswal, 1996; Nioi et al., 2003; Aleksunes and Manautou, 2007).

Our laboratory has used a battery of 15 chemical activators to identify hepatic phase-I and –II enzymes and transport genes as target genes of AhR, CAR, PXR, PPARα, and Nrf2 in male mice. For example, isoforms of the phase-I enzyme aldehyde dehydrogenase (Aldh) are strongly induced by ligands of CAR, PXR, and PPARα (Alnouti and Klaassen, 2008b). Similarly, phase-II enzymes including sulfotransferases (Sults), UDP-glucuronosyltransferases (Ugts), and glutathione-*S*-transferases (Gsts) are increased by chemical activators of all 5 transcription factors (Alnouti and Klaassen, 2008a; Knight et al., 2008; Buckley and Klaassen, 2009a; Yeager et al., 2009). Evidence also suggests that transport genes including isoforms of the ATP-binding cassette (Abc) and the solute carrier (Slc) families are up- or down-regulated by chemicals that activate AhR, CAR, PXR, PPARα, and Nrf2 (Cheng et al., 2005; Maher et al., 2005; Cheng and Klaassen, 2006; Moffit et al., 2006; Klaassen and Aleksunes, 2010). These studies have primarily been performed in male wild-type C57BL/6 mice and do not account for gender differences in responsiveness, nor confirm the dependence of these mRNA changes on specific transcription factors.

The extent to which phase-I and –II enzymes and transport genes are coordinately regulated in networks by ligand-activated transcription factors has not been explored in depth. Xenobiotics may not only act on their target receptor, but also activate other transcription factors. For example, 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) induces Cyp2b10 as well as Cyp3a11, suggesting that this chemical may activate both CAR and PXR signaling pathways (Petrick and Klaassen, 2007). Alternatively, chemicals may be specific for a single transcription factor and it is the target gene that can be transcriptionally activated by multiple transcription factors. Therefore, we have developed a systematic approach to identify the *in vivo* target genes of AhR, CAR, PXR, PPARα, and Nrf2 in response to chemical activators. The purpose of the present study was to 1) comprehensively evaluate the

transcriptional profiles of hepatic drug metabolizing and transport genes in male and female mice in response to pharmacological activation, and 2) utilize transcription factor-null mice to delineate the signaling pathways involved in transcriptional activation and repression. Five chemicals were selected as prototypical activators for these experiments: TCDD (AhR), TCPOBOP (CAR), PCN (PXR), CFB (PPARα), and OPZ (Nrf2). Selection of enzyme and transporter genes was based upon up- or down-regulation in male wild-type mice treated with these activators in previous studies (Cheng et al., 2005; Maher et al., 2005; Alnouti and Klaassen, 2008b; 2008a; Knight et al., 2008; Buckley and Klaassen, 2009a).

Materials and Methods

Animals. Eight to ten week-old male and female C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). AhR-null mice (>99% congenic for C57BL/6 background) were obtained from Jackson Laboratories, Inc. (Bar Harbor, ME) and were described previously (Schmidt *et al.*, 1996). Breeder pairs from the CAR-null mouse line on the C57BL/6 background were obtained from Dr. Ivan Rusyn (University of North Carolina, Chapel Hill, NC) that were engineered by Tularik, Inc. (South San Francisco, CA), as described previously (Ueda *et al.*, 2002). Nrf2-null breeding pairs were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) (Chan *et al.*, 1996) and were backcrossed into the C57BL/6 background to >99% congenicity, as determined by the speed congenics group at Jackson Laboratories, Inc. Breeders of PPARα-null mice were originally engineered in the laboratory of Dr. Frank J. Gonzalez at the National Institutes of Health/National Cancer Institute, Bethesda, MD (Lee *et al.*, 1995) and backcrossed into the C57BL/6 strain (Akiyama *et al.*, 2001). PXR-null breeder pairs were engineered and backcrossed into the C57BL/6 background (Staudinger et al., 2001a). Mice were allowed food (Harlan-Teklab 8064, Madison, WI) and water *ad libitum*, and were acclimated to the housing facility for at least one week prior to treatment. Mice were treated i.p. with either vehicle control (corn oil) or activators of AhR, CAR, Nrf2, PPARα, or PXR, as detailed in Table 1. Activators and dosing regimens were selected based on previous studies (Cheng *et al.*, 2005). Mice were dosed once daily for 4 consecutive days. On day 5, livers were removed, snap-frozen in liquid nitrogen, and stored at -80°C. Groups of 4-5 mice were used in each treatment group, with the exception of AhR-null female mice where only 3 animals were available for treatment with vehicle. The Institutional Animal Care and Use Committee at the University of Kansas Medical Center approved these studies.

RNA Isolation and Messenger RNA Quantification. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX). The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm, and purity confirmed by 260/280 nm ratio using a Nanodrop 2000

(Thermo Fisher Scientific, Wilmington, DE). The RNA integrity was assessed by visualization of 18S and 28S rRNA bands on formaldehyde-agarose gels. The hepatic mRNA expression was determined by the Quantigene® Plex 2.0 Reagent System (Affymetrix Inc., Santa Clara, CA). Panomics plex sets were used: 2.0 panels 21085 and 21086. Samples were analyzed using a Bio-Plex System Array reader (Bio-Rad, Hercules, CA). Five hundred nanograms of total RNA were used for each plex set. Subsequent steps have been reported previously (Aleksunes et al., 2009).

Statistical Analysis. The software program GraphPad Prism© version 5 (Graphpad Software, La Jolla, CA) was used for statistical analysis. Differences among individual groups were evaluated by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Results

Hepatic Regulation of Cytochrome P450 Enzymes and Nqo1 mRNA. The prototypical target genes of each transcription factor were induced in male and female wild-type mice after chemical activation, with no change observed in null mice: AhR – Cyp1a2 (2.5- to 5-fold); CAR – Cyp2b10 (30- to 180-fold); PXR – Cyp3a11 (1.5- to 3-fold); PPARα – Cyp4a14 (3- to 200-fold); Nrf2 – Nqo1 (9- to 10-fold) (Fig. 1). The five chemical inducers were not entirely specific as they increased target genes of the other transcription factors, however, induction was often to a much lesser extent. In addition to AhR-mediated regulation, Cyp1a2 mRNA was increased approximately 2- to 2.5-fold in livers of wild-type mice treated with TCPOBOP and OPZ. Cyp2b10 mRNA was also elevated in male wild-type mice treated with TCDD (4.7-fold) and female wild-type mice treated with PCN (15-fold); however, to a lesser degree than observed in TCPOBOP-treated wild-type mice. Cyp3a11 mRNA was also up-regulated in livers of wild-type mice, but not null mice, treated with TCPOBOP (2- to 3-fold), CFB (males only, 1.3-fold), and OPZ (females only, 1.6-fold). CFB and OPZ increased Cyp4a14 mRNA 3- and 5-fold in female wild-type mice, respectively, whereas TCPOBOP decreased Cyp4a14 mRNA to 22% of vehicle-treated female mice. In addition to OPZ, other chemical activators such as TCDD, TCPOBOP (males only), or CFB (males only), modestly increased Nqo1 mRNA in wild-type, but not AhR-, CAR-, or PPARα-null mice.

In general, basal mRNA expression of Cyp enzymes was similar between wild-type and null mice with some exceptions (Fig. 1). AhR-null mice had lower basal Cyp1a2 mRNA (25% of wild-types). Constitutive expression of Cyp3a11 mRNA was slightly higher (1.5- to 2-fold) in PXR- (female mice), PPARα-, and Nrf2-null mice.

Hepatic Regulation of Aldehyde Dehydrogenase mRNAs. Up-regulation of Aldh1a1 mRNA was observed in livers of wild-type mice treated with TCPOBOP (2-fold male and female), PCN (3.5-fold females only), CFB (1.6-fold male and female), or OPZ (2.3-fold male and female), but not in the

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respective transcription factor-null mice (Fig. 2). Induction of Aldh1a7 mRNA was observed in wild-type mice given TCPOBOP (3-fold) or PCN (females only, 2.8-fold), and to a lesser extent in mice treated with TCDD (females only, 1.3-fold), CFB (males only, 1.6-fold), or OPZ (females only, 1.7-fold). Of note, Aldh1a7 mRNA also tended to be increased in Nrf2-null mice treated with OPZ, further suggesting multiple transcription factors are involved in regulating this gene. In wild-type male mice, Aldh3a2 mRNA was modestly increased and decreased by CFB and TCDD, respectively, with no change in PPARα- and AhR-null mice (Fig. 3). The remaining Aldh members (4a1, 6a1, 7a1, an 9a1) exhibited only minor changes in livers of wild-type mice in response to TCPOBOP, TCDD, or CFB (Fig. 3).

Compared to vehicle-treated wild-type mice, the constitutive mRNA expression of Aldh1a1 and 1b1 were elevated in PXR- and AhR-null mice, respectively (Fig. 2). A variety of other Aldh isoforms were also differentially expressed in control wild-type and transcription factor-null mice in one gender or the other. It should be noted that Aldh2, 3a1, and 8a1 are largely unchanged by activator treatment (Alnouti and Klaassen, 2008b) and were excluded from the present study.

