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2 **Impact of neonatal activation of nuclear receptor CAR (Nr1i3) on *Cyp2* gene expression in adult mouse**

3 **liver**

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15 Key words: TCPOBOP, phenobarbital, epigenetic reprogramming, growth hormone, Cyp2b10
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17 Running head: Neonatal activation of CAR (Nr1i3) in mouse liver
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38   **Abstract:** Perinatal exposure to environmental chemicals is proposed to reprogram development and

39   alter disease susceptibility later in life. Supporting this, neonatal activation of the nuclear receptor CAR

40   (Nr1i3) by TCPOBOP induces persistent expression of mouse hepatic *Cyp2* genes into adulthood,

41   attributed to long-term epigenetic memory of the early life exposure [Hepatology (2012) 56:1499-1509].

42   Here, we confirm that the same high-dose (15x ED50) neonatal TCPOBOP exposure used in that work

43   induces prolonged (12 weeks) increases in hepatic *Cyp2* expression; however, we show that the

44   persistence of expression can be fully explained by the persistence of residual TCPOBOP in liver tissue.

45   When the long-term presence of TCPOBOP in tissue was eliminated by decreasing the neonatal

46   TCPOBOP dose 22-fold (0.67x ED50), strong neonatal increases in hepatic *Cyp2* expression were still

47   obtained but did not persist into adulthood. Furthermore, the neonatal ED50-range TCPOBOP exposure

48   did not sensitize mice to a subsequent, low-dose TCPOBOP treatment. In contrast, neonatal treatment

49   with phenobarbital, a short half-life ($t_{1/2}$=8 h) agonist of CAR and of PXR (Nr1i2), induced high-level

50   neonatal activation of *Cyp2* genes and also altered their responsiveness to low-dose phenobarbital

51   exposure at adulthood by either increasing (*Cyp2b10*) or decreasing (*Cyp2c55*) expression. Thus,

52   neonatal xenobiotic exposure can reprogram hepatic *Cyp2* genes and alter their responsiveness to

53   exposures later in life. These findings highlight the need to carefully consider xenobiotic dose, half-life

54   and persistence in tissue when evaluating the long-term effects of early life environmental chemical

55   exposures.

56

57   **Introduction**

58

59   Many environmental chemicals dysregulate gene expression, most notably in hepatocytes, by

60   mechanisms that involve activation of members of the Nuclear Receptor superfamily (Toporova and

61   Balaguer 2020; Waxman 1999). CAR and other nuclear receptors are activated by a wide range of

62   structurally diverse foreign chemicals, including many industrial pollutants and pharmaceuticals

63   (Baldwin and Roling 2009; Chang and Waxman 2006; Hernandez et al. 2009; Kobayashi et al. 2015;

64   Omiecinski et al. 2011). CAR also regulates normal physiological pathways, including hepatic energy

65   homeostasis, cell proliferation and inflammation (Cai et al. 2021) and may thereby impact

66   pathophysiological conditions such as fatty liver disease, diabetes, and hepatocellular carcinoma (Cave

67   et al. 2016; Dong et al. 2009; Phillips et al. 2007).

68

69 Under normal cellular conditions, CAR is sequestered in the inactive state in the cytoplasm as a multi-

70 protein complex, including heat shock protein 90 and cytoplasmic CAR retention protein (Kobayashi et

71 al. 2003; Timsit and Negishi 2014; Yoshinari et al. 2003). CAR agonist ligands, such as TCPOBOP, bind to

72 the ligand-binding domain of CAR, leading to dissociation of its cytoplasmic chaperones and

73 translocation of CAR to the nucleus, where it heterodimerizes with retinoid X receptor (RXR)

74 (Mackowiak and Wang 2016). The CAR/RXR heterodimer subsequently recruits coactivators and binds to

75 specific response elements in genomic DNA, followed by the transcriptional activation of many genes,

76 including phase I and phase II enzymes of drug metabolism and transporters that regulate the

77 metabolism of endogenous and exogenous chemicals (Mackowiak and Wang 2016; Qatanani and Moore

78 2005). CAR activation is associated with epigenetic changes in mouse liver proximal to CAR binding sites

79 (Niu et al. 2018; Tian et al. 2018) and nearby CAR responsive genes (Rampersaud et al. 2019). These

80 changes are apparent as early as 3 h after exposure to TCPOBOP and include both increases and

81 decreases in chromatin accessibility, which can be monitored as changes in DNase hypersensitivity

82 (Lodato et al. 2018; Vitobello et al. 2019). Changes in histone methylation and acetylation, and changes

83 in DNA methylation, have also been linked to the activation and repression of CAR target genes in

84 mouse liver (Lempiainen et al. 2011; Rampersaud et al. 2019).

85

86 Early developmental exposure to xenobiotics, including chemicals that can activate CAR, has been

87 proposed to lead to neonatal reprogramming (neonatal imprinting) in a way that can alter metabolic

88 function and gene expression in liver and other tissues (Cave 2020; Küblbeck et al. 2020; Piekos et al.

89 2017). Potential effects on life expectancy (Agrawal and Shapiro 2005) and disease susceptibility later in

90 life have also been reported (Hochberg et al. 2011). The molecular mechanisms that underlie the early

91 developmental lesions that lead to adult pathophysiology are likely to be epigenetic in nature but are

92 poorly understood (Moggs and Terranova 2018; Nahar et al. 2014; Treviño et al. 2020). For example,

93 when CAR is activated in neonatal mouse liver by the CAR-specific agonist TCPOBOP, a persistent

94 increase in expression was reported for two CAR target genes, *Cyp2b10* and *Cyp2c37*, via a proposed

95 epigenetic memory of neonatal CAR exposure (Chen et al. 2012). However, the long half-life apparent

96 for liver metabolic activities induced by TCPOBOP (Poland et al. 1980) raises the question of whether

97 continued presence of TCPOBOP in mouse tissue, rather than an epigenetic memory, drives the

98 observed persistence of CAR target gene induction.

99

100   Here, we show that neonatal exposure to TCPOBOP at a dose widely used by many investigators, 3

101   mg/kg, induces long-term, persistent activation of CAR-responsive genes in the liver, but also results in

102   the persistence of TCPOBOP in liver tissue at a concentration we find is sufficiently high to account for

103   the prolonged increase in expression of *Cyp2* family genes in the liver. Additionally, we characterize the

104   impact of neonatal exposure to phenobarbital, which activates CAR indirectly via changes in CAR

105   phosphorylation (Negishi et al. 2020). Our findings show that neonatal exposure to phenobarbital, which

106   lacks the complications of long-term persistence in tissue owing to its comparatively short half-life (~8 h)

107   (Markowitz et al. 2010) compared to TCPOBOP, sensitizes mouse liver to a subsequent exposure to

108   phenobarbital in adulthood. Thus, such short-lived chemicals are more amenable for studying

109   mechanisms by which CAR and other xenobiotic sensors translate early environmental stimuli to

110   persistent changes in gene expression.

111

112   **Materials and Methods**

113

114   Animals - All mouse work was carried out in compliance with procedures approved by the Boston

115   University Institutional Animal Care and Use Committee (protocol # PROTO201800698), and in

116   compliance with ARRIVE 2.0 Essential 10 guidelines (Percie du Sert et al. 2020), including study design,

117   sample size, randomization, experimental animals and procedures, and statistical methods. Adult male

118   and female CD-1 mice (8-10 weeks of age) were purchased from Charles River Laboratories (Wilmington,

119   MA) and housed in the Boston University Laboratory Animal Care Facility. Mice were kept on a 12-hour

120   light cycle (7:30 AM – 7:30 PM). For mouse breeding, one adult male and one adult female mouse were

121   housed together until a vaginal plug was observed, at which time the mice were separated. The first day

122   pups were observed was designated as postnatal day 1 (PND1). Litters were housed with dams until the

123   mice were weaned on PND21. Mice were treated with TCPOBOP (1,4-Bis-[2-(3,5-

124   dichloropyridyloxy)]benzene; Sigma-Aldrich, St. Louis, MO) dissolved in a 1% DMSO solution in

125   tocopherol-stripped corn oil (Fisher Scientific), or with phenobarbital (sodium salt; Sigma-Aldrich)

126   dissolved in 0.9% sodium chloride solution, or with vehicle (control) by intraperitoneal injection

127   between 8:00 AM and 8:45 AM on the day(s) of treatment at doses stated in the text. Treatments were

128   administered to mice at the ages ranging from PND4 to 7 weeks of age, as specified for each study. Mice

129   were euthanized after a time period ranging from 3 h to 51 h after TCPOBOP or vehicle (control)

130   injection, and at a fixed time of day (between 11:00 AM and 11:45 AM) to minimize gene expression

131   variations between mice due to the strong circadian effects on gene expression in liver (Kettner et al.

132   2016). Where indicated, pregnant dams were given either drinking water supplemented with 0.05%

133   (w/w) phenobarbital or drinking water as a control. A small piece of each liver was snap frozen in liquid

134   nitrogen then stored at -80C prior to extraction of RNA for qPCR analysis and of TCPOBOP for LC/MS

135   analysis.

136

137   <u>Neonatal mouse exposure models</u> - Mice were exposed to either TCPOBOP or phenobarbital during the

138   perinatal period and, where indicated, again later in life, using one of the following four exposure

139   schedules. In Study A (Fig. 1), PND4 pups were given TCPOBOP by i.p. injection at 3 mg/kg or vehicle

140   (control). Mice were given a second injection of TCPOBOP (3 mg/kg) or vehicle in week 3 (on a day

141   between PND20 and PND23) and euthanized 3 h later. In Study B (Fig. 3), PND4 pups were given

142   TCPOBOP at 133 μg/kg (0.67x ED50) or vehicle. Mice were given a second injection of TCPOBOP, at 40

143   μg/kg (0.2x ED50), or vehicle, in week 7 (on days PND46-PND52) and euthanized 51 h later. In Study C

144   (Fig. 5), newborn mice were exposed to phenobarbital given to nursing dams through their drinking

145   water (0.05% (w/v) phenobarbital) to give a 6 consecutive-day perinatal exposure (PND2 through PND7).

146   In week 7 (on days PND49-PND52), mice were given phenobarbital by i.p. injection at 10 mg/kg/day, or

147   vehicle (control), on each of 3 consecutive days and euthanized 3 h after the last injection. Finally, in

148   Study D (Fig. S2), PND4 pups were given two i.p. injections of phenobarbital, each at 40 mg/kg, or

149   vehicle, on PND4 and again on PND5. In week 7 (on days PND49-50), the mice were injected with

150   phenobarbital at 10 mg/kg/day i.p., or vehicle, on each of 3 consecutive days and euthanized 3 h after

151   the last injection.

152

153   <u>RNA purification and RT-qPCR</u> – A portion of each liver (0.1-0.2 g) was homogenized for 30 sec in 1 mL of

154   TRIzol using a Polytron homogenizer. The TRIzol homogenate was processed according to the

155   manufacturer's protocol (Life Technologies) to purify total liver RNA. The purified RNA was treated with

156   DNase I (Promega) to remove genomic DNA contaminants and then converted to cDNA using Applied

157   Biosystems High Fidelity RT kit (ThermoFisher). Quantitative real time PCR (qPCR) primer pairs specific

158   for mature mRNA were designed to be in adjacent exons of each target gene, with amplicons spanning

159   long introns to reduce the likelihood that amplification of contaminating genomic DNA would contribute

160   to the qPCR signal. Primer pairs specific for primary (unspliced) RNA transcripts were designed to have

161   amplicon span either an exon-injunction or an intron-exon junction of the target gene. qPCR was

162   performed using Power SYBR Green PCR Master Mix (ThermoFisher). Relative RNA expression levels

163   were calculated using the ΔΔCt method and normalized to the expression of 18S ribosomal RNA. Primer

164   sequences used to amplify each gene are shown in Table S1.

165

166   TCPOBOP extraction and LC/MS - A piece of each frozen liver was added to PBS (0.5 g liver/0.5 mL PBS)

167   and immediately homogenized using a Polytron homogenizer for 30 sec. The homogenate (~ 1 mL) was

168   shaken with 5 mL of hexane for 1 h at room temperature. The hexane layer was transferred to a clean

169   glass tube, and 5 mL of fresh hexane was added to the remaining homogenate and vortexed on a flat

170   platform for 1 h at room temperature. The hexane layers were combined and evaporated under a gentle

171   stream of nitrogen gas. The residue was dissolved in ~0.1-0.2 mL of 4% DMSO (Sigma-Aldrich) in

172   acetonitrile (Fisher Scientific) and stored at -20C until LC/MS analysis. HPLC was performed using a C18

173   column run at a flow rate of 0.6 ml/min of 30% water and 70% acetonitrile. The HPLC-separated peaks

174   were further analyzed using a Waters QTof Premier mass spectrometer in the Boston University

175   Chemical Instrumentation Core. Standard curves for TCPOBOP quantification were generated using pure

176   TCPOBOP dissolved in 4% DMSO in acetonitrile. The prominent TCPOBOP total ion count peak at 402.96

177   m/s was used to calculate the concentration of TCPOBOP present in each liver extract.

178

179   Statistical analysis – Data are presented as mean +/- SEM for the number of individual livers (biological

180   replicates) specified in each figure legend. Significance was assessed by two-tailed t-test for pairwise

181   comparisons specified in each figure legend and implemented in GraphPad Prism.

182

183   **Results**

184

185   **Responsiveness of neonatal mice to TCPOBOP**. We investigated the effects of neonatal exposure to

186   TCPOBOP (3 mg/kg on PND4) on liver expression of *Cyp2b10* and *Cyp2c55*, which are both induced >50-

187   fold within 3 h of TCPOBOP treatment in adult mouse liver (Lodato et al. 2017). TCPOBOP stimulated a

188   persistent induction of both Cyp2 genes, as well as of lnc5998, a lncRNA that is highly TCPOBOP-

189   inducible and is divergently transcribed from *Cyp2b10* (Lodato et al. 2017) (Fig. 1, bar 2 vs bar 1). Large,

190   significant inductions were also seen in livers of mice given a single injection of TCPOBOP in week 3 and

191   euthanized 3 h later (bar 3), but the extent of induction was lower than was seen in the PND4-treated

192   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

193   4). Similar results were observed in female mice (Fig. 1A-C vs Fig. 1D-F). The increased effectiveness of

194   TCPOBOP at inducing gene expression in mice receiving both TCPOBOP treatments was much greater for

195   *Cyp2c55* and *lnc5998* than for *Cyp2b10*, which both respond to a single injection of TCPOBOP more

196   slowly than *Cyp2b10* (Lodato et al. 2017), and hence are not maximally induced at the 3 h time point.

197

198   Although all three genes were significantly induced by both neonatal and week 3 TCPOBOP exposure,

199   we did not observe a further increase beyond that induced by neonatal TCPOBOP in either sex when the

200   mice were challenged with a second injection of TCPOBOP at 3 weeks of age (Fig. 1, bar 4 vs bar 2).

201   Given the long half-life of TCPOBOP, about 2 weeks in adult mice (Poland et al. 1980), these findings

202   suggest that the transcriptional activation of these CAR target genes reaches its maximal level following

203   the first TCPOBOP injection on PND4, and that the higher expression at week 3 seen in PND4-treated

204   mice compared to week 3 (3 h)-treated mice (bar 2 vs. bar 3) reflects the much longer effective

205   exposure time in the PND4 treatment group (17 days vs 3 h).

206

207   **Optimization of neonatal and TCPOBOP dose**. We sought to distinguish any potential long-term

208   reprogramming effects of neonatal TCPOBOP exposure from effects due to the residual TCPOBOP that

209   may persist in liver or other tissues. We first optimized the dose of TCPOBOP used in the initial, neonatal

210   exposure. We reasoned that the dose needs to be high enough to activate a robust liver gene response

211   but sufficiently low to be effectively cleared within a few weeks, i.e., to a level low enough to discern the

212   impact of a second TCPOBOP exposure later in life. PND4 mice were given TCPOBOP at doses of 0.33x,

213   0.67x and 1x the ED50 dose, based on the ED50 value of 0.2 mg TCPOBOP/kg body weight reported for

214   induction of hepatic cytochrome P450-dependent aminopyrine N-demethylase activity in 6 week female

215   mouse liver (Poland et al. 1980). Of note, the ED50 value of 0.2 mg/kg is 15-fold lower than the standard

216   dose of 3 mg/kg TCPOBOP that is widely used in mouse liver studies of CAR target gene induction,

217   including Fig. 1 and in (Chen et al. 2012). Fig. 2 shows that the expression of *Cyp2b10* and *Cyp2c55* was

218   detectable but minimally responsive to an ED50 dose of TCPOBOP after 3 h and then increased

219   dramatically by the 27 h time point (Fig. 2A, Fig. 2B). Given the rapid induction of both genes within 3 h

220   when mice are given the saturating dose of 15x ED50 (i.e., 3 mg/kg) (Lodato et al, 2017), we surmise

221   that the slower induction time course seen with ED50-range doses of TCPOBOP (Fig. 2) reflects the

222   relatively long time required for TCPOBOP to biodistribute to the liver and generate a tissue level

223   sufficient to meet the threshold concentration for strong activation of CAR and its target genes. Very

224   similar patterns were seen for the dose-response and time course of induction of the primary, unspliced

225   transcripts *Cyp2b10* and *Cyp2c55* (Fig. 2C, Fig. 2D), which are indicative of relative rates of gene

226   transcription due to the expected short half-life of such transcripts (Gaidatzis et al. 2015). The apparent

227 increases in *Cyp2* gene transcription rates from 3 h to 27 h, as well as the TCPOBOP dose-dependent

228 increases seen at 27 h, are consistent with an increase in the abundance of transcriptionally active

229 hepatic CAR−TCPOBOP complexes from 3 h to 27 h.

230

231 The induction of Cyp2b10 mature mRNA and its transcription rate (i.e., primary transcript level) 27 h

232 after TCPOBOP exposure on PND4 showed a strong decrease 17 d later, on PND21 (Fig. 2A, Fig. 2C). The

233 magnitude of this decrease is consistent with the 14-day half-life for TCPOBOP elimination from the liver

234 (Poland et al. 1980) when taking into account the substantial increase in body size and liver weight from

235 PND4 to PND21, which will effectively dilute the residual TCPOBOP concentration in liver. Thus, 17 days

236 after TCPOBOP injection on PND4 at a dose of 0.67x ED50 (0.133 mg/kg), there is sufficient elimination

237 of TCPOBOP to decrease Cyp2b10 expression significantly. The transcriptional rate (primary transcript

238 level) of *Cyp2c55* was also low after 17 days compared to 27 h. In contrast, mature Cyp2c55 mRNA levels

239 were elevated after 17 days (i.e., on PND21), both with and without TCPOBOP, indicating there is a

240 developmental accumulation in the basal level of Cyp2c55 mRNA, a general characteristic of many genes

241 during this period of liver maturation (Gunewardena et al. 2015). Based on these findings, we selected

242 an 0.67x ED50 dose of TCPOBOP (0.133 mg/kg) to evaluate the potential of neonatal TCPOBOP exposure

243 for reprogramming later in life.

244

245 **Optimization of adult challenge TCPOBOP dose**. Next, we sought to identify a suitable dose of TCPOBOP

246 to use for a subsequent exposure, when adult mice exposed to TCPOBOP neonatally are challenged with

247 a second TCPOBOP injection. We reasoned that the second, challenge dose of TCPOBOP needs to induce

248 CAR-responsive genes significantly, but to a level that is less than maximal, which would allow us to

249 detect any additive or synergistic gene induction due to the impact of the prior, neonatal exposure.

250 Male mice, 7-weeks of age, were treated with TCPOBOP at 0.05x, 0.2x, and 1x ED50 doses and livers

251 were harvested two days later after 51 h. This time point was chosen to give sufficient time for

252 TCPOBOP to biodistribute to the liver and induce gene expression. Fig. S1 shows that TCPOBOP at a 0.2x

253 ED50 dose (0.04 mg/kg) increased *Cyp2* gene expression significantly, but to a level that was sub-

254 maximal; thus, gene responses were 5.4 to 5.6-fold lower (Cyp2b10) or 3.4 to 8.5-fold lower (Cyp2c55)

255 than at the 1x ED50 dose for both mature and primary gene transcripts. Non-linear dose-responses were

256 apparent when comparing the 0.05x and 0.2x ED50 doses, which could be due to the retention in fat

257 (Poland et al. 1980) of a higher fraction of the administered TCPOBOP dose when mice are treated at the

258 lowest dose.

259

260   **Combination of neonatal TCPOBOP with adult rechallenge exposure**. Based on the above dose-

261   response studies, we injected mice with TCPOBOP at 0.67x ED50 on PND4 and then re-challenged the

262   mice with a second TCPOBOP exposure at 0.2x ED50 in week 7. Livers were harvested 51 h later and

263   analyzed for expression of CAR-responsive genes. PND4 TCPOBOP treatment alone had no discernable

264   effect on *Cyp2b10* or *Cyp2c55* expression at 7 weeks, in either males or females (Fig. 3, bar 2 vs bar 1).

265   The low, sub-maximally inducing re-challenge dose of TCPOBOP given in week 7 had the expected short-

266   term inductive effect on CAR target gene expression (Fig. 3, bar 3 vs bar 1), but did not result in any

267   additive or synergistic response when given to mice treated with TCPOBOP neonatally (Fig. 3, bar 4 vs

268   bar 3). Thus, neonatal exposure to TCPOBOP does not lead to persistent induction of these CAR target

269   genes, nor does it sensitize these genes to a subsequent exposure at adulthood in either sex.

270

271   Given the absence of any reprogramming effects of neonatal TCPOBOP exposure when mice were

272   exposed to low dose (0.67x ED50) TCPOBOP neonatally (Fig. 3), we sought to confirm the persistent

273   induction of *Cyp2* family genes that was previously seen at week 12 when neonatal mice were treated

274   with TCPOBOP at a high dose, 15x ED50 (3 mg/kg) (Chen et al. 2012). Male and female PND4 pups were

275   treated with TCPOBOP (3 mg/kg), and livers were collected at weeks 3, 7 and 12. Gene expression levels

276   were compared to those seen in livers of week 7 male mice treated with a range of TCPOBOP doses (0,

277   0.2x, 1x, and 15x ED50) and euthanized 51 h later (Fig. 4A). Neonatal TCPOBOP exposure at 15x ED50

278   induced persistent expression of *Cyp2b10* and *Cyp2c55* after both 7 and 12 weeks at levels significantly

279   higher than the control group (Fig. 4B and Fig. 4C, groups G-J vs A). Moreover, the expression levels at

280   week 7 were very similar to those seen in livers of 7-week mice exposed to low dose TCPOBOP (0.2x

281   ED50) for 51 h (group B). Next, we employed LC/MS analysis to measure hepatic TCPOBOP

282   concentrations to determine whether the persistent expression of these genes can be explained by

283   TCPOBOP remaining in the liver at 7 weeks. Fig. 4D shows that residual TCPOBOP persists in liver tissue

284   at week 7 and at week 12 at a level similar to the level found 51-h after TCPOBOP dosing at 0.2x ED50

285   (groups G-J vs A), which is also the neonatal dose that induces an equivalent level of persistent *Cyp2*

286   expression (i.e., G-J vs A in Fig. 4B, Fig. 4C). Thus, the persistent expression of both *Cyp2* genes under the

287   conditions of TCPOBOP treatment used here and by others (Chen et al. 2012) can be fully explained by

288   the continued presence of TCPOBOP in liver tissue even 12 weeks after the initial dosing.

289

290 **Long-term effects of neonatal phenobarbital exposure**. Taken together the studies above demonstrate

291 that: 1) neonatal exposure to a low dose TCPOBOP (0.67x ED50) does not lead to persistent *Cyp2* gene

292 expression at week 7; and 2) neonatal exposure to a high dose of TCPOBOP (15x ED50) does not allow us

293 to evaluate its potential reprogramming effects later in life due to the persistent elevation of TCPOBOP

294 levels in the liver. It remains possible, however, that *Cyp2* genes can be reprogrammed for persistent

295 expression, but that this requires a high level of CAR activation in neonatal liver. We tested this

296 hypothesis in neonatal mice exposed to phenobarbital, a CAR agonist with a much shorter half-life than

297 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

298 strain) (Markowitz et al. 2010).

299

300 For this study, we widened the window of neonatal phenobarbital exposure to encompass days PND2

301 through PND7 to include critical early time periods that may be required for gene reprogramming, as

302 has been reported for several chemical exposures (Hanson and Gluckman 2014; Hanson and Skinner

303 2016; Vickers 2011). Phenobarbital was delivered via the drinking water consumed by the dams, an

304 established route for perinatal exposures (Waalkes et al. 2003) that enables drug delivery to neonatal

305 mice by lactation (Asoh et al. 1999). Pups were weaned on PND21 and then left untreated until a

306 second, challenge exposure to phenobarbital was given by i.p. injection at 7 weeks of age. We found

307 that early phenobarbital exposure from PND2-PND7 did not lead to persistent expression of *Cyp2b10* or

308 *Cyp2c55* at week 7 (Fig. 5, bar 2 vs bar 1). Further, in females, we observed a moderate but significant

309 increase in expression of the mature *Cyp2b10* transcript after phenobarbital re-challenge in week 7. The

310 same trend was seen in males for the mature *Cyp2b10* transcript, and in both sexes for the primary

311 *Cyp2b10* transcript, but without reaching statistical significance (Fig. 5A-5D). In contrast, *Cyp2c55*

312 showed a significant decrease in expression in female but not male liver after the phenobarbital

313 rechallenge, as was seen for both the mature and the primary transcripts.

314

315 These results were largely confirmed in a second study, where neonatal mice were injected with

316 phenobarbital at 40 mg/kg/day on days PND4 and PND5, and then re-challenged with phenobarbital at

317 10 mg/kg/day on 3 consecutive days in Week 7 (Fig. S2). Again, neonatal phenobarbital exposure alone

318 did not lead to persistent expression after 7 weeks, but when combined with adult phenobarbital

319 exposure in week 7, increased the level of *Cyp2b10* mature transcript in male mice significantly higher

320 than that seen in mice given the week 7 exposure alone (Fig. S2, bar 4 vs bar 3). In females but not

321 males, *Cyp2c55* showed a decreased response to the adult phenobarbital re-challenge, very similar to

322   the decrease seen in Fig. 5F and Fig. 5H, but this effect did not reach statistical significance. We conclude

323   that neonatal exposure to a high dose of phenobarbital induces a moderate reprogramming of these

324   *Cyp2* genes in week 7. These reprogramming effects of early phenobarbital exposure are not indirect

325   responses to changes in CAR expression, whose expression did not change significantly with these

326   treatments (Fig. S3A).

327

328   **Impact on sex-specific liver gene expression**. Perinatal exposure of rats to phenobarbital can cause

329   long-term dysfunctions such as changes in circulating hormone levels, decreased levels of hepatic

330   monoamine oxidase and infertility (Agrawal et al. 1995; Gupta et al. 1982; Soliman and Richardson

331   1983). In particular, neonatal phenobarbital exposure was found to alter pituitary secretion patterns and

332   circulating blood levels of growth hormone later in life, and correspondingly, it alters adult liver

333   expression patterns of *Cyp* genes that show sex-dependent, growth hormone-regulated expression

334   patterns (Agrawal and Shapiro 2003). Here, we investigated the effects of both neonatal and 7-week

335   phenobarbital exposure on the adult liver expression of two sex-specific, growth hormone-regulated

336   genes, the female-specific *A1bg* and the male-specific *Cyp7b1* (Fig. S5). Neonatal phenobarbital

337   exposure had no effect on the expression of either gene at 7 weeks of age. Furthermore, adult exposure

338   to phenobarbital, at 7 weeks of age, led to only a small increase in *Cyp7b1* expression. These findings

339   suggest that in the mouse model, early phenobarbital exposure does not lead to major changes in

340   plasma growth hormone profiles known to regulate these sex-specific genes. These findings, in turn,

341   suggest that the effects of neonatal phenobarbital on *Cyp2* genes, described above, are unlikely to be an

342   indirect response to circulating growth hormone levels.

343

344

345   **Discussion**

346

347   Exposure to endocrine-active environmental chemicals during the perinatal period, a critical window of

348   developmental plasticity, has been proposed to reprogram development and alter disease susceptibility

349   later in life. Many environmental chemicals disrupt gene expression by interaction with xenobiotic

350   sensors from the Nuclear Receptor superfamily, including CAR, which coordinates cellular and

351   transcriptional responses affecting hepatic drug metabolism, energy homeostasis and tumor

352   development. A prior study found that TCPOBOP injection in PND4 mice induced long-term hepatic

353   expression of several CAR target genes from the *Cyp2* family. Elevated expression persisted into

354   adulthood in association with long-term epigenetic changes (Chen et al. 2012), which could involve

355   activation of an epigenetic switch (Lempiainen et al. 2011). However, given the long biological half-life of

356   TCPOBOP ($t_{1/2}$ ~14 days) (Poland et al. 1980), we asked whether persistence of TCPOBOP in mouse

357   tissue, rather than an epigenetic memory, might drive the persistence of gene expression. We confirmed

358   that the neonatal TCPOBOP exposure regimen used by (Chen et al. 2012) does indeed induce long-term

359   increases in liver *Cyp2* expression lasting at least 12 weeks; however, the persistence of expression was

360   readily explained by the persistence of TCPOBOP in liver tissue at a level sufficient to account for the

361   prolonged increase in expression that we observed. We were able to avoid the long-term persistence of

362   TCPOBOP in liver tissue by decreasing the neonatal TCPOBOP exposure dose 22-fold, from a dose of 15x

363   ED50 used in (Chen et al. 2012)) to a dose of 0.67x ED50. However, although strong neonatal increases

364   in hepatic *Cyp2* expression were still achieved, they did not persist into adulthood. Moreover, this early

365   ED50-range exposure to TCPOBOP did not sensitize mice to a subsequent, low-dose TCPOBOP dosing.

366   Thus, tissue persistence of TCPOBOP, rather than an epigenetic memory, drives the persistence of the

367   elevated *Cyp2* gene expression seen in mouse liver. These findings highlight the importance of carefully

368   considering both dose and pharmacokinetics of elimination from tissue depots when evaluating

369   chemicals such as TCPOBOP for potential reprogramming effects of early life exposures.

370

371   Persistent local epigenetic changes were also reported by (Chen et al. 2012) in the neonatal TCPOBOP

372   exposure model; however, those same epigenetic changes are also induced by short-term TCPOBOP

373   exposure (Rampersaud et al. 2019). Consequently, we can attribute them to the ongoing activation of

374   CAR by residual TCPOBOP in liver tissue, rather than to a long-term epigenetic memory of the initial

375   exposure. TCPOBOP has also been shown to accumulate in mouse maternal adipose tissue, from where

376   it can be transferred to pups by lactation to activate CAR-responsive genes in offspring livers (Dietrich et

377   al. 2018). Exposures via that route may appear to give rise to transgenerational effects on gene

378   expression or epigenetics, when in fact they are directly linked to the parental exposure. For TCPOBOP

379   and other lipophilic chemicals, it can thus be difficult to distinguish true long-term gene dysregulation

380   from long-term changes due to ongoing exposure via tissue depots that persist in liver, fat or elsewhere.

381   For such chemicals, reducing the exposure dose to effectively shorten the overall exposure period, as

382   was done here for TCPOBOP, is one approach to determine a chemical's intrinsic potential for persistent

383   biological effects, albeit with the caveat that in some cases a higher dose may be needed to elicit a

384   robust, long-term epigenetic response.

385

386   Accordingly, we used phenobarbital to test whether a threshold level of CAR activation, perhaps not

387   reached by the 0.67x ED50 dose of TCPOBOP, may be required for long-term *Cyp2* reprogramming. This

388   short half-life CAR agonist ($t_{1/2}$ ~8 hr in adult mice) enabled us to achieve high level neonatal CAR

389   activation without the persistent exposure that is unavoidable when using correspondingly high doses of

390   TCPOBOP due to its ~50-fold longer half-life. Neonatal phenobarbital induced long-term changes in the

391   responsiveness of *Cyp2* genes to a second, low dose exposure to phenobarbital at adulthood.

392   Specifically, we observed moderate increases in the responsiveness of *Cyp2b10* to low dose

393   phenobarbital at week 7, as well as decreased responsiveness of *Cyp2c55* in female but not male liver,

394   as was seen in two neonatal exposure models with different designs. These findings support earlier work

395   in the rat model, where neonatal phenobarbital administration led to 30-40% over-induction of rat

396   hepatic *CYP2B1* and *CYP2B2* expression when the rats were rechallenged as adults with low doses of the

397   barbiturate (Agrawal and Shapiro 1996). Persistent induction of mouse hepatic CAR target genes was

398   previously described following neonatal phenobarbital exposure (Tien et al. 2015), however, we found

399   that the LD50-range dose of phenobarbital used in that study (>200 mg/kg on PND5) was severely toxic

400   with some lethality (unpublished experiments) and is thus not pharmacologically relevant. Further

401   investigation will be required to elucidate underlying mechanisms, including why the early life exposure

402   to phenobarbital employed in our study has opposite effects on these *Cyp2b10* and *Cyp2c55*, both of

403   which are themselves strongly induced by the initial phenobarbital treatment. Moreover, as

404   phenobarbital activates both CAR and the related nuclear receptor PXR, and with significant overlap

405   between their target genes (Cui and Klaassen 2016), it will be important to determine which receptor

406   mediates the long-term gene responses to neonatal phenobarbital exposure described here.

407

408   We initially selected TCPOBOP for studying long-term effects of early CAR activation due to its high

409   specificity for a single nuclear receptor, CAR (Tojima et al. 2012; Tzameli et al. 2000), and for the

410   unusually strong gene responses it can induce, as exemplified by the *Cyp2* genes examined here. While

411   TCPOBOP is a specific activating ligand of rodent but not human CAR owing to species-specific

412   differences in CAR's ligand binding domain (Mackowiak and Wang 2016), DNA-binding and the

413   associated key genomic and epigenetic effects of CAR activation are most likely conserved across

414   mammalian species. Studies of indirect CAR agonists, such as those reported here for phenobarbital,

415   may be more readily extrapolated across species due to conservation of the overall signaling pathways

416   through which they activate CAR. Phenobarbital activates CAR by inhibiting epidermal growth factor

417   receptor signaling (Chai et al. 2016), which ultimately leads to dephosphorylation of CAR-threonine 38

418   and CAR nuclear translocation (Mackowiak and Wang 2016; Qatanani and Moore 2005). One limitation,

419   however, is that multiple receptors are often activated by xenobiotics that dysregulate gene expression

420   in the liver (i.e., both CAR and PXR in the case of phenobarbital), which as noted complicates

421   mechanistic studies of any downstream epigenetic actions. Epigenetic reprogramming may also occur in

422   adult mouse exposure models, where long-term treatment with phenobarbital induces many novel

423   differentially methylated and hydroxymethylated genomic regions that strongly correlate with

424   transcriptional responses and are not found after a short-term exposure (Thomson et al. 2013).

425

426   Finally, our findings have implications for studies such as those of the Target II consortium (Wang et al.

427   2018), where multiple environmental chemical exposures are being investigated in perinatal mouse

428   models, including evaluation of changes in gene expression, changes in chromatin accessibility and other

429   epigenetic changes at adulthood. Understanding the mechanisms underlying these processes will

430   provide important insight into how exposure to environmental chemicals and pharmaceuticals in early

431   life can influence long-term health outcomes and disease risk. Challenges going forward will include

432   distinguishing long vs short term effects for chemicals with long half-lives, determining which receptors

433   mediate the effects observed and elucidation of underlying mechanisms, including mechanisms driving

434   epigenetic changes that are expected to be a main driver of long-term phenotypes seen following many

435   environmental chemical exposures.

436

437

438   **Conflicts of interest** – The authors declare that they have no conflicts of interest

439

440   **Authors' contributions** – AS and DJW jointly conceived of the study. All of the experimental work, data

441   analysis and figure preparation was performed by AS. DJW drafted the manuscript with input and review

442   by AS. DJW provided guidance and supervised the overall project and revised and finalized the

443   manuscript for publication. Both authors reviewed and approved the final manuscript.

444

449

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602
603

604   **Figure legends**

605

606   **Fig. 1**. **Impact of neonatal TCPOBOP exposure with repeat dosing in week 3 on CAR-responsive genes**.

607   Male and female pups were injected with TCPOBOP (3 mg/kg) or vehicle (control) on PND4, and in week

608   3, were again treated with TCPOBOP (3 mg/kg), or vehicle (control). All mice were euthanized in week 3,

609   3 h after the final injection (**Study A design**). Shown are relative RNA levels of each gene determined by

610   RT-qPCR analysis of RNA extracted from each liver, with values normalized to that of the vehicle-only

611   group (first bar) set to a value of 1.0. **A-C**, gene expression in male livers; **D-F**, gene expression in female

612   livers. Data are shown as mean +/- SEM (n = 3, 4, 4, 4 individual males, for bars 1-4, respectively, and n =

613   2, 5, 1, 5 individual females, for bars 1-4, respectively). Data were analyzed by pair-wise t-test for two

614   separate comparisons: *, effects of TCPOBOP exposure on PND4 (bar 2 vs bar 1, and bar 4 vs bar 3); and

615   †, effects of TCPOBOP in week 3 (bar 3 vs bar 1, and bar 4 vs bar 2). TC, TCPOBOP. The same patterns

616   were seen in both sexes, but the small sample size in the week 3 alone female group precluded a full

617   statistical analysis. Significance: * or †, $p < 0.05$; ** or ††, $p < 0.01$; *** or †††, $p < 0.001$.

618

619   **Fig. 2**. **TCPOBOP dose-response in neonatal mouse liver**. Male and female PND4 pups were injected

620   with TCPOBOