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Research article

Impact of Neonatal Activation of Nuclear Receptor CAR (Nr1i3) on *Cyp2* Gene Expression in Adult Mouse Liver

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ABSTRACT

Perinatal exposure to environmental chemicals is proposed to reprogram development and alter disease susceptibility later in life. Supporting this, neonatal activation of the nuclear receptor constitutive androstane receptor (CAR) (*Nr1i3*) by TCPOBOP was previously reported to induce persistent expression of mouse hepatic *Cyp2* genes into adulthood, and was attributed to long-term epigenetic memory of the early life exposure. Here, we confirm that the same high-dose neonatal TCPOBOP exposure studied previously (3 mg/kg, 15x ED50) does indeed induce prolonged (12 weeks) increases in hepatic *Cyp2* expression; however, we show that the persistence of expression can be fully explained by the persistence of residual TCPOBOP in liver tissue. When the long-term presence of TCPOBOP in tissue was eliminated by decreasing the neonatal TCPOBOP dose 22-fold (0.67× ED50), strong neonatal increases in hepatic *Cyp2* expression were still obtained but did not persist into adulthood. Furthermore, the neonatal ED50-range TCPOBOP exposure did not sensitize mice to a subsequent, low-dose TCPOBOP treatment. In contrast, neonatal treatment with phenobarbital, a short half-life ($t_{1/2} = 8$ h) agonist of CAR and PXR (*Nr1i2*), induced high-level neonatal activation of *Cyp2* genes and also altered their responsiveness to low-dose phenobarbital exposure at adulthood by either increasing (*Cyp2b10*) or decreasing (*Cyp2c55*) expression. Thus, neonatal xenobiotic exposure can reprogram hepatic *Cyp2* genes and alter their responsiveness to exposures later in life. These findings highlight the need to carefully consider xenobiotic dose, half-life, and persistence in tissue when evaluating the long-term effects of early life environmental chemical exposures.

Key words: TCPOBOP; phenobarbital; epigenetic reprogramming; growth hormone; Cyp2b10; Cyp2c.

Many environmental chemicals dysregulate gene expression, most notably in hepatocytes, by mechanisms that involve the activation of members of the nuclear receptor superfamily (Toporova and Balaguer, 2020; Waxman, 1999). CAR (constitutive androstane receptor, *Nr1i3*) and other nuclear receptors are activated by a wide range of structurally diverse foreign chemicals, including many industrial pollutants and pharmaceuticals (Baldwin and Roling, 2009; Chang and Waxman, 2006; Hernandez *et al.*, 2009; Kobayashi *et al.*, 2015; Omiecinski *et al.*, 2011). CAR also regulates normal physiological pathways, including hepatic energy homeostasis, cell proliferation, and inflammation (Cai *et al.*, 2021) and may thereby impact pathophysiological conditions such as fatty liver disease, diabetes, and hepatocellular carcinoma (Cave *et al.*, 2016; Dong *et al.*, 2009; Phillips *et al.*, 2007).

Under basal cellular conditions, CAR is sequestered in the inactive state in the cytoplasm as a multi-protein complex containing heat shock protein 90 and cytoplasmic CAR retention protein (Kobayashi *et al.*, 2003; Timsit and Negishi, 2014; Yoshinari *et al.*, 2003). CAR agonist ligands are often lipophilic and can diffuse through the plasma membrane to bind cytoplasmic CAR via its ligand-binding domain. This leads to dissociation of CAR's cytoplasmic chaperones followed by translocation of CAR to the nucleus, where CAR heterodimerizes with retinoid X receptor (RXR) (Mackowiak and Wang, 2016). In the nucleus, CAR/RXR heterodimers recruit coactivators and bind to specific response elements in genomic DNA (Niu *et al.*, 2018; Tian *et al.*, 2018) followed by transcriptional activation of many genes, including phase I and phase II enzymes of drug metabolism and transporters that regulate the metabolism of

endogenous and exogenous chemicals (Mackowiak and Wang, 2016; Qatanani and Moore, 2005). CAR activation is associated with epigenetic changes in mouse liver proximal to CAR-binding sites and nearby CAR responsive genes (Rampersaud *et al.*, 2019; Tian *et al.*, 2018). These changes are apparent as early as 3 h after exposure to TCPOBOP and include both increases and decreases in chromatin accessibility, which can be monitored as changes in DNase hypersensitivity (Lodato *et al.*, 2018; Vitobello *et al.*, 2019). Changes in histone methylation and acetylation, and changes in DNA methylation, have also been linked to the activation and repression of CAR target genes in mouse liver (Lempiainen *et al.*, 2011; Rampersaud *et al.*, 2019).

Early developmental exposure to xenobiotics, including chemicals that can activate CAR, has been proposed to lead to neonatal reprogramming (neonatal imprinting) in a way that can alter metabolic function and gene expression in liver and other tissues (Cave, 2020; Kublbeck *et al.*, 2020; Piekos *et al.*, 2017). Potential effects on life expectancy (Agrawal and Shapiro, 2005) and disease susceptibility later in life have also been reported (Hochberg *et al.*, 2011). The molecular mechanisms that underlie the early developmental lesions that lead to adult pathophysiology are likely to be epigenetic in nature but are poorly understood (Moggs and Terranova, 2018; Nahar *et al.*, 2014; Trevino *et al.*, 2020). For example, when CAR is activated in neonatal mouse liver by the CAR-specific agonist ligand TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene) (Poland *et al.*, 1980; Tzameli *et al.*, 2000), a persistent increase in expression was reported for 2 CAR target genes, *Cyp2b10* and *Cyp2c37*, via a mechanism proposed to involve an epigenetic memory of neonatal CAR exposure (Chen *et al.*, 2012). However, the long half-life reported for liver metabolic activities induced by TCPOBOP (Poland *et al.*, 1980) raises the question of whether the continued presence of TCPOBOP in mouse tissue, rather than an epigenetic memory, drives the observed persistence of CAR target gene induction.

Here, we show that neonatal exposure to TCPOBOP at a dose widely used by many investigators, 3 mg/kg, induces long-term, persistent activation of CAR-responsive genes in the liver, but also results in the persistence of TCPOBOP in liver tissue at a concentration we demonstrate is sufficiently high to account for the prolonged increase in expression of *Cyp2* family genes in the liver 12 weeks later. Additionally, we characterize the impact of neonatal exposure to phenobarbital, which activates CAR indirectly via changes in CAR phosphorylation (Negishi *et al.*, 2020). Our findings show that neonatal exposure to phenobarbital, which lacks the complications of long-term persistence in tissue owing to its much shorter half-life (Markowitz *et al.*, 2010) compared with TCPOBOP (Poland *et al.*, 1980) (8–16 h vs 2 weeks), sensitizes mouse liver to a subsequent exposure to phenobarbital in adulthood. Thus, such short-lived chemicals are more amenable for studying mechanisms by which CAR and other xenobiotic sensors translate early environmental stimuli to persistent changes in gene expression.

MATERIALS AND METHODS

Animals. All mouse work was carried out in compliance with procedures approved by the Boston University Institutional Animal Care and Use Committee (protocol No. PROTO201800698) and in compliance with ARRIVE 2.0 Essential 10 guidelines (Percie du Sert *et al.*, 2020), including study design, sample size, randomization, experimental animals and procedures, and statistical methods. Adult male and female CD-1 mice (8–10 weeks of age) were purchased from Charles River Laboratories (Wilmington, Massachusetts) and housed in the Boston University Laboratory Animal Care Facility. Mice were kept on a 12-h light cycle (7:30 AM–7:30 PM). For mouse breeding, 1 adult male and 1 adult female mouse were housed together until a vaginal plug was observed, at which time the mice were separated. The first day pups were observed was designated as postnatal day 1 (PND1). Litters were housed with dams until the mice were weaned on PND21. Mice were treated with TCPOBOP (Sigma-Aldrich, St. Louis, Missouri) dissolved in a 1% DMSO solution in tocopherol-stripped corn oil (Fisher Scientific), or with phenobarbital (sodium salt; Sigma-Aldrich) dissolved in 0.9% sodium chloride solution, or with vehicle (control) by intraperitoneal injection between 8:00 AM and 8:45 AM on the day(s) of treatment at doses stated in the text. Treatments were administered to mice at ages ranging from PND4 to 7 weeks of age, as specified for each study. Mice were euthanized at time points specified for each study and at a fixed time of day (between 11:00 AM and 11:45 AM) to minimize gene expression variations between mice due to the strong circadian effects on gene expression in liver (Kettner *et al.*, 2016). Where indicated, nursing dams were given drinking water supplemented with 0.05% (w/w) phenobarbital or drinking water as a control. A small piece of each liver was snap frozen in liquid nitrogen then stored at −80°C followed by RNA extraction for RT-qPCR analysis and TCPOBOP extraction for LC/MS analysis.

Neonatal mouse exposure models. Mice were exposed to either TCPOBOP or phenobarbital during the perinatal period and, where indicated, were given a second exposure later in life using one of the following 4 study designs. In Study A (Figure 1, below), PND4 pups were given TCPOBOP by i.p. injection at 3 mg/kg or vehicle (control). Mice were given a second injection of TCPOBOP (3 mg/kg) or vehicle in week 3 (on a day between PND20 and PND23) and euthanized 3 h later. In a variation of this study design (Figure 4, below), PND4-exposed mice were euthanized after 3, 7, or 12 weeks without a second TCPOBOP exposure. In Study B (Figure 3, below), PND4 pups were given TCPOBOP at 133 μg/kg (0.67× ED50) or vehicle. Mice were given a second injection of TCPOBOP, at 40 μg/kg (0.2× ED50), or vehicle, in week 7 (injection given on one of the days between PND46 and PND52) and euthanized 51 h later. In Study C (Figure 5, below), newborn mice were exposed to phenobarbital given to nursing dams through their drinking water (0.05% [w/v] phenobarbital) for a 6 consecutive-day perinatal exposure period (PND2 through PND7). In week 7 (beginning on one of the days between PND49 and PND52), the mice were given phenobarbital by i.p. injection at 10 mg/kg/day, or vehicle (control), on each of 3 consecutive days and euthanized 3 h after the last injection. Finally, in Study D (Supplementary Figure S2), PND4 pups were given 2 i.p. injections of phenobarbital, each at 40 mg/kg, or vehicle, on PND4 and again on PND5. In week 7 (beginning on PND49 or PND50), the mice were injected with phenobarbital at 10 mg/kg/day i.p., or vehicle, on each of 3 consecutive days and euthanized 3 h after the last injection.

RNA purification and RT-qPCR. A portion of each liver (0.1–0.2 g) was homogenized for 30 s in 1 ml of TRIzol using a Polytron homogenizer. The TRIzol homogenate was processed according to the manufacturer's protocol (Life Technologies) to purify total liver RNA. The purified RNA was treated with DNase I (Promega) to remove genomic DNA contaminants and then converted to cDNA using Applied Biosystems High Fidelity RT kit (ThermoFisher). Quantitative real-time PCR (qPCR) primer pairs specific for mature mRNA were located in adjacent exons of each target gene, with the exons selected to give amplicons spanning long introns to reduce

the likelihood that amplification of contaminating genomic DNA would contribute to the qPCR signal. Primer pairs specific for primary (unspliced) RNA transcripts were designed to have amplicons that span either an exon-injunction or an intron-exon junction of the target gene. qPCR was performed using Power SYBR Green PCR Master Mix (ThermoFisher). Relative RNA expression levels were calculated using the ΔΔCt method and normalized to the expression of 18S ribosomal RNA. Primer sequences used to amplify each gene are shown in Supplementary Table S1.

TCPOBOP extraction and LC/MS. A piece of each frozen liver was added to phosphate-buffered saline (0.5 g liver/0.5 ml buffer) and immediately homogenized using a Polytron homogenizer for 30 s. The homogenate (~1 ml) was shaken with 5 ml of hexane for 1 h at room temperature. The hexane layer was transferred to a clean glass tube, and 5 ml of fresh hexane was added to the remaining homogenate and vortexed on a flat platform for 1 h at room temperature. The hexane layers were combined and evaporated under a gentle stream of nitrogen gas. The residue was dissolved in ~0.1–0.2 ml of 4% DMSO (Sigma-Aldrich) in acetonitrile (Fisher Scientific) and stored at −20°C until LC/MS analysis. HPLC was performed using a C18 column run at a flow rate of 0.6 ml/min of 30% water and 70% acetonitrile. The HPLC-separated peaks were further analyzed using a Waters QTof Premier mass spectrometer in the Boston University Chemical Instrumentation Core. Standard curves for TCPOBOP quantification were generated using pure TCPOBOP dissolved in 4% DMSO in acetonitrile. The prominent TCPOBOP total ion count peak at 402.95 m/s was used to calculate the concentration of TCPOBOP present in each liver extract. Supplementary Figure S4 presents details on the identification and quantification of TCPOBOP, including mass spectra, details on the parent ions used for single ion monitoring, and standard curves used for quantification.

Statistical analysis. Data are presented as mean ± SEM for the number of individual livers (biological replicates) specified in each figure legend. Significance was assessed by a 2-tailed *t*-test for pairwise comparisons specified in each figure legend and implemented in GraphPad Prism.

RESULTS

Responsiveness of Neonatal Mice to TCPOBOP

We investigated the effects of neonatal exposure to TCPOBOP (3 mg/kg on PND4) on liver expression of *Cyp2b10* and *Cyp2c55*, which are both induced >50-fold within 3 h of TCPOBOP treatment in adult mouse liver (Lodato *et al.*, 2017). TCPOBOP stimulated a persistent induction of both *Cyp2* genes, as well as of lnc5998, a long-non-coding (lnc)RNA that is divergently transcribed from *Cyp2b10* and is highly inducible by TCPOBOP (Lodato *et al.*, 2017) (Figure 1, bar 2 vs bar 1). Large, significant inductions were also seen in livers of mice given a single injection of TCPOBOP in week 3 and euthanized 3 h later (bar 3), but the extent of induction was lower than was seen in the PND4-treated mice (bar 3 vs bar 2) or in mice exposed to TCPOBOP on both PND4 and at 3 weeks of age (bar 3 vs bar 4). Similar results were observed in female mice (Figs. 1A–C vs Figs. 1D–F). The increased effectiveness of TCPOBOP at inducing gene expression in mice receiving both TCPOBOP treatments was much greater for *Cyp2c55* and *lnc5998* than for *Cyp2b10* (bar 4 vs bar 3), which both respond to a single injection of TCPOBOP more slowly than *Cyp2b10* (Lodato *et al.*, 2017), and hence are not maximally induced at the 3 h time point.

Although all 3 genes were significantly induced by both the neonatal exposure and the week 3 TCPOBOP exposure, we did not observe a further increase in expression above the level reached with neonatal TCPOBOP alone when the mice were given a second injection of TCPOBOP at 3 weeks of age (Figure 1, bar 4 vs bar 2), in either sex). Given the long half-life of TCPOBOP, about 2 weeks in adult mice (Poland *et al.*, 1980), these findings suggest that the transcriptional activation of these CAR target genes reaches its maximal level once TCPOBOP biodistribution to the liver has occurred following the first injection on PND4, and that the lower expression seen in the mice treated in week 3 (bar 3 vs bar 2) is due to the incomplete biodistribution in the 3 h period between injection and tissue collection in the week 3 treatment group.

Optimization of Neonatal TCPOBOP Dose

We sought to distinguish any potential long-term reprogramming effects of neonatal TCPOBOP exposure from effects due to the residual TCPOBOP that may persist in liver or other tissues. We first optimized the dose of TCPOBOP used in the initial, neonatal exposure. We reasoned that the dose needs to be high enough to activate a robust liver gene response but sufficiently low to be effectively cleared within a few weeks, ie, reduced to a level low enough to discern the impact of a second TCPOBOP exposure later in life. PND4 mice were given TCPOBOP at doses of 0.33×, 0.67×, and 1× the ED50 dose, based on the ED50 value of 0.2 mg TCPOBOP/kg body weight reported for induction of hepatic cytochrome P450-dependent aminopyrine N-demethylase activity in 6-week female mouse liver (Poland *et al.*, 1980). Of note, the ED50 value of 0.2 mg/kg is 15-fold lower than the standard dose of 3 mg/kg TCPOBOP that is widely used in mouse liver studies of CAR target gene induction, including our studies in Figure 1 and in Chen *et al.* (2012). Figure 2 shows that the expression of *Cyp2b10* and *Cyp2c55* was detectable but minimally responsive to an ED50 dose of TCPOBOP after 3 h and then increased dramatically by the 27 h time point (Figs. 2A and B). Given the rapid induction of *Cyp2b10* within 3 h of when mice are given the saturating dose of 15× ED50 (ie, 3 mg/kg) (Lodato *et al.*, 2017), we surmise that the slower induction time course seen with ED50-range doses of TCPOBOP (Figure 2) reflects the relatively long time required for TCPOBOP to biodistribute to the liver and generate a tissue level sufficient to meet the threshold concentration for strong activation of CAR and its target genes. Very similar patterns were seen for both the time course and for the dose-response of induction of the primary, unspliced transcripts *Cyp2b10* and *Cyp2c55* (Figs. 2C and D), which are indicative of relative rates of gene transcription due to the expected short half-life of such transcripts (Gaidatzis *et al.*, 2015). Thus, the striking increases in mature RNA for *Cyp2b10* and *Cyp2c55* from 3 h to 27 h seen in Figure 2 reflect increases in *Cyp2* gene transcription rates, rather than a time-dependent accumulation of the mature RNAs in the liver. Similarly, the TCPOBOP dose-dependent increases in both the primary and the mature transcripts seen at 27 h are consistent with an increase in the abundance of transcriptionally active hepatic CAR-TCPOBOP complexes from 3 h to 27 h. Other factors, including changes in CAR levels, could also contribute to this apparent increase in transcription rate.

The induction of Cyp2b10 mature mRNA and its transcription rate (ie, primary transcript level) seen 27 h after TCPOBOP exposure on PND4 was followed by a strong decrease 17 days later, on PND21 (Figs. 2A and C). The magnitude of this decrease is consistent with the 2 weeks half-life for TCPOBOP elimination from the liver (Poland *et al.*, 1980) when taking into account the

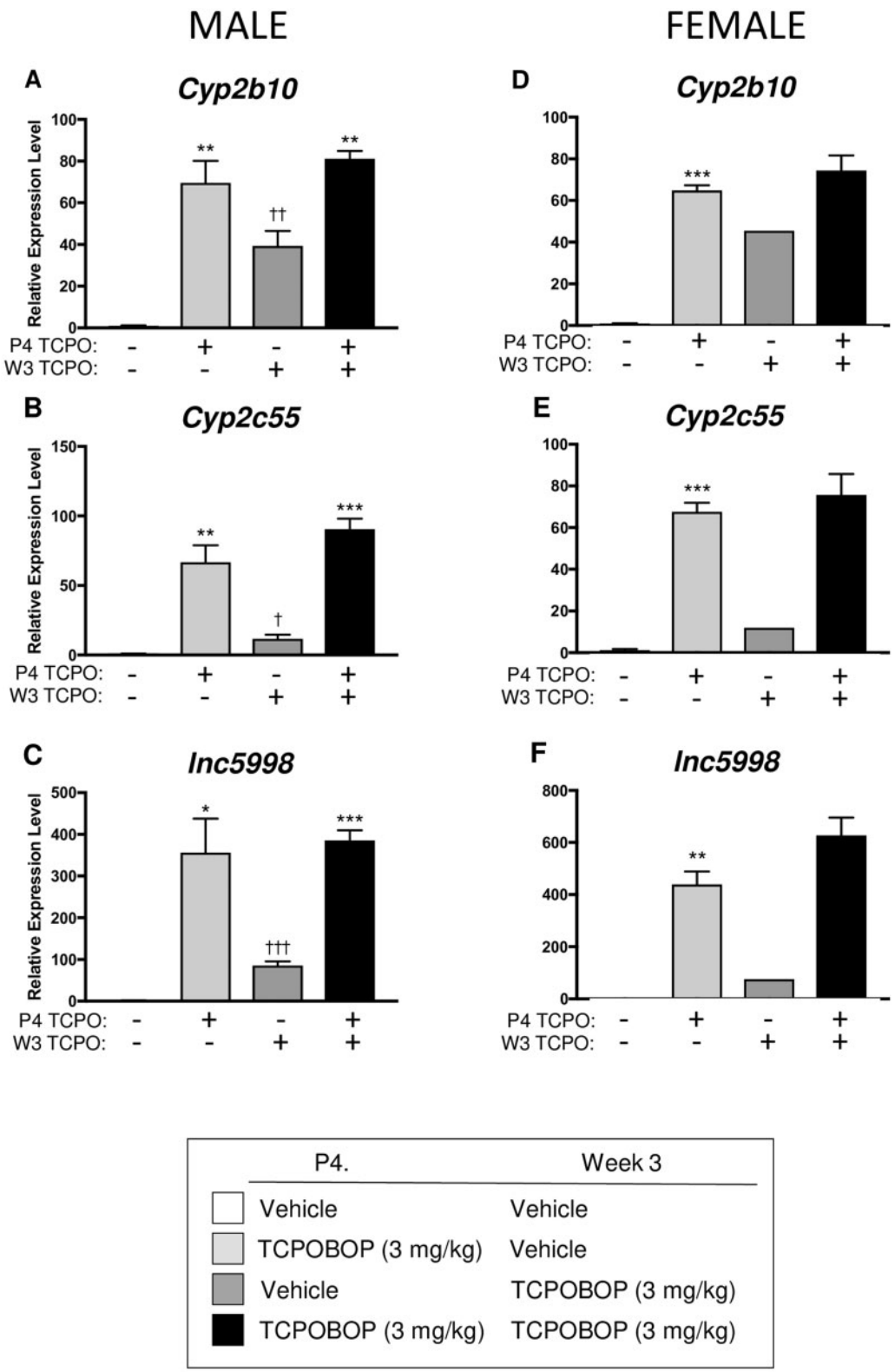

Figure 1. Impact of neonatal TCPOBOP exposure with repeat dosing in week 3 on the expression of CAR-responsive genes. Male and female pups were injected with TCPOBOP (3 mg/kg) or vehicle (control) on PND4, and in week 3, were again treated with TCPOBOP (3 mg/kg), or vehicle (control). All mice were euthanized in week 3, 3 h after the final injection ("Study A design"). Shown are relative RNA levels of each gene determined by RT-qPCR analysis of RNA extracted from each liver, with values normalized to that of the vehicle-only group (first bar) set to a value of 1.0. A–C, gene expression in male livers; D–F, gene expression in female livers. Data are shown as mean ± SEM ($n = 3, 4, 4, 4$ individual males, for bars 1–4, respectively, and $n = 2, 5, 1, 5$ individual females, for bars 1–4, respectively). Data were analyzed by pair-wise *t*-test for 2 separate comparisons: *Effects of TCPOBOP exposure on PND4 (bar 2 vs bar 1, and bar 4 vs bar 3); and †Effects of TCPOBOP in week 3 (bar 3 vs bar 1, and bar 4 vs bar 2). The same patterns were seen in both sexes, but the small sample size in the week 3 alone female group (bar 3) precluded a full statistical analysis. Significance: * or $^{\dagger}p < .05$; ** or $^{\dagger\dagger}p < .01$; *** or $^{\dagger\dagger\dagger}p < .001$.

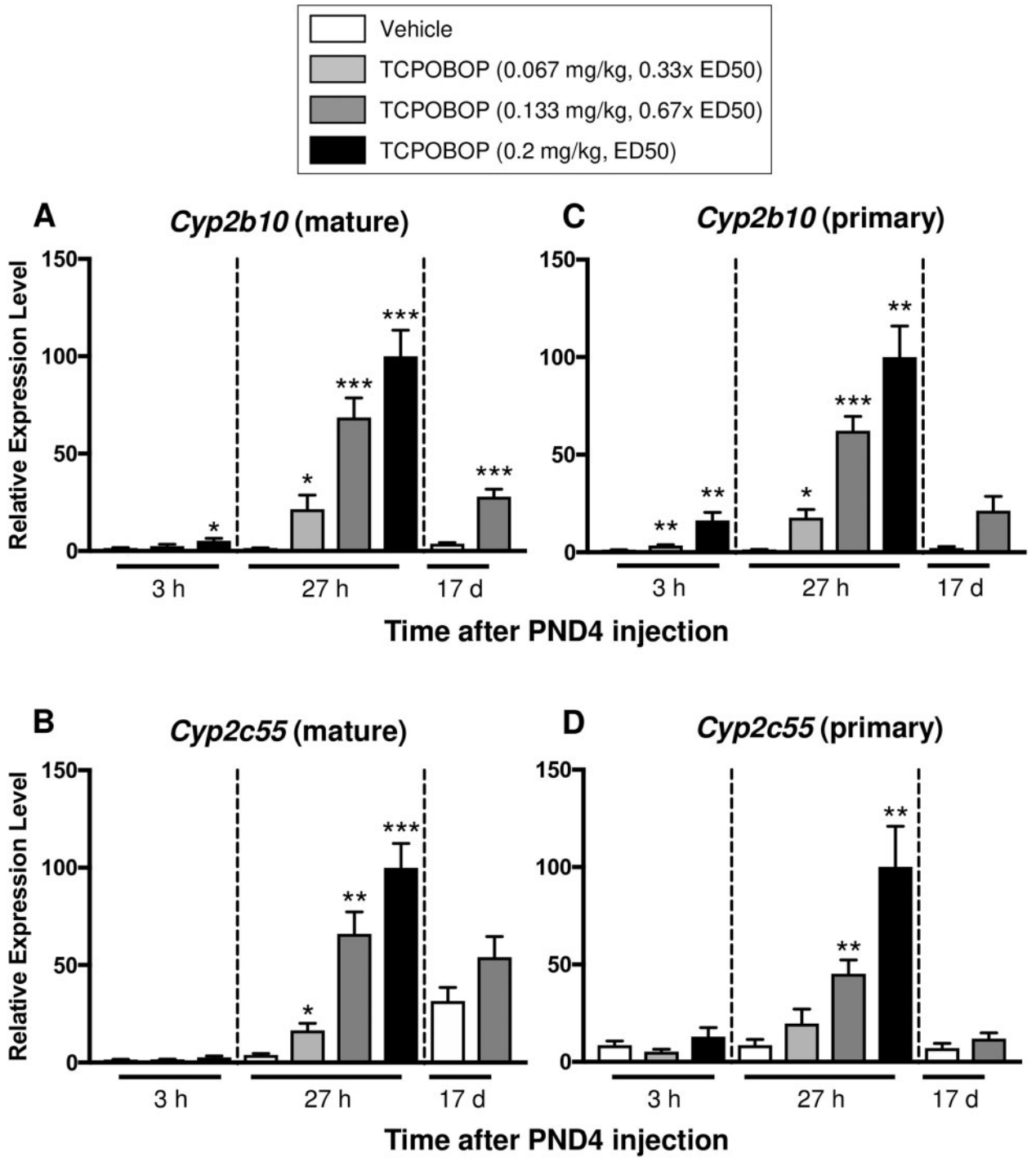

Figure 2. TCPOBOP dose-response in neonatal mouse liver. Male and female PND4 pups were injected with TCPOBOP at 0, 66.7 μg/kg (0.33× ED50), 133 μg/kg (0.67× ED50), or 200 μg/kg (ED50) and euthanized after 3 h (on PND4; 0 and 0.33x doses), after 27 h (on PND5), or after 17 days (on PND21; 0 and 0.67x doses). Shown are gene expression data for the mature and primary RNA transcripts of *Cyp2b10* and *Cyp2c55* for $n = 4$ to $n = 8$ livers per group. Data presentation as in Figure 1, with each experimental condition compared with its age-matched control (first bar at each time point): $^{*}p < .05$; $^{**}p < .01$; and $^{***}p < .001$. All Y-axis values are relative to the 27 h 0.2 mg/kg TCPOBOP group, which was set = 100 in each panel.

substantial increase in body size and liver weight from PND4 to PND21, which will effectively dilute the residual TCPOBOP concentration in liver. Thus, 17 days after TCPOBOP injection on PND4 at a dose of 0.67× ED50 (0.133 mg/kg), there is sufficient elimination of TCPOBOP to decrease *Cyp2b10* expression significantly. The transcriptional rate (primary transcript level) of *Cyp2c55* was also low after 17 days compared with 27 h. However, mature Cyp2c55 mRNA levels were elevated after 17 days (ie, on PND21), both with and without TCPOBOP, indicating there is a developmental accumulation in the basal level of Cyp2c55 mRNA, a general characteristic of many genes during this period of liver maturation (Gunewardena *et al.*, 2015). Based

on these findings, we selected an 0.67× ED50 dose of TCPOBOP (0.133 mg/kg) to evaluate the potential of neonatal TCPOBOP exposure for reprogramming later in life.

Optimization of Adult Challenge TCPOBOP Dose

Next, we sought to identify a suitable dose of TCPOBOP to use for a subsequent exposure, when adult mice exposed to TCPOBOP neonatally will be challenged with a second TCPOBOP injection. We reasoned that the second, challenge dose of TCPOBOP needs to induce CAR-responsive genes significantly, but to a level that is less than maximal, which would allow us to detect any additive or synergistic gene induction due to any long-term effects of the prior, neonatal exposure. Male mice, 7 weeks of age, were treated with TCPOBOP at 0.05×, 0.2×, and 1× ED50 doses, and livers were harvested 2 days later (51 h). This time point was chosen to give sufficient time for TCPOBOP to biodistribute to the liver and induce gene expression. Supplementary Figure S1 shows that TCPOBOP at the 0.2× ED50 dose (0.04 mg/kg) increased *Cyp2* gene expression significantly, but to a level that was sub-maximal; thus, gene responses were 5.4- to 5.6-fold lower (*Cyp2b10*) or 3.4- to 8.5-fold lower (*Cyp2c55*) than at the 1× ED50 dose for both mature and primary gene transcripts. Nonlinear dose-responses were apparent when comparing the 0.05× and 0.2× ED50 doses, which could be due to the retention in fat (Poland *et al.*, 1980) of a higher fraction of the administered TCPOBOP dose when mice are treated at the lowest dose.

Combination of Neonatal TCPOBOP With Adult Rechallenge Exposure

Based on the above dose-response studies, we injected mice with TCPOBOP at 0.67× ED50 on PND4 and then rechallenged the mice with a second TCPOBOP exposure at 0.2× ED50 in week 7. Livers were harvested 51 h later and analyzed for expression of CAR-responsive genes. PND4 TCPOBOP treatment alone had no discernable effect on *Cyp2b10* or *Cyp2c55* expression at 7 weeks, in either males or females (Figure 3, bar 2 vs bar 1). The low, sub-maximally inducing rechallenge dose of TCPOBOP given in week 7 had the expected short-term inductive effect on CAR target gene expression (Figure 3, bar 3 vs bar 1), but did not result in any additive or synergistic response when given to mice treated with TCPOBOP neonatally (Figure 3, bar 4 vs bar 3). Thus, neonatal exposure to TCPOBOP does not lead to persistent induction of these CAR target genes, nor does it sensitize these genes to a subsequent exposure at adulthood in either sex.

Given the absence of any reprogramming effects of neonatal TCPOBOP exposure when mice were exposed to low-dose (0.67× ED50) TCPOBOP neonatally (Figure 3), we sought to confirm the persistent induction of *Cyp2* family genes that was previously seen at week 12 when neonatal mice were treated with TCPOBOP at a high dose, 15× ED50 (3 mg/kg) (Chen *et al.*, 2012). Male and female PND4 pups were treated with TCPOBOP (3 mg/kg), and livers were collected at weeks 3, 7, and 12. Gene expression levels were compared with those seen in livers of week 7 male mice treated with a range of TCPOBOP doses (0, 0.2×, 1×, and 15× ED50) and euthanized 51 h later (Figure 4A). Neonatal TCPOBOP exposure at 15× ED50-induced persistent expression of *Cyp2b10* and *Cyp2c55* after both 7 and 12 weeks at levels significantly higher than the control group (Figs. 4B and C, groups G–J vs A). Moreover, the expression levels at week 7 were very similar to those seen in livers of 7-week mice exposed to low-dose TCPOBOP (0.2× ED50) for 51 h (group B). Next, we employed LC/MS analysis to measure hepatic TCPOBOP concentrations to determine whether the persistent expression of these genes can be explained by TCPOBOP remaining in the liver at 7 weeks. Figure 4D shows that residual TCPOBOP persists in liver tissue at week 7 and at week 12 at a level similar to the level found 51-h after TCPOBOP dosing at 0.2× ED50 (groups G–J vs B), which is also the neonatal dose that induces an equivalent level of persistent *Cyp2* expression (ie, groups G–J vs B in Figs. 4B and C). Thus, the persistent expression of both *Cyp2* genes under the conditions of TCPOBOP treatment used here and by others (Chen *et al.*, 2012) can be fully explained by the continued presence of TCPOBOP in liver tissue even 12 weeks after the initial dosing.

Long-Term Effects of Neonatal Phenobarbital Exposure

Taken together the studies above demonstrate that (1) neonatal exposure to a low-dose TCPOBOP (0.67× ED50) does not lead to persistent *Cyp2* gene expression at week 7; and (2) neonatal exposure to a high dose of TCPOBOP (15× ED50) does not allow us to evaluate its potential reprogramming effects later in life due to the persistent elevation of TCPOBOP levels in the liver. It remains possible, however, that *Cyp2* genes can be reprogrammed for persistent expression, but that this requires a high level of CAR activation in neonatal liver. We tested this hypothesis in neonatal mice exposed to phenobarbital, a CAR agonist with a much shorter half-life than TCPOBOP ($t_{1/2}$ [phenobarbital] = 15.8 h in PND19 mice [CD-1 strain], and $t_{1/2}$ = 7.5 h in adult mice [NMRI strain]) (Markowitz *et al.*, 2010).

For this study, we widened the window of neonatal phenobarbital exposure to encompass days PND2 through PND7 to include critical early time periods that may be required for gene reprogramming, as has been reported for several chemical exposures (Hanson and Gluckman, 2014; Hanson and Skinner, 2016; Vickers, 2011). Phenobarbital was delivered via the drinking water consumed by the dams, an established route for perinatal exposures (Waalkes *et al.*, 2003) that enables drug delivery to neonatal mice by lactation (Asoh *et al.*, 1999). Pups were weaned on PND21 and then left untreated until a second, challenge exposure to phenobarbital was given by i.p. injection at 7 weeks of age. We found that early phenobarbital exposure from PND2 to PND7 did not lead to persistent expression of *Cyp2b10* or *Cyp2c55* at week 7, except in the case of mature Cyp2c55 RNA in female liver (Figure 5, bar 2 vs bar 1). Furthermore, in females, we observed a moderate but significant increase in expression of the mature *Cyp2b10* transcript after phenobarbital rechallenge in week 7. The same trend was seen in males for the mature *Cyp2b10* transcript, and in both sexes for the primary *Cyp2b10* transcript, but without reaching statistical significance (Figs. 5A–D). In contrast, *Cyp2c55* showed a significant decrease in expression in female but not male liver after the phenobarbital rechallenge, as confirmed for both the mature and the primary transcripts.

These results were largely verified in a second study, where neonatal mice were injected with phenobarbital at 40 mg/kg/day on days PND4 and PND5, and then rechallenged with phenobarbital at 10 mg/kg/day on 3 consecutive days in week 7 (Supplementary Figure S2). Again, neonatal phenobarbital exposure alone did not lead to persistent expression after 7 weeks, but when combined with adult phenobarbital exposure in week 7, increased the level of *Cyp2b10* mature transcript in male mice significantly higher than that seen in mice given the week 7 exposure alone (Supplementary Figure S2, bar 4 vs bar 3). In females but not males, *Cyp2c55* showed a decreased response to the adult phenobarbital rechallenge, very similar to the

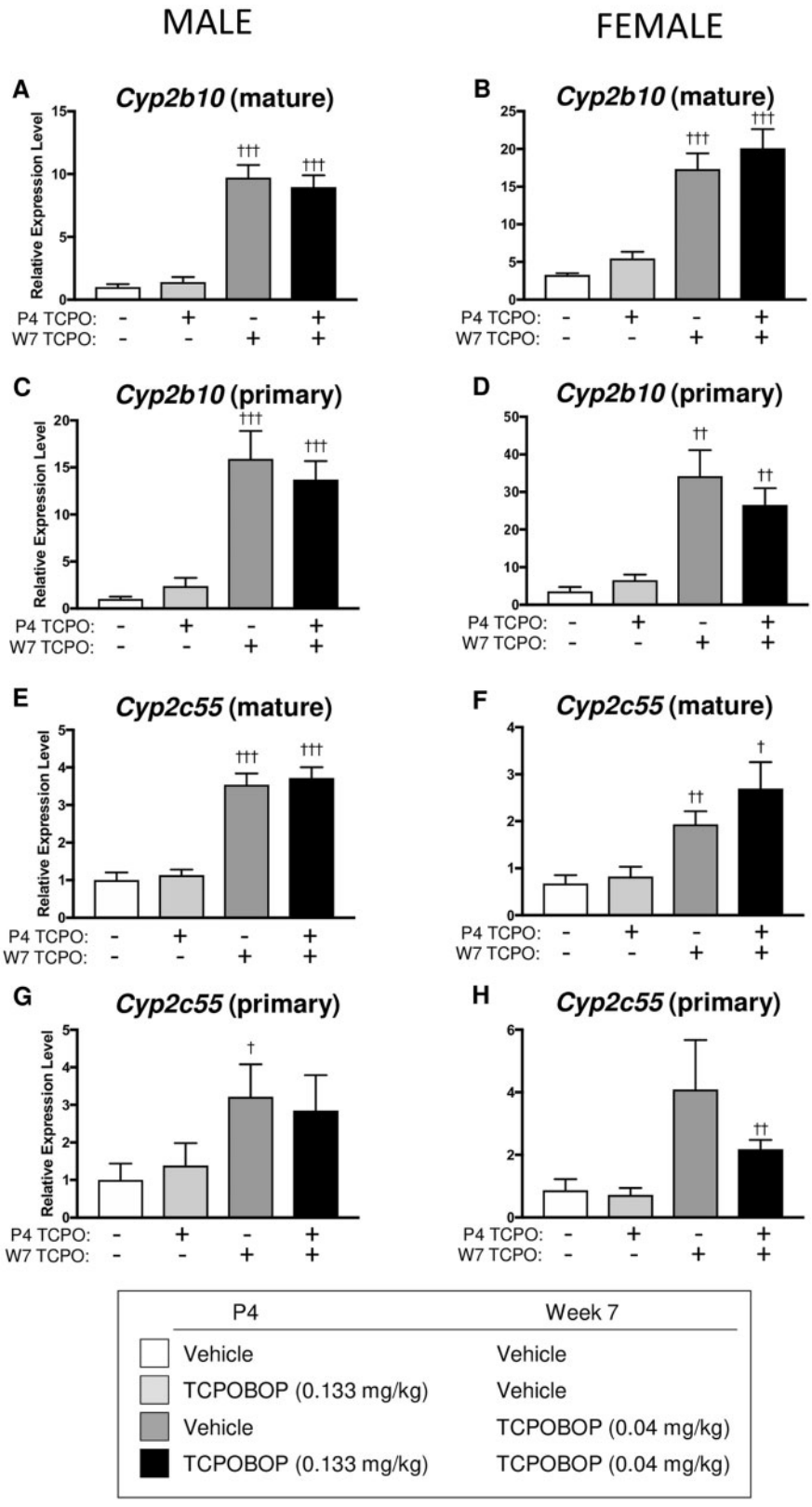

Figure 3. Neonatal TCPOBOP exposure followed by second TCPOBOP exposure in week 7. Male and female PND4 pups were injected with TCPOBOP at 133 μg/kg (0.67× ED50), or vehicle (control), and in week 7 were rechallenged with a second dose of TCPOBOP at 40 μg/kg (0.2× ED50) or with vehicle (control). All mice were euthanized in week 7, 51 h after the last injection ("Study design B"). Shown are the expression levels of the mature and primary transcripts of *Cyp2b10* and *Cyp2c55*, as indicated, for $n = 5$–7 livers per group. Data presentation as in Figure 1, comparing the effects of TCPOBOP exposure in week 7 alone (bar 3 vs bar 1) or in combination with TCPOBOP exposure on PND4 (bar 4 vs bar 2): $^{\dagger}p < .05$; $^{\dagger\dagger}p < .01$; and $^{\dagger\dagger\dagger}p < .001$. The effects of TCPOBOP exposure on PND4 were not significant for any of the genes in these livers, collected at age 7 weeks (bar 2 vs bar 1, and bar 4 vs bar 3). Y-axis values are expressed relative to the vehicle-treated control male group for each gene.

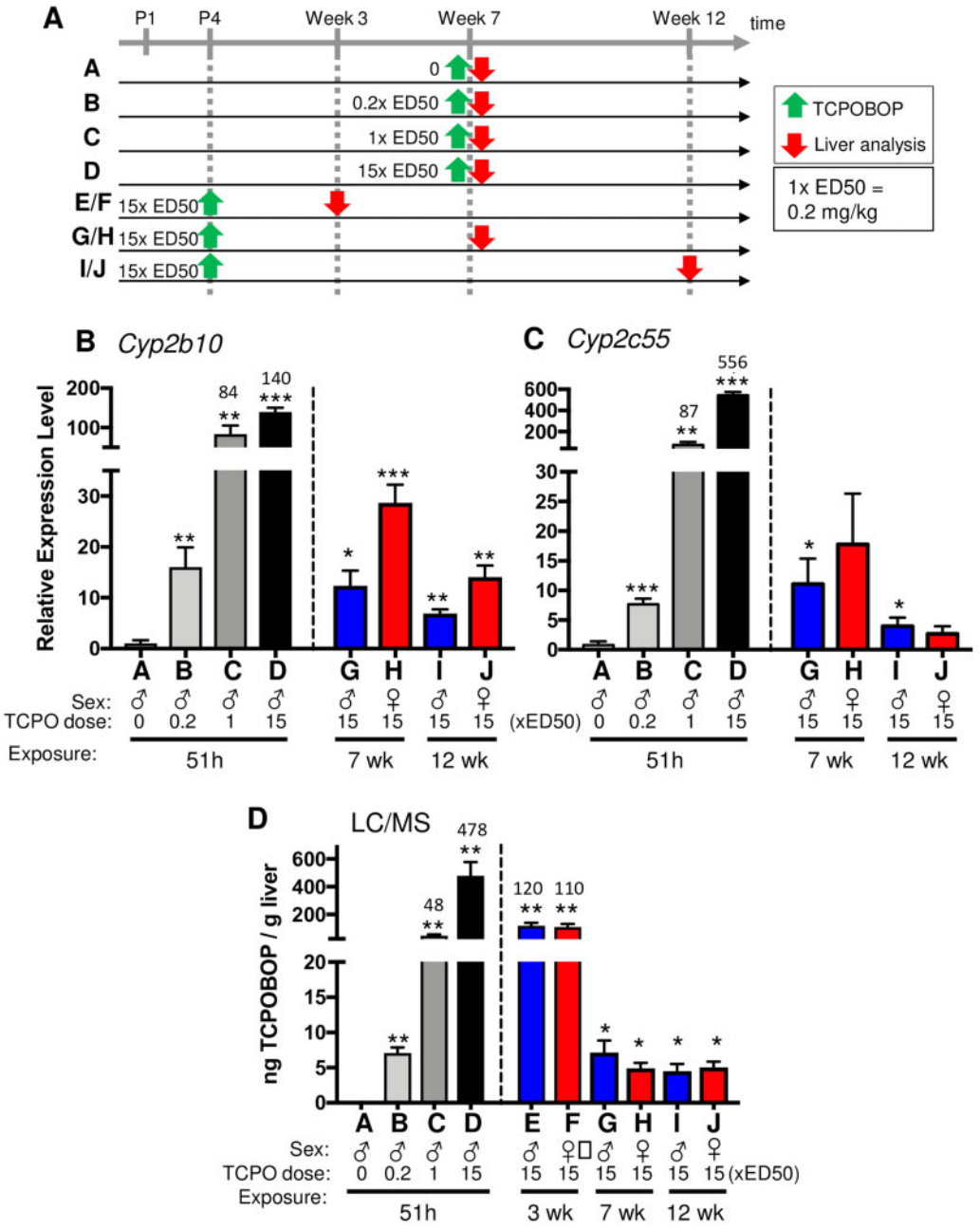

Figure 4. Neonatal exposure to TCPOBOP (3 mg/kg) results in significant residual TCPOBOP and persistent *Cyp2* gene expression after 7 and 12 weeks. A, Time course and experimental design. Green arrows, TCPOBOP treatment; red arrows, collection of liver for analysis. Male and female PND4 pups were injected with 3 mg/kg of TCPOBOP (15× ED50 dose) and were euthanized in week 3, 7 or 12 (groups E–J, green and red arrows). For comparison, 7-week-old male mice were injected with TCPOBOP at 0, 0.2×, 1×, and 15× ED50 doses (0, 0.04, 0.2, or 3 mg/kg, respectively) and were euthanized 51 h later (groups A–D; gray and black bars in panels B–D). Further details about each group are shown along the x-axis of panels B–D. B and C, RT-qPCR analysis of liver RNA, with expression levels of *Cyp2b10* and *Cyp2c55* normalized to group A (vehicle control). D, TCPOBOP was extracted from 0.5 g of each liver with hexane and quantified by LC/MS. In (B–D), data shown are mean and SEM, $n = 4$ livers per group. Values above the tallest bars indicate the actual y-axis values. Significance compared with vehicle control (group A): $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$. Please refer to the online article for colored figure.

decrease seen in Figure 5F and H, but this effect did not reach statistical significance. We conclude that neonatal exposure to a high dose of phenobarbital induces a moderate reprogramming of the expression of these *Cyp2* genes in week 7. These reprogramming effects of early phenobarbital exposure are not indirect responses to changes in CAR expression, which did not change significantly with these treatments (Supplementary Figure S3A).

Impact on Sex-Specific Liver Gene Expression

Perinatal exposure of rats to phenobarbital can cause long-term dysfunctions such as changes in circulating hormone levels, decreased levels of hepatic monoamine oxidase and infertility (Agrawal *et al.*, 1995; Gupta *et al.*, 1982; Soliman and Richardson, 1983). In particular, neonatal phenobarbital exposure was found to alter pituitary secretion patterns and circulating blood levels of growth hormone later in life, and as a consequence, it alters

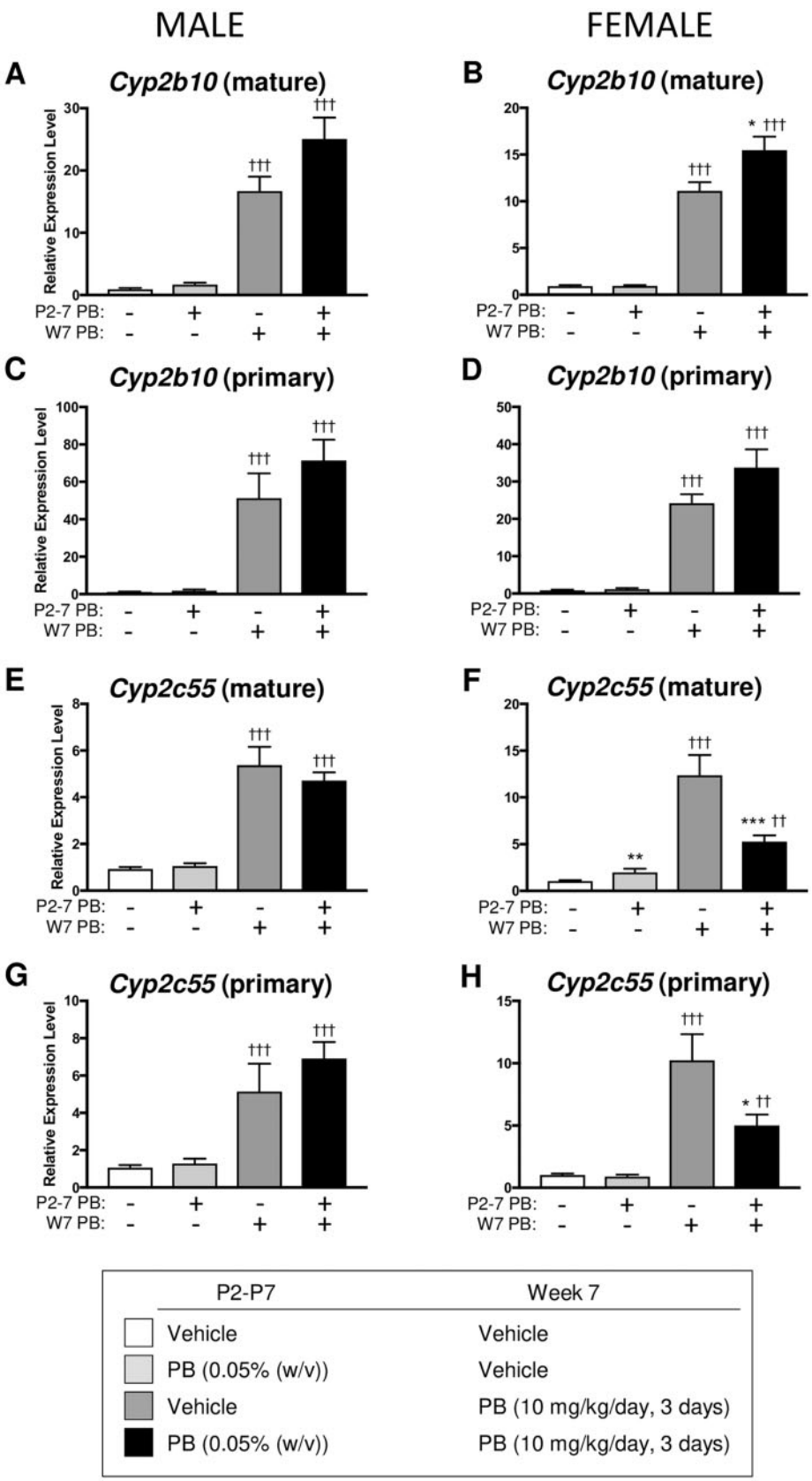

Figure 5. Neonatal phenobarbital exposure (0.05% [w/v] in drinking water of the dams) from PND2 to PND7, with a second phenobarbital exposure (10 mg/kg, daily for 3 days), or vehicle control, given in week 7. All mice were euthanized in week 7, 3 h after the last injection ("Study design C"). Shown are RT-qPCR expression data for mature and primary transcripts for *Cyp2b10* and *Cyp2c55*, normalized to the vehicle control (first bar in each set), mean ± SEM ($n = 21, 12, 5, 12$ individual males, for bars 1–4, respectively, and $n = 23, 8, 8, 14$ individual females, for bars 1–4, respectively). Data presentation as in Figure 1, comparing the effects of phenobarbital exposure in week 7 alone (bar 3 vs bar 1) or in week 7 in combination with PND2–PND7 phenobarbital exposure (bar 4 vs bar 2): *Comparison of bar 2 vs bar 1, and of bar 4 vs bar 3; and †Comparison of bar 3 vs bar 1, and of bar 4 vs bar 2. Significance: * or $^{\dagger}p < .05$; ** or $^{\dagger\dagger}p < .01$; *** or $^{\dagger\dagger\dagger}p < .001$.

adult liver expression patterns of *Cyp* genes that show sex-dependent, growth hormone-regulated expression patterns (Agrawal and Shapiro, 2003). Here, we investigated the effects of both neonatal and 7-week phenobarbital exposure on the adult liver expression of 2 sex-specific, growth hormone-regulated genes, the female-specific *A1bg* and the male-specific *Cyp7b1* (Supplementary Figure S3). Neonatal phenobarbital exposure had no effect on the expression of either gene at 7 weeks of age. Furthermore, adult exposure to phenobarbital, at 7 weeks of age, led to only a small increase in *Cyp7b1* expression. These findings suggest that in the mouse model, early phenobarbital exposure does not lead to major changes in plasma growth hormone profiles known to regulate these sex-specific genes. These findings, in turn, suggest that the effects of neonatal phenobarbital on *Cyp2* genes, described above, are unlikely to be an indirect response to a change in circulating growth hormone levels.

DISCUSSION

Exposure to endocrine-active environmental chemicals during the perinatal period, a critical window of developmental plasticity, has been proposed to reprogram development and alter disease susceptibility later in life. Many environmental chemicals disrupt gene expression by interaction with xenobiotic sensors from the Nuclear Receptor superfamily, including CAR, which coordinates cellular and transcriptional responses affecting hepatic drug metabolism, energy homeostasis, and tumor development. A prior study found that TCPOBOP injection in PND4 mice-induced long-term hepatic expression of several CAR target genes from the *Cyp2* family. Elevated expression persisted into adulthood in association with long-term epigenetic changes (Chen *et al.*, 2012), which could involve the activation of an epigenetic switch (Lempiainen *et al.*, 2011). However, given the long biological half-life of TCPOBOP ($t_{1/2} \sim 2$ weeks) (Poland *et al.*, 1980), we asked whether the persistence of TCPOBOP in mouse tissue, rather than an epigenetic memory, might drive the persistence of gene expression. We confirmed that the neonatal TCPOBOP exposure regimen used by Chen *et al.* (2012) does indeed induce long-term increases in liver *Cyp2* expression lasting at least 12 weeks; however, the persistence of expression was readily explained by the persistence of TCPOBOP in liver tissue at a level sufficient to account for the prolonged increase in expression that we observed. We were able to avoid the long-term persistence of TCPOBOP in liver tissue by decreasing the neonatal TCPOBOP exposure dose 22-fold, from a dose of 15× ED50 used in Chen *et al.* (2012) to a dose of 0.67× ED50. However, although strong neonatal increases in hepatic *Cyp2* expression were still achieved, they did not persist into adulthood. Moreover, this early ED50-range exposure to TCPOBOP did not sensitize mice to a subsequent, low-dose TCPOBOP treatment. We conclude that tissue persistence of TCPOBOP, rather than an epigenetic memory, drives the persistence of elevated *Cyp2* gene expression seen in mouse liver. These findings highlight the importance of carefully considering both dose and pharmacokinetics of elimination from tissue depots when evaluating chemicals such as TCPOBOP for potential reprogramming effects of early life exposures.

Persistent local epigenetic changes were reported by Chen *et al.* (2012) in the neonatal TCPOBOP exposure model; however, we previously reported that those same epigenetic changes are also induced by short-term TCPOBOP exposure (Rampersaud *et al.*, 2019). Consequently, we can attribute those epigenetic changes to the ongoing activation of CAR by residual TCPOBOP in liver tissue, rather than to a long-term epigenetic memory of the initial exposure. TCPOBOP has also been shown to accumulate in mouse maternal adipose tissue, from where it can be transferred to pups by lactation to activate CAR-responsive genes in the liver (Dietrich *et al.*, 2018). Exposures via that route may appear to give rise to transgenerational effects on gene expression or epigenetics, when in fact they are directly linked to the parental exposure. For TCPOBOP and other lipophilic chemicals, it can thus be difficult to distinguish true long-term gene and epigenetic dysregulation from long-term changes due to ongoing exposure via tissue depots that persist in liver, fat or elsewhere. For such chemicals, reducing the exposure dose to effectively shorten the overall exposure period, as was done here for TCPOBOP, is one approach to determine a chemical's intrinsic potential for persistent biological effects, albeit with the caveat that in some cases a higher dose may be needed to elicit a robust, long-term epigenetic response.

We used phenobarbital to test whether a threshold level of CAR activation, perhaps not reached by TCPOBOP at a 0.67× ED50 dose, may be required for long-term *Cyp2* reprogramming. This short half-life CAR agonist ($t_{1/2} = 7.5$ h in adult mice) (Markowitz *et al.*, 2010) enabled us to achieve high-level neonatal CAR activation without the persistent exposure that is unavoidable when using correspondingly high doses of TCPOBOP due to its 40- to 50-fold longer half-life. Neonatal phenobarbital exposure induced long-term changes in the responsiveness of *Cyp2* genes to a second, low-dose exposure to phenobarbital at adulthood. Specifically, we observed moderate increases in the responsiveness of *Cyp2b10* to low-dose phenobarbital at week 7, as well as decreased responsiveness of *Cyp2c55* in female but not male liver, as was seen in 2 neonatal exposure models with different designs. These findings support earlier work in the rat model, where neonatal phenobarbital administration led to 30–40% over-induction of rat hepatic CYP2B1 and CYP2B2 RNA and protein in rats rechallenged as adults with low doses of the barbiturate (Agrawal and Shapiro, 1996). Persistent induction of mouse hepatic CAR target gene RNA and protein was previously described following neonatal phenobarbital exposure (Tien *et al.*, 2015), however, we found that the LD50-range dose of phenobarbital used in that study (>200 mg/kg on PND5) was severely toxic with some lethality (unpublished experiments) and is thus not pharmacologically relevant. Further investigation will be required to verify the present results at the level of Cyp2 protein and functional activity, and to elucidate underlying mechanisms, including why the early life exposure to phenobarbital employed in our study has opposite effects on *Cyp2b10* and *Cyp2c55*, both of which are themselves strongly induced by the initial phenobarbital treatment. Moreover, as phenobarbital activates both CAR and the related nuclear receptor PXR, and with significant overlap between their target genes (Cui and Klaassen, 2016), it will be important to determine which receptor mediates the long-term gene responses to neonatal phenobarbital exposure described here.

We initially selected TCPOBOP for studying long-term effects of early CAR activation due to its high specificity for a single nuclear receptor, CAR (Tojima *et al.*, 2012; Tzameli *et al.*, 2000), and for the unusually strong gene responses it can induce, as exemplified by the *Cyp2* genes examined here. Although TCPOBOP is a specific activating ligand of rodent but not human CAR owing to species-specific differences in CAR's ligand-binding domain (Mackowiak and Wang, 2016), DNA-binding and the associated key genomic and epigenetic effects of CAR activation are most likely conserved across mammalian species. Studies of indirect CAR agonists, such as those reported here for phenobarbital,

may be more readily extrapolated across species due to conservation of the overall signaling pathways through which they activate CAR. Phenobarbital activates CAR by inhibiting epidermal growth factor receptor signaling (Chai *et al.*, 2016), which ultimately leads to dephosphorylation of CAR-threonine 38 and CAR nuclear translocation (Mackowiak and Wang, 2016; Qatanani and Moore, 2005). One limitation, however, is that multiple receptors are often activated by xenobiotics that dysregulate gene expression in the liver (ie, both CAR and PXR in the case of phenobarbital), which complicates mechanistic studies of any downstream epigenetic actions. Epigenetic reprogramming may also occur in adult mouse exposure models, where long-term treatment with phenobarbital induces many novel differentially methylated and hydroxymethylated genomic regions that strongly correlate with transcriptional responses and are not found after a short-term exposure (Thomson *et al.*, 2013).

Finally, our findings have implications for studies such as those of the Target II consortium (Wang *et al.*, 2018), where multiple environmental chemical exposures are being investigated in perinatal mouse models, including evaluation of changes in gene expression, changes in chromatin accessibility, and other epigenetic changes at adulthood. Understanding the mechanisms underlying these processes will provide important insight into how exposure to environmental chemicals and pharmaceuticals in early life can influence long-term health outcomes and disease risk. Challenges going forward will include distinguishing long- versus short-term effects for chemicals with long half-lives, determining which receptors mediate the effects observed and elucidation of underlying mechanisms, including mechanisms driving epigenetic changes that are expected to be a main driver of long-term phenotypes seen following many environmental chemical exposures.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.S. and D.J.W. jointly conceived of the study. All of the experimental work, data analysis, and figure preparation were performed by A.S. D.J.W. drafted the manuscript with input from A.S. D.J.W. provided guidance and supervised the overall project and prepared the final manuscript for publication. Both authors reviewed and approved the manuscript.

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