Hepatic Regulation of UDP-Glucuronysltransferase mRNAs. Hepatic Ugt expression was inducible (Figs. 4 and 5). TCDD increased Ugt1a1, 1a6, 1a9, 2b34 (females only), and 2b35 mRNA by 2- to 5-fold in wild-type, but not in AhR-null mice. Ugt1a1 and 2b34 mRNA levels were elevated in both male and female wild-type mice treated with TCPOBOP, with no change observed in CAR-null mice. Interestingly, TCPOBOP induced mRNA expression Ugt1a9, 2b35, and 2b36 in male mice only. Activation of PXR with PCN increased hepatic Ugt1a1, 1a5, and 1a9 mRNAs in both genders of wild-type, but not PXR-null mice. CFB treatment caused slight increases in Ugt1a5 and Ugt1a9 mRNA levels in male mice only. OPZ increased mRNA expression of Ugt2b34 (females only, 1.4-fold) and 2b36 (males only, 1.6-fold) in wild-type, but not Nrf2-null mice.

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Down-regulation of Ugt2a3 in response to TCDD (males only), TCPOBOP, or OPZ (females only) was also observed in wild-type mice. Whereas Ugt2a3 mRNA was unchanged in AhR-null and PPARα-null mice, it was reduced in Nrf2-null mice administered the chemical activator, suggesting Nrf2-independent regulation of this gene. In addition, Ugt2b1 mRNA was reduced approximately 40% in livers of male wild-type mice treated with TCDD or CFB.

Transcription factor-null mice treated with vehicle only demonstrated some differences in basal Ugt mRNA expression (Figs. 4 and 5). For example, Ugt2a3 mRNA was elevated in male and female PPARα-null mice, whereas Ugt1a6 and 2b1 were reduced in Nrf2-null mice. Additional gender-specific basal differences were also noted for the other Ugt isoforms. Ugt1a2, 1a7, 1a10, and 3a1/2 were unchanged by these chemical activators (Buckley and Klaassen, 2009a), and were not included in the present study.

Hepatic Regulation of Sulfotransferase mRNAs. Similar to a prior publication, mRNA of most Sult isoforms was higher in female than male mice (Fig. 6) (Alnouti and Klaassen, 2006). In addition to Sult isoforms, mRNA expression of 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2), which generates the sulfate source for Sult enzymes, was also quantified. TCPOBOP increased mRNA expression of all Sult enzymes as well as Papss2 in wild-type, but not in CAR-null mice. Likewise, other chemical activators elevated Sult mRNA including Sult2a2 (PCN, females, 1.8-fold), Sult3a1 (OPZ, females, 1.8-fold), Sult5a1 (TCDD, 3-fold), and Papss2 (PCN, females, 2.5-fold, and CFB, males, 1.4-fold) in livers of wild-type mice, with little to no change in transcription factor-null mice. Down-regulation of Sult3a1 (20% of control) or 5a1 (58% of control) was observed in wild-type female mice in response to TCDD or CFB administration, respectively.

Compared to wild-type mice, some Sult genes were differentially expressed in vehicle-treated transcription factor-null mice. Notably, Sult5a1 and Papss2 mRNA were elevated in PPARα- and PXR-

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null mice respectively. The majority of other basal differences in Sult isoforms in the various transcription factor-null mice were observed in female mice. Sult1a1, 1b1, 1c1, 1c2, 1d1, 2b1, and 4a1 are largely unchanged by these microsomal activators (Alnouti and Klaassen, 2008a) and excluded from the present study.

Hepatic Regulation of Glutathione *S*-Transferase mRNAs. Messenger RNA expression of Gsta and Gstm isoforms was highly inducible by multiple chemical activators (Figs. 7 and 8). The most consistent up-regulation of Gst isoforms in both sexes was observed in response to TCPOBOP, PCN, or OPZ. TCPOBOP increased (between 1.6- and 23-fold) Gsta1, a4, t1, and Gstm1-m4 in wild-type, but not in CAR-null mice. Similarly, PCN increased Gsta1 (16-fold in males, 113-fold in females) and Gstm1-m3 mRNAs (2- to 50-fold). OPZ elevated Gsta1, a4, and Gstm1-4 between 2.3- and 33-fold in male and female wild-type mice. TCDD and CFB also induced Gst mRNA expression, but often to a smaller extent and only in one sex of mice. Most Gst mRNA elevations occurred only in wild-type mice. Notable exceptions include Gsta1 and m2-4 mRNAs, which were also induced in Nrf2-null and PXR-null mice treated with activators, albeit to a lesser degree.

Constitutive mRNA expression of hepatic Gst isoforms, as assessed in vehicle-treated mice, were largely similar between wild-type and transcription factor-null mice (Figs. 7 and 8). In fact, no Gst isoform was consistently altered in both male and female transcription factor-null mice. Instead, male AhR-null mice exhibited higher Gsta1, a4, and mGst3 mRNA and female PXR-null mice had elevated levels of Gsta4, m1, m2, and m4 mRNA. Because Gsta3, k1, m5, m6, p1/2, t3, z1, and mGst1 mRNA exhibit little to no change in response to chemical activator treatment (Knight et al., 2008), they were not included in this study.

Hepatic Regulation of SLC and ABC Transporter mRNAs. Slc transporters, namely organic anion transporting polypeptide (Oatp) 1a1 and 1a4, were differentially regulated by knocking out various

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transcription factors and in response to chemical activators (Fig. 9). Other prominent liver-expressed transporters including Oatp1b2 and 2b1 exhibited little to no change after chemical activator treatment (Cheng et al., 2005) and were not included in the present study. One intriguing observation was the marked TCDD-mediated down-regulation of Oatp1a1 mRNA to 13% and 3% of vehicle-treated controls in male and female wild-type mice, respectively. Additional chemical activators reduced Oatp1a1 mRNA in only one gender, including TCPOBOP (males), PCN (females), or OPZ (females). In each case, down-regulation of Oatp1a1 was observed only in wild-type mice, but not in transcription factor-null mice. In contrast, Oatp1a4 mRNA was markedly induced by PCN treatment in both sexes between 4.4- and 5.4-fold as well as in male mice treated with TCPOBOP (2.8-fold). Multidrug and toxin extrusion protein 1 (Mate1) mRNA expression was only slightly induced 1.4-fold by CFB in wild-type male mice.

Induction of Mrp2 (2-fold), Mrp3 (3- to 4-fold), and Mrp4 (6.7- to 11-fold) mRNA was observed in male and female TCPOBOP-treated wild-type mice, with no change in expression in CAR-null mice. Similar up-regulation of Mrp2 mRNA, albeit to a much lower degree (1.5-fold), was detected in livers of wild-type mice treated with TCDD (females only), PCN (females only), or CFB (males only). Mrp3 mRNA was induced 2- to 6-fold in a transcription factor-dependent manner in response to TCDD (females only), PCN, CFB (males only), or OPZ. In addition to TCPOBOP, OPZ markedly increased Mrp4 mRNA in livers of wild-type mice, suggesting CAR and Nrf2 are key transcription factors for this gene. CFB also induced mRNA expression of Abcg5 2.8-fold in wild-type, but not in PPARα-null male mice.

It should be noted that higher basal Oatp1a1 mRNA expression (1.5-fold) was observed in vehicle-treated PPARα-null male mice and lower Oatp1a1 mRNA was detected in vehicle-treated AhR-, CAR-, PXR-, and Nrf2-null male or female mice. Messenger RNA expression of Oatp1a4 was higher basally in AhR- (males only, 2.4-fold) and PPARα-null mice (3-fold). Constitutive mRNA expression of

hepatic multidrug resistance-associated proteins 2-4 (Mrp2-4) isoforms, as assessed in vehicle-treated mice, were largely similar between wild-type and transcription factor-null mice (Fig. 10).

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Discussion

The current study utilized a systematic approach to investigate 5 transcription factors in the *in vivo* regulation of drug metabolizing and transport genes in response to microsomal enzyme inducers. Mice lacking these transcription factors are useful tools in evaluating the physiological and chemical regulation of drug processing genes. Use of null mice demonstrated that the majority of the transcriptional changes in response to chemical treatment were dependent upon expression of particular transcription factors. As expected, prototypical genes for each transcription factor (Cyps and Nqo1) were induced in response to corresponding chemical activators. Similar to prior work in this laboratory, the pharmacological inducers used in this study are not entirely specific to one target Cyp gene but produce minor increases in other Cyp isoforms (Petrick and Klaassen, 2007). For example, TCPOBOP activates its target gene, Cyp2b10, but also Cyp1a2, Cyp3a11, and Nqo1 (Fig. 1). In each of these cases, TCPOBOP-mediated induction was dependent upon CAR expression. Collectively, the results of this study 1) document the critical role of transcription factors in the basal expression of some drug metabolism and transport genes (Table 2), 2) provide evidence of transcription factor-dependent regulation of phase-I and –II enzymes and transport genes in response to chemical activation (Table 3), and 3) identify the influence of gender on the transcriptional regulation of drug disposition genes.

Constitutive expression of a few hepatic genes was altered in the absence of a transcription factor (Table 2). The genes that were consistently increased in both male and female transcription factor-null mice include Aldh1b1 in AhR-null mice, Aldh1a1 and Papss2 in PXR-null mice, Cyp3a11, Ugt2a3, Sult5a1, and Oatp1a4 in PPARα-null mice, as well as Cyp3a11 and 4a14 in Nrf2-null mice. Conversely, lower basal mRNA expression in both sexes was observed for Cyp1a2 and Oatp1a1 in AhR-null mice and Ugt1a6 and 2b1 in Nrf2-null mice. It is unclear why the remaining differences in constitutive expression occur in one gender but not the other. This laboratory has previously investigated gender-divergent regulation of Ugt and Sult enzymes (Buckley and Klaassen, 2009b; Alnouti and Klaassen, 2011). Sult enzymes are critically regulated by the stimulatory effects of estrogens and growth hormone secretion

patterns and suppressed by androgens. Interestingly, the differences in basal Sult mRNA expression in PXR- and PPARα-null mice were largely observed in female mice. It is important to also note that low basal Ugt expression in male Nrf2-null mice corresponds with a prior study from this laboratory (Yeager et al., 2009). In order to better understand the mechanisms underlying gender-specific patterns in basal metabolic and transport genes, future studies should focus on sex and growth hormone signaling in the various transcription factor-null mice.

The use of both male and female mice in the current study provides novel insight into the genes that are consistently regulated by activation of transcription factors, regardless of gender (Table 3). Activation of CAR signaling using TCPOBOP resulted in the up-regulation of the largest number of genes in a CAR-dependent manner including Cyp1a2, 2b10, 3a11, Aldh1a1, 1a7, Ugt1a1, 2b34, Sult1e1, 3a1, 5a1, Papss2, Gsta1, a4, t1, m1-m4, and Mrp2-4. This wide range of target genes points to the critical role of this transcription factor as well as the potency of TCPOBOP to transactivate CAR-mediated gene expression. AhR and PXR signaling are important in up-regulating various isoforms of the Ugt and Mrp families. PXR was also a consistent inducer of Gst enzymes. Activation of Nrf2 using OPZ treatment was mostly limited to regulating Gst and Mrp genes. The broad regulation of Mrp transport genes by each transcription factor has been reviewed previously (Klaassen and Slitt, 2005; Klaassen and Aleksunes, 2010). Collectively, this study highlights the role of each transcription factor to particularly regulate different families of drug metabolism genes.

There were a few genes that were down-regulated in both male and female mice treated with chemical activators. The most notable genes include the reduced expression of Ugt2a3 mRNA in TCPOBOP-treated mice and Oatp1a1 mRNA in TCDD-treated mice. Work from this laboratory previously observed these changes in male mice (Cheng et al., 2005; Buckley and Klaassen, 2009a), however, the consistent down-regulation of both genes in female mice points to a critical role for AhR and CAR in regulating the levels of target genes.

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Similar to constitutive expression patterns, the inducible regulation of some genes demonstrated gender-specific patterns. Because of the preferentially higher expression of Sult isoforms in female mice, it was not surprising that the majority of transcriptional changes (induction of Sult2a2 and 3a1) were observed in this gender. Moreover, up-regulation of Aldh1a7 mRNA was observed in female wild-type mice treated with TCDD, PCN, or OPZ and in male wild-type mice treated with CFB (Figure 2). TCPOBOP-mediated up-regulation of Aldh1a7 was observed in both genders. Likewise, most genes induced by TCPOBOP were similarly increased in both male and female wild-type livers, suggesting that hormonal differences may not be critical in regulating CAR target genes.

Overlap in responses between different transcription factors is evident in the current study. Similar to prior work, up-regulation of Nqo1 was observed in response to activation of AhR, CAR, PPARα, and Nrf2 in wild-type, but not in transcription factor-null mice (Maher et al., 2007; Merrell et al., 2008; Yeager et al., 2009). These data suggest cross-regulation of Nqo1 and other drug disposition genes by interrelated signaling pathways (Slitt et al., 2006; Kohle and Bock, 2009). This overlap in responses is often termed “cross-talk” and was evident for a number of genes. Work in our laboratory has demonstrated roles for both Nrf2 and PPARα in up-regulation of Mrp3 and 4 mRNA in response to chemicals such as perfluorooctanoic acid (Maher et al., 2008). Additional work is necessary to delineate whether regulation occurs directly or indirectly and the temporal sequence in which pathways participate in transcriptional up-regulation.

The results of this study correspond with prior studies using prototypical inducers in male wild-type mice. Strong concordance was notable for Cyp, Sult, Gst, and Mrp mRNA regulation (Maher et al., 2005; Petrick and Klaassen, 2007; Alnouti and Klaassen, 2008a; Knight et al., 2008). There were a limited number of genes in which induction was noted in a prior publication but not the current study. For example, Aldh1b1 mRNA was modestly up-regulated by chemical activators of all five transcription

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factors (Alnouti and Klaassen, 2008b). However, in the current study, only non-significant increases were observed in male mice (Fig. 2). Likewise, CFB was previously shown to up-regulate Aldh4a1, 6a1, and 7a1 mRNA that were not observed in the present work (Fig. 3). Similarly, Ugt1a6 mRNA was induced by ligands of PXR, PPARα, and Nrf2 (Buckley and Klaassen, 2009a) that was not detected in this study (Fig. 4). Attempts were made to control the experimental design between the current and prior studies including animal strain, age, and dosing time. However, there were minor differences including the dose and route of administration of oltipraz (75 mg/kg ip in the current study versus 150 mg/kg po in the prior study) (Alnouti and Klaassen, 2008a). The quantification of mRNA expression in both studies were based upon the branched DNA signal amplification assay however, the current study used a multiplex format with re-designed probes. These differences may account for some minor differences between the current and prior studies.

Prototypical chemical inducers were used in the present study, however, these data are applicable to other xenobiotics and pathological states. It is known that activation of CAR and PXR protects against bile acid toxicity and cholestasis in mice (Staudinger et al., 2001b; Wagner et al., 2005). The transcriptional pathways that are activated in response to injury via CAR and PXR may be better understood in the context of data generated in the present study. Likewise, the United States Environmental Protection Agency's ToxCast program utilizes screening mechanisms for identifying interactions of environmental chemicals and xenobiotics with key nuclear receptors, including those in this study (Martin et al., 2010; Rotroff et al., 2010). The responses of wild-type and transcription factor–null mice in response to chemical inducers is of potential importance to better understand the toxicological regulation of these pathways by chemicals and toxicants.

Extrapolation of the current data to humans is supported in part by *in vitro* exposure of human hepatocytes to activators of key pathways. For example, similar to the rodent data, treatment of human hepatocytes with phenobarbital (CAR agonist), rifampin (PXR agonist), or OPZ induces mRNA

expression of CYP2B6, 3A4, or NQO1, respectively, as well as up-regulation of MRP2 by all three chemicals (Jigorel et al., 2006). Exposure of human hepatocytes to AhR, CAR, or PXR ligands up-regulates UGT activity in human hepatocytes in a manner similar to our *in vivo* rodent mRNA data (Soars et al., 2004). Notably, differences in receptor regulation between rodents and humans (such as for PPARα) and responsiveness to xenobiotic activation between species (such as for CAR and PXR) are limitations to extrapolation of the present data. Likewise, responses documented in this work may be dependent upon the specific chemicals, doses, routes of administration, and durations selected for the current study. Additional studies will also be needed to confirm protein and functional changes in each of these disposition pathways. However, the fact that preclinical studies are typically performed in rodents strongly support dissecting the transcriptional networks of AhR, CAR, PXR, PPARα, and Nrf2 in mice.

Using prototypical chemical activators and transcription factor-null mice, the present study documents the transcriptional responses of male and females to chemical treatment and identifies the dependence of these responses on the function of key hepatic transcription factors. In general, differential expression of target genes in wild-type mice treated with prototypical chemical activators was largely absent in transcription factor-null mice. Collectively, these studies provide a comprehensive understanding of the basal and inducible regulation of phase-I and –II enzymes and transport genes in livers of mice via AhR-, CAR-, PXR-, PPARα-, and Nrf2-mediated pathways.

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Author Contributions

Participated in research design: Aleksunes, Klaassen
Conducted experiments: Aleksunes
Contributed new reagents or analytic tools: Klaassen
Performed data analysis: Aleksunes
Wrote or contributing to the writing of manuscript: Aleksunes, Klaassen

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Footnotes

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Figure Legends

Figure 1. Hepatic mRNA expression of cytochrome P450 (Cyp) enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 2. Hepatic mRNA expression of aldehyde dehydrogenase (Aldh) family 1 enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 3. Hepatic mRNA expression of aldehyde dehydrogenase (Aldh) family 3-9 enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 4. Hepatic mRNA expression of UDP-glucuronosyltransferases (Ugt) family 1 enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 5. Hepatic mRNA expression of UDP-glucuronosyltransferases (Ugt) family 2 enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

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Figure 6. Hepatic mRNA expression of sulfotransferase (Sult) and phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2) enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 7. Hepatic mRNA expression of glutathione S-transferase (Gst) enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 8. Hepatic mRNA expression of glutathione S-transferase (Gst) enzymes in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females

are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 9. Hepatic mRNA expression of uptake transporters in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression of organic anion transporter polypeptides (Oatp) and multidrug and toxin extrusion (Mate) transporters was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

Figure 10. Hepatic mRNA expression of efflux transporters in livers of wild-type and transcription factor-null mice after chemical activation. Messenger RNA expression of Abc (ATP binding cassette) transporters, including the multidrug resistance-associated protein (Mrp) transporters, was quantified using total hepatic RNA from control- and inducer-treated wild-type and transcription factor-null mice on day 5. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and presented as mean relative expression ± SE. For each graph, males are on the left and females are on the right. Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to control mice of that genotype. Daggers (†) represent a statistically significant difference ($p < 0.05$) between wild-type and null control mice. Double daggers (‡) represent a statistically significant difference ($p < 0.05$) between wild-type and null inducer-treated mice.

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Table 1. Dosing Regimens in Wild-Type and Transcription Factor-Null Mice

Compound	Transcription Factor	Dose	Vehicle	Route
TCDD	AhR	40 µg/kg	Corn Oil	ip
TCPOBOP	CAR	300 µg/kg	Corn Oil	ip
PCN	PXR	200 mg/kg	Corn Oil	ip
CFB	PPARα	500 mg/kg	Corn Oil	ip
OPZ	Nrf2	75 mg/kg	Corn Oil	ip

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Table 2. Constitutive Expression of Metabolism and Transport Genes in Livers from Transcription Factor-Null Mice Relative to Wild-Type Mice[a]

	Transcription Factor-Null Mice				
	AhR	**CAR**	**PXR**	**PPARα**	**Nrf2**
Phase-I Enzymes					
Cyp	↓ **Cyp1a2**	↑ Cyp4a14 (♂)	↑ Cyp3a11 (♀)	↑ Cyp1a2 (♀) ↑ **Cyp3a11**	↑ **Cyp3a11** ↑ **Cyp4a14**
Aldh	↑ **Aldh1b1** ↓ Aldh1a1 (♀) ↓ Aldh3a2 (♂)	↓ Aldh1a1 (♀) ↓ Aldh6a1 (♀) ↓ Aldh7a1 (♀)	↑ **Aldh1a1**	↓ Aldh1a1 (♀) ↑ Aldh1b1 (♀) ↑ Aldh7a1 (♂) ↓ Aldh9a1 (♀)	↓ Aldh1a1 (♀) ↑ Aldh1b1 (♀) ↑ Aldh4a1 (♀) ↓ Aldh6a1 (♂)
Phase-II Enzymes					
Ugt	↓ Ugt1a5 (♀)	↓ Ugt1a6 (♀)		↑ Ugt1a6 (♂) ↑ **Ugt2a3** ↑ Ugt2b35 (♂) ↑ Ugt2b36 (♂)	↓ **Ugt1a6** ↓ **Ugt2b1** ↓ Ugt2b34 (♀) ↓ **Ugt2b35** ↓ **Ugt2b36**
Sult	↓ Sult3a1 (♀) ↑ Papss2 (♂)		↑ Sult2a2 (♀) ↑ Sult3a1 (♀) ↑ **Papss2**	↑ Sult1e1 (♀) ↑ **Sult5a1** ↑ Papss2 (♀)	↓ Sult3a1 (♀) ↓ Sult5a1 (♀)
Gst	↑ Gsta1 (♂) ↑ Gsta4 (♂) ↑ mGst3 (♂)	↑ Gsta4 (♂)	↑ Gsta4 (♀) ↑ Gstm1 (♀) ↑ Gstm2 (♀) ↑ Gstm4 (♀)	↑ Gsta4 (♂)	↓ Gstm1 (♀)
Transporters					
Slco	↓ **Oatp1a1** ↑ Oatp1a4 (♂)	↓ Oatp1a1 (♂)	↓ Oatp1a1 (♀)	↑ Oatp1a1 (♂) ↑ **Oatp1a4**	↓ Oatp1a1 (♀)
Abc	↓ Mrp2 (♀) ↑ Mrp4 (♂) ↑ Abcg5 (♂)			↓ Abcg5 (♀)	

[a] Basal expression of genes is shown as increased (↑) or decreased (↓) relative to wild-type mice. Bolded font denotes consistent changes in both male and female mice.

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Table 3. Summary of Chemical- and Transcription Factor-Dependent mRNA Changes[a]

	AhR		CAR		PXR		PPARα		Nrf2	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Phase-I Enzymes										
Cyp1a2	↑↑	↑	↑	↑	-	-	-	-	↑	↑
Cyp2b10	↑↑	-	↑↑↑	↑↑↑	-	↑↑	-	-	-	-
Cyp3a11	-	-	↑↑	↑	↑	↑	↑	-	-	↑
Cyp4a11	-	-	-	↓↓↓	-	-	↑↑↑	↑↑	-	↑↑
Nqo1	↑↑	↑↑	↑	-	-	-	↑↑	-	↑↑	↑↑
Aldh1a1	-	-	↑	↑	-	↑↑	↑	↑	↑	↑
Aldh1a7	-	↑	↑	↑↑	-	↑	↑	-	-	↑
Aldh1b1	-	-	-	-	-	-	-	-	-	-
Aldh3a2	↓↓↓	-	-	-	-	-	↑	-	-	-
Aldh4a1	-	-	-	↓	-	-	-	-	-	-
Aldh6a1	-	-	-	↓↓	-	-	-	-	-	-
Aldh7a1	↑	↑	-	↓	-	-	-	-	-	-
Aldh9a1	-	-	-	-	-	-	↑	-	-	-
Phase-II Enzymes										
Ugt1a1	↑	↑	↑	↑	↑	↑	-	-	-	-
Ugt1a5	-	-	-	-	↑	↑	↑	-	-	-
Ugt1a6	↑	↑↑	-	-	-	-	-	-	-	↑
Ugt1a9	↑↑	↑↑	↑	-	↑	↑↑	↑	-	-	-
Ugt2a3	↓↓	-	↓↓	↓↓	-	-	-	-	-	↓↓
Ugt2b1	↓↓↓	-	-	-	-	-	↓↓↓	-	-	-
Ugt2b34	-	↑	↑	↑	-	↑	-	-	-	↑
Ugt2b35	↑	↑	↑↑	-	-	-	-	-	-	-
Ugt2b36	-	-	↑	-	-	-	-	-	↑	-
Sult1e1	-	-	↑↑↑	↑↑	-	-	-	-	-	-
Sult2a2	-	-	-	↑	-	↑↑	-	-	-	-
Sult3a1	-	↓↓↓	↑↑↑	↑↑	-	-	-	-	-	↑
Sult5a1	↑	↑↑	↑	↑	-	-	-	↓↓	-	-
Papss2	-	↓↓	↑	↑	-	↑	↑	-	-	-
Gsta1	-	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	-	↑↑	↑↑↑
Gsta4	-	↑	↑	↑	-	↑	-	-	↑	↑
Gstt1	↓↓	-	↑	↑	-	-	-	-	-	-
Gstt2	-	↑	-	-	-	-	↑	-	-	-
mGst3	-	-	-	-	-	-	↑↑	↑	↑	↑
Gstm1	-	↑	↑↑	↑	↑	↑↑	↑	-	↑↑	↑
Gstm2	-	↑	↑↑	↑↑	↑	↑↑	-	-	↑↑	↑↑
Gstm3	↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑	-	↑↑	↑↑
Gstm4	-	-	↑	↑	-	↑	↑	↑	↑↑	↑↑
Transporters										
Oatp1a1	↓↓↓	↓↓↓	↓↓↓	-	-	↓↓	-	-	-	↓↓↓
Oatp1a4	-	-	↑	-	↑↑	↑↑	-	-	-	-
Mate1	-	-	-	-	-	-	↑	-	-	-
Mrp2	-	↑	↑	↑	-	↑	↑	-	-	-
Mrp3	-	↑	↑↑	↑	↑	↑↑	↑	-	↑	↑↑
Mrp4	↑	↑↑	↑↑	↑↑	-	-	↑	↑	↑↑	↑↑
Abcg5	-	-	-	-	-	-	↑	-	-	-

[a] Increases in mRNA expression are denoted as <3-fold (↑), 3- to 30-fold (↑↑), and >30-fold (↑↑↑). Reductions in mRNA expression are denoted as levels decreased to >75% of control (↓), 50-75% of control (↓↓), and <50% of control (↓↓↓).

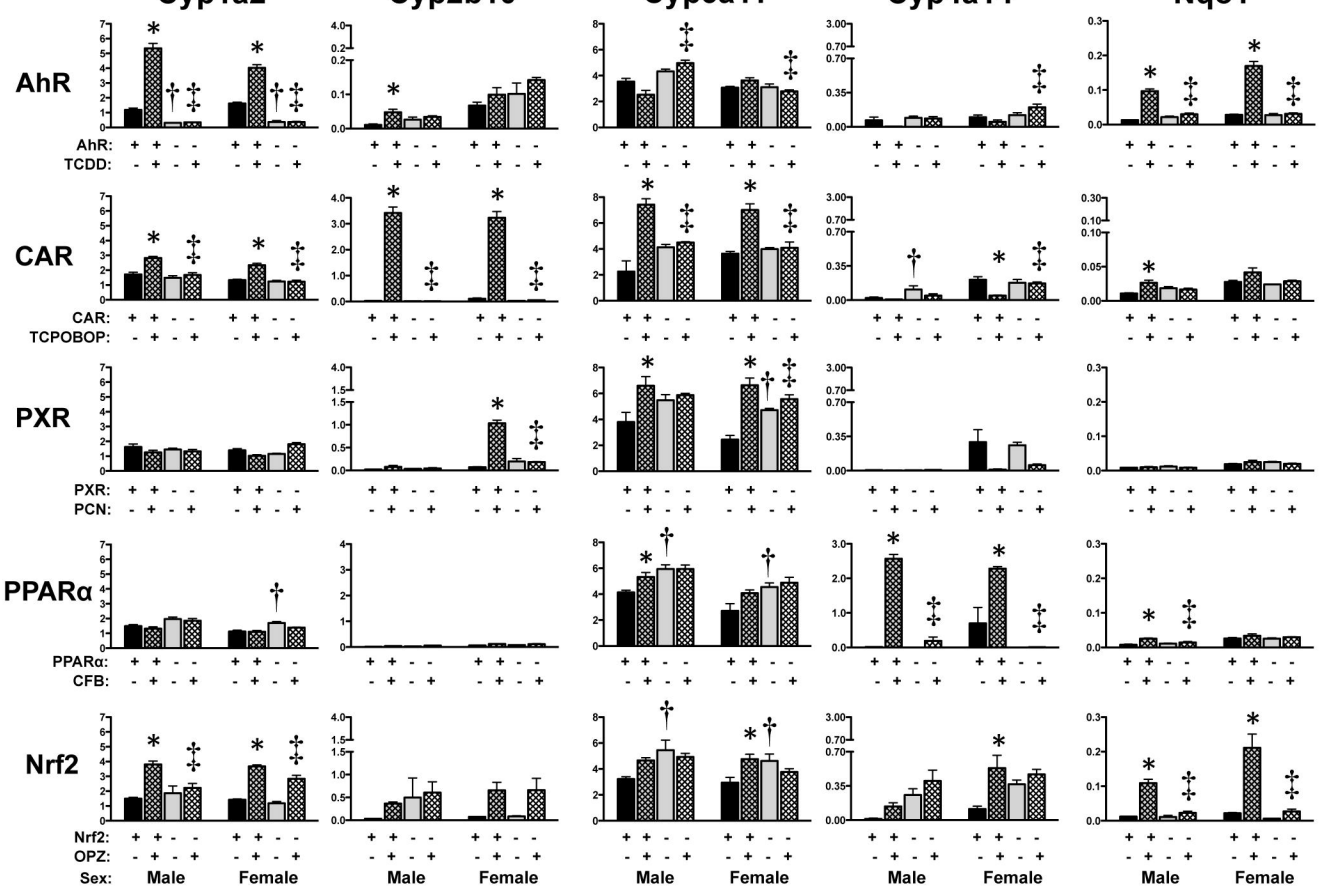
Cyp1a2
Cyp2b10
Cyp3a11
Cyp4a14
Nqo1
AhR
CAR
PXR
PPARα
Nrf2
mRNA Expression (GAPDH Corrected)
AhR:
TCDD:
CAR:
TCPOBOP:
PXR:
PCN:
PPARα:
CFB:
Nrf2:
OPZ:
Sex:
Male
Female

FIGURE 1

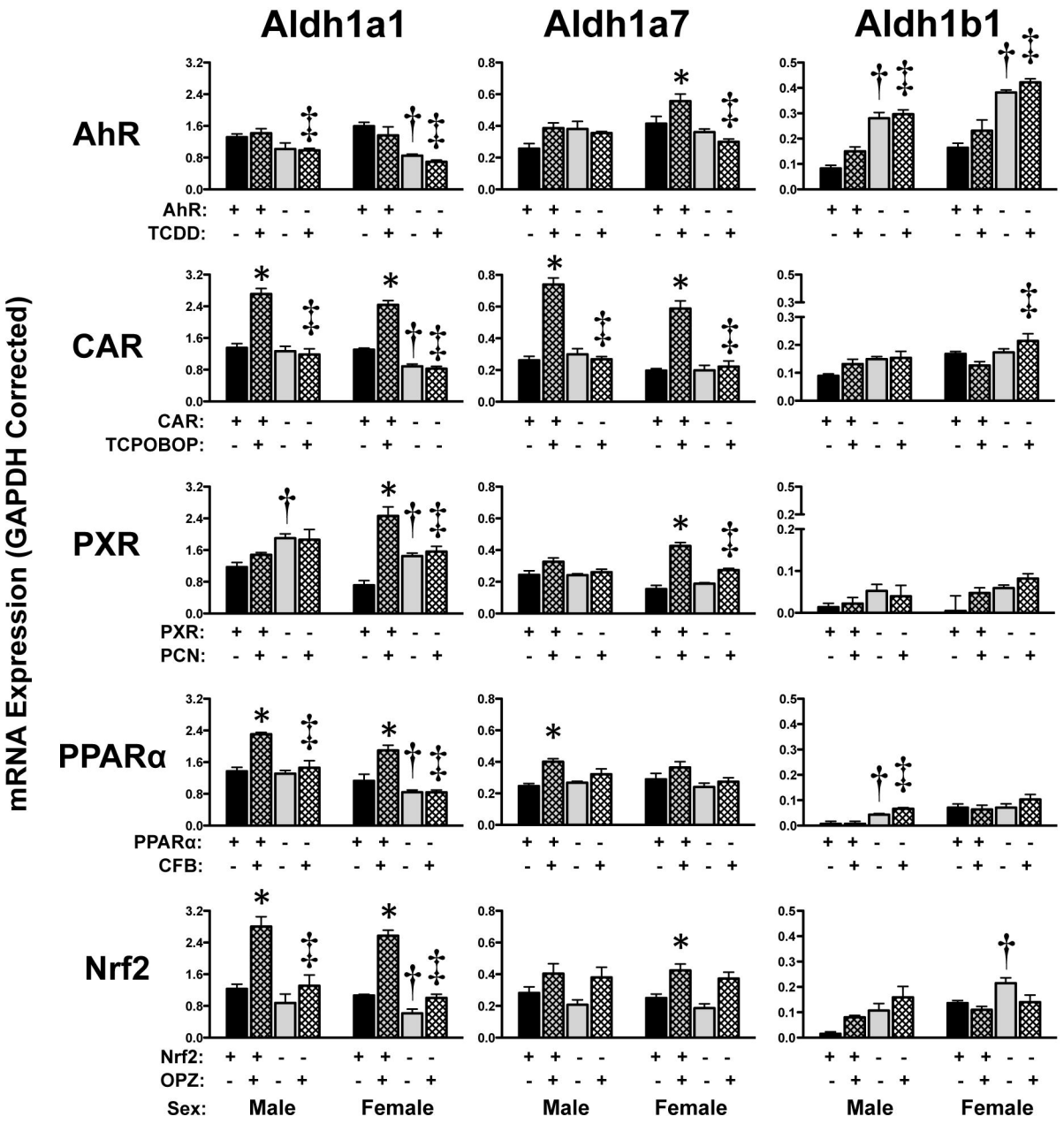

FIGURE 2

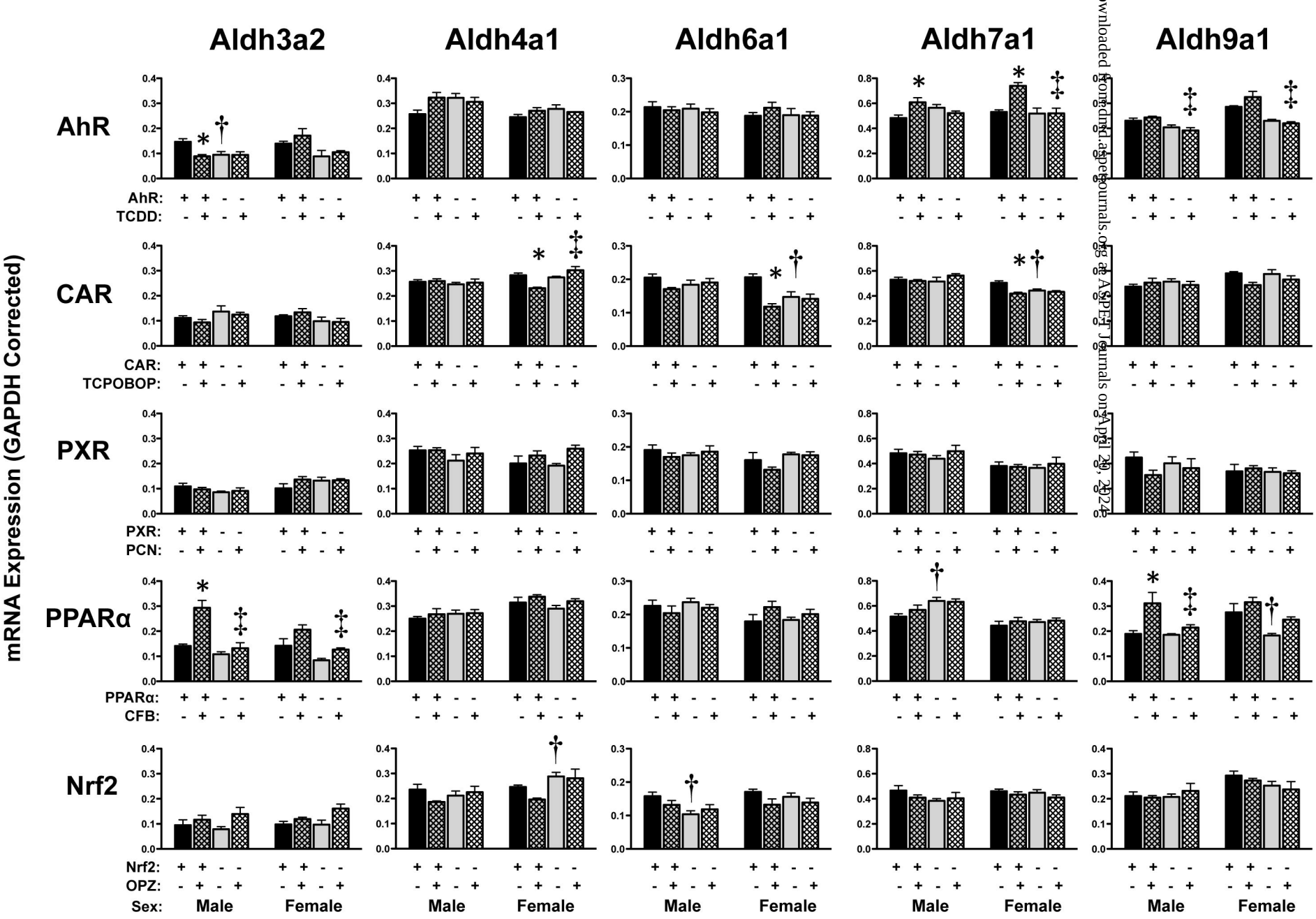

FIGURE 3

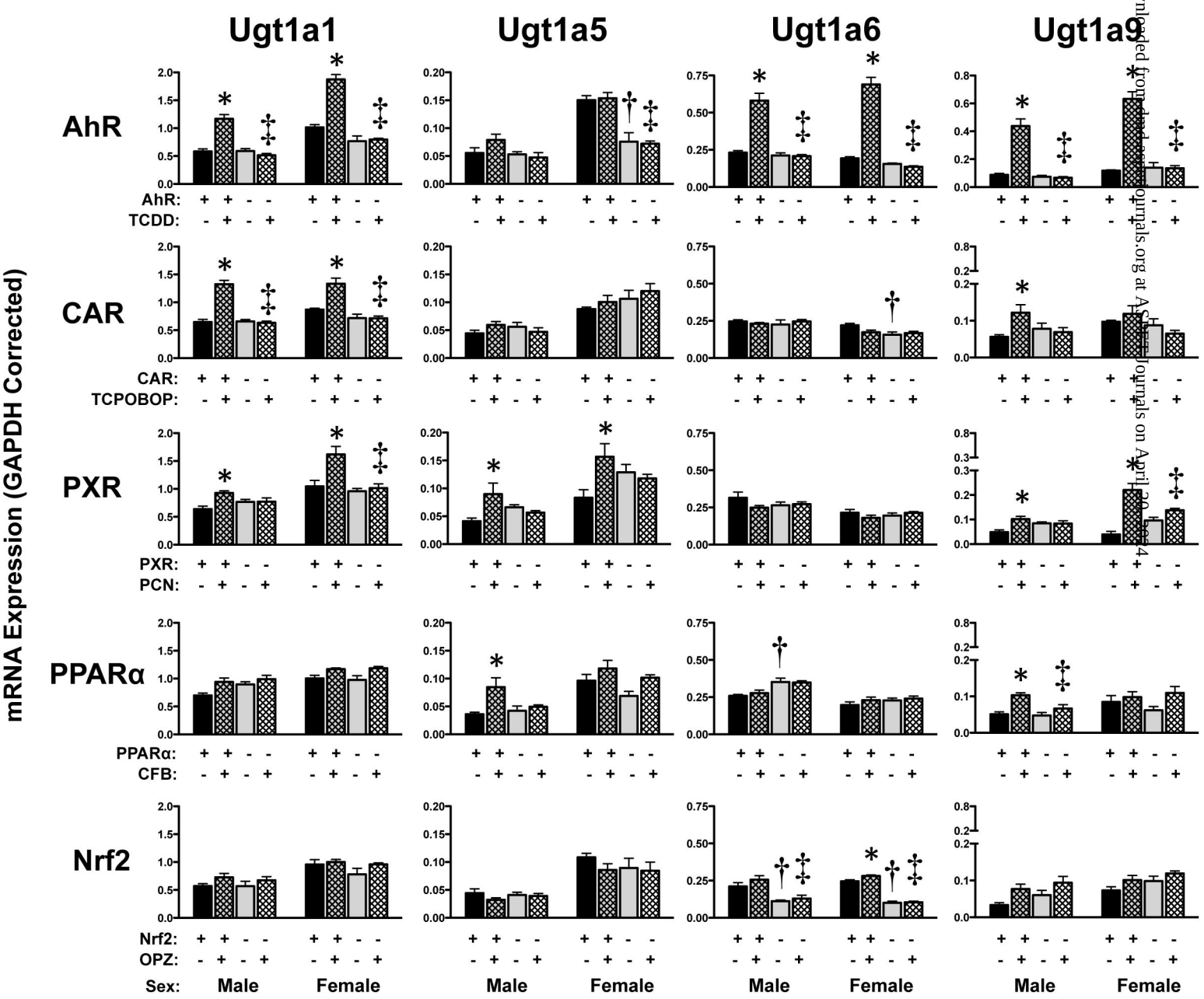

FIGURE 4

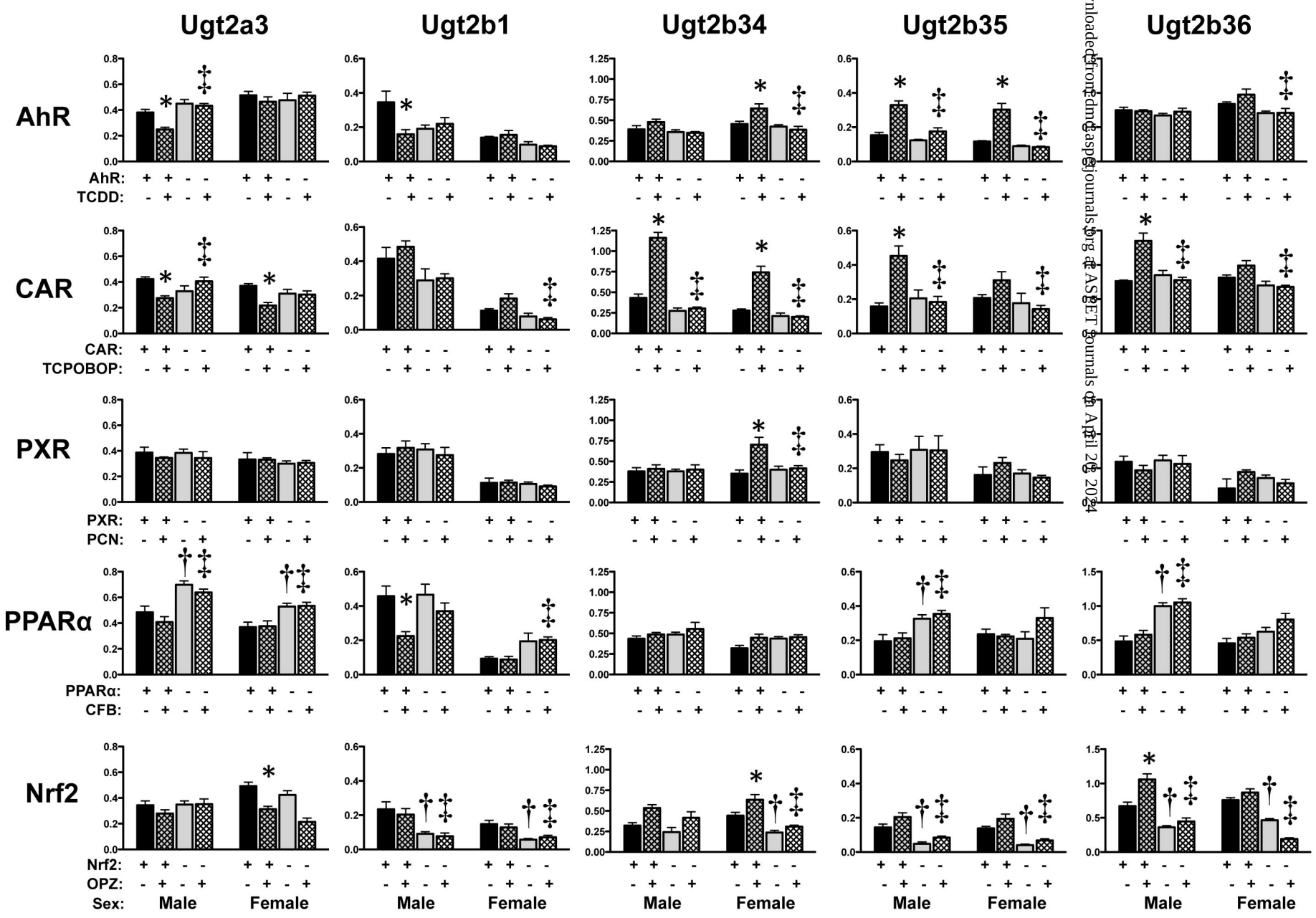

FIGURE 5

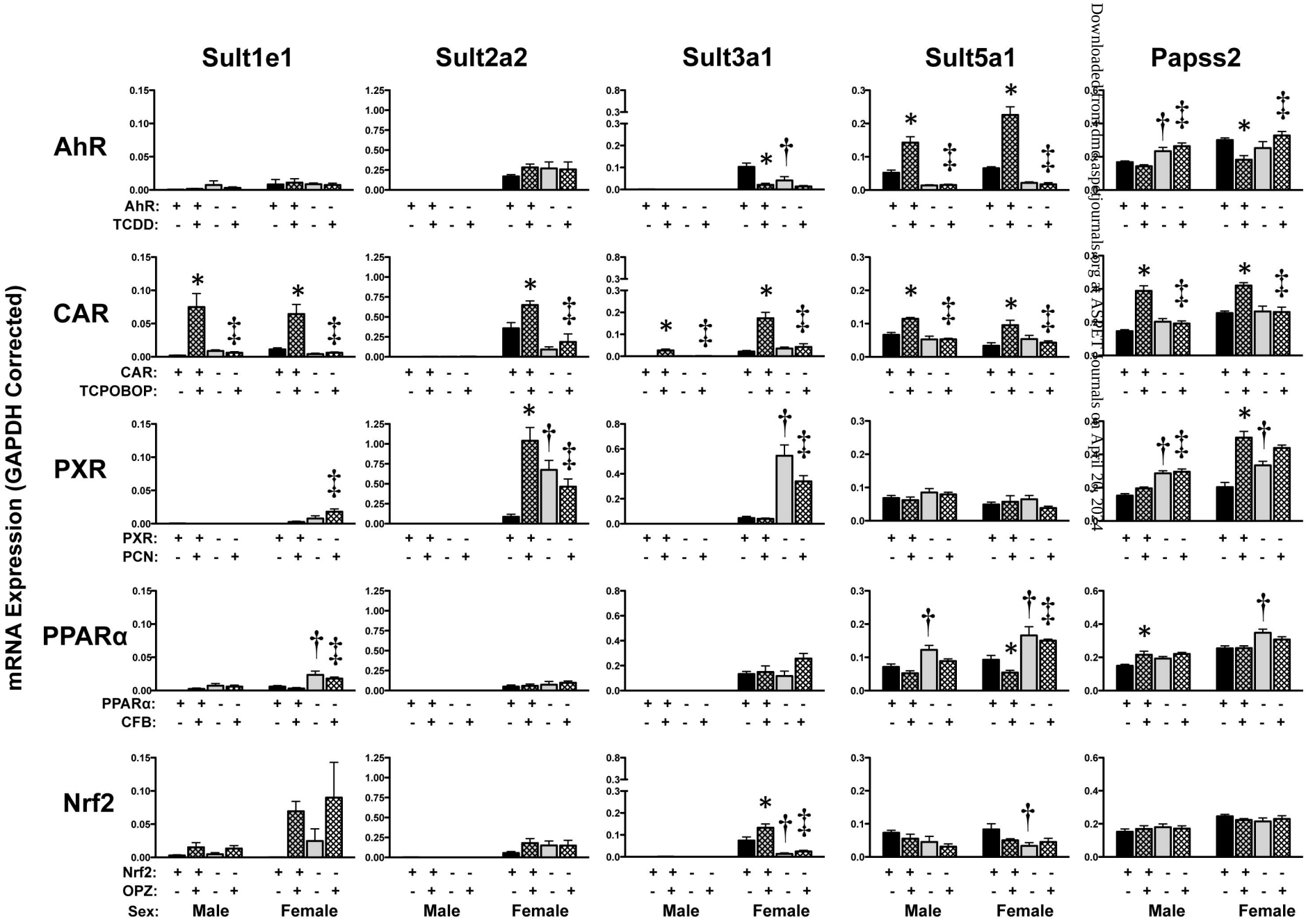

FIGURE 6

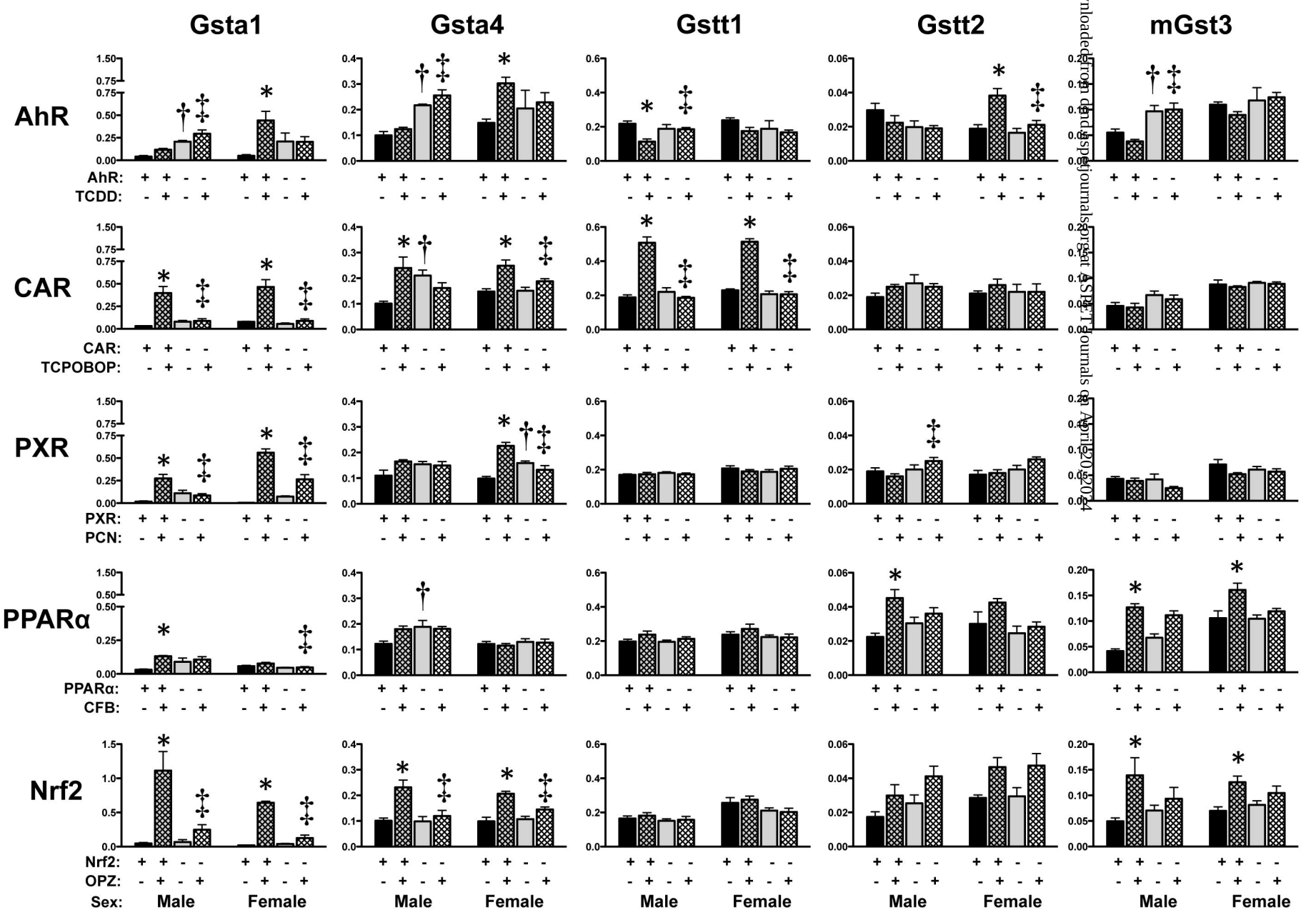

FIGURE 7

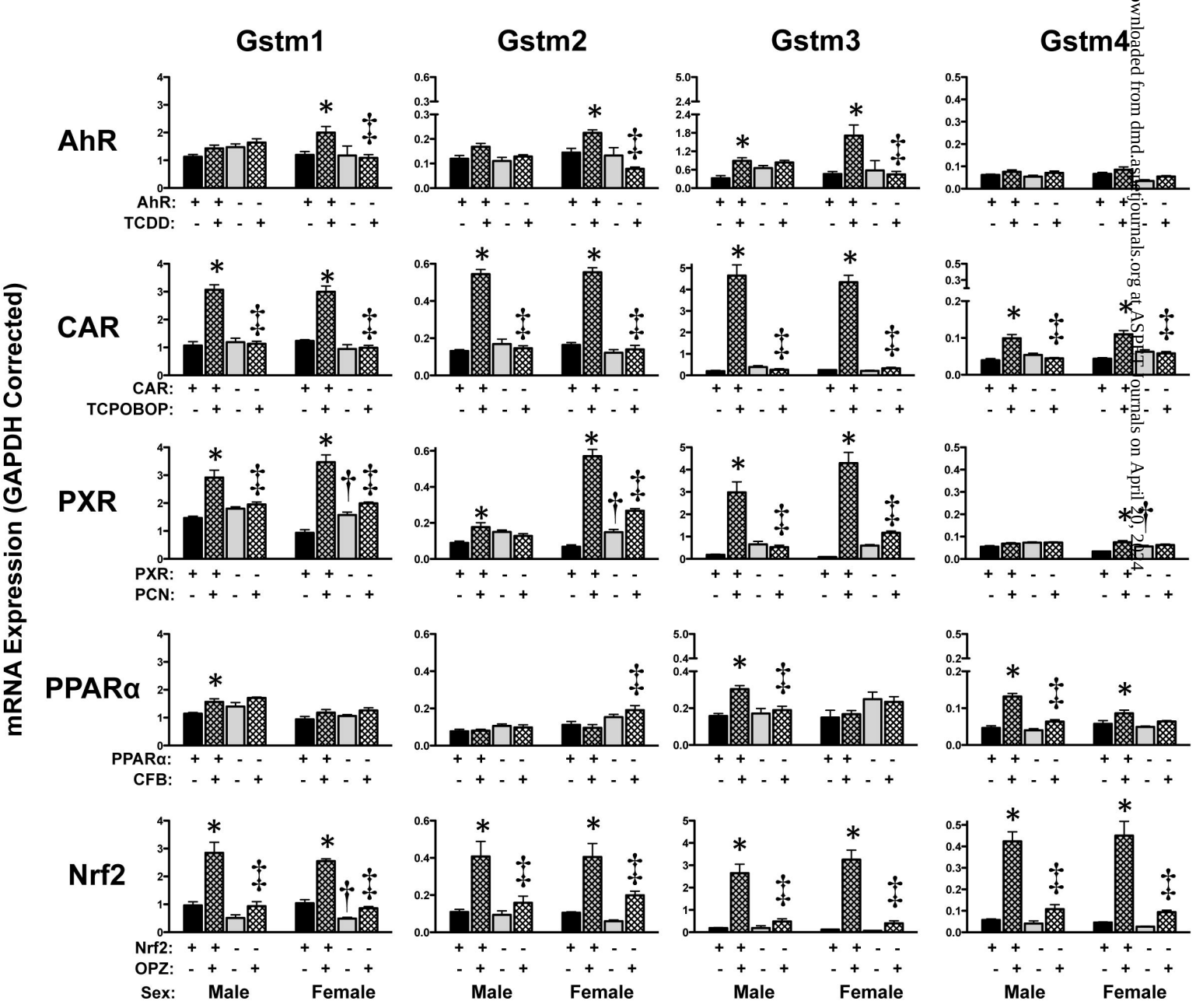

FIGURE 8

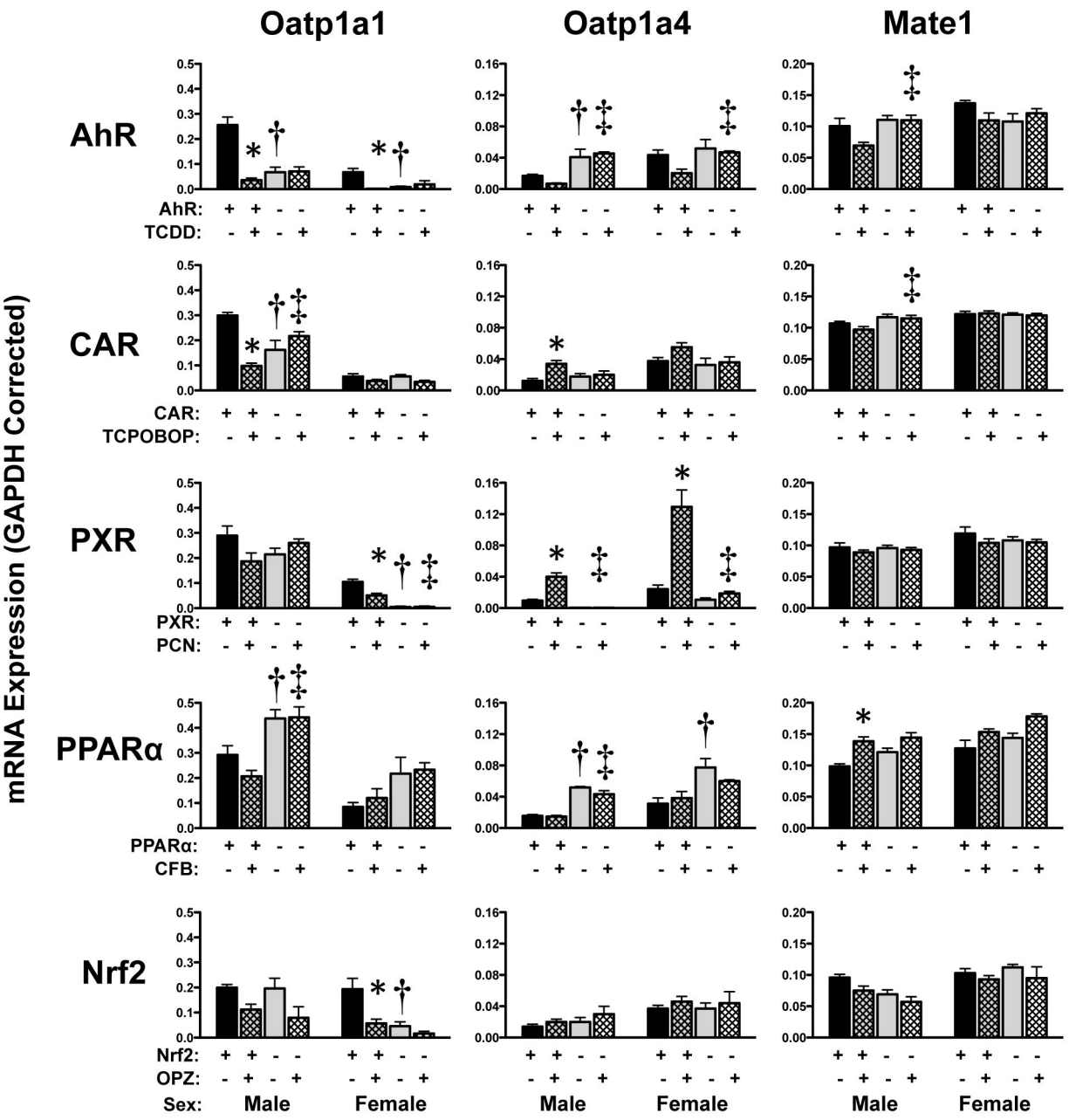

FIGURE 9

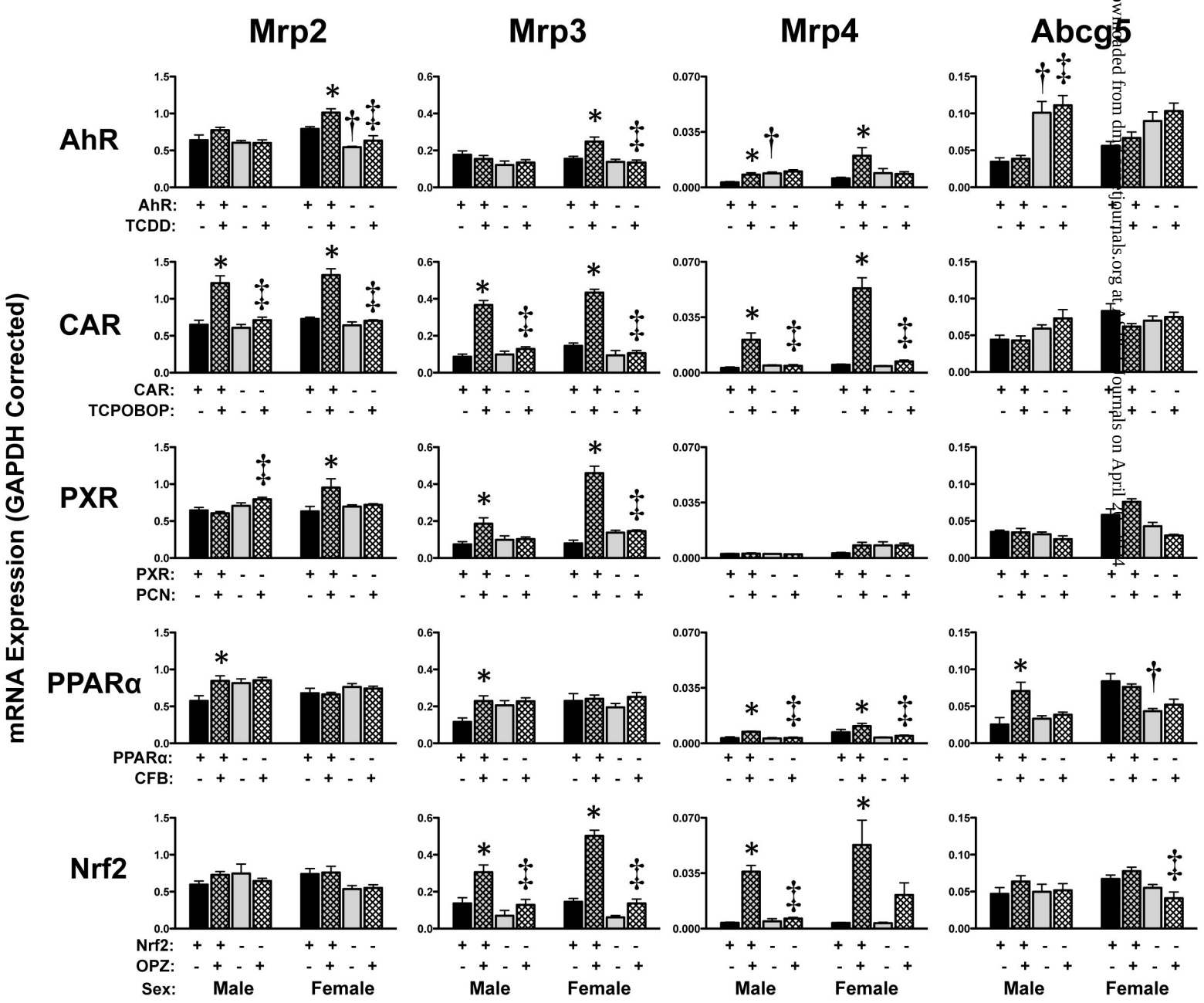

FIGURE 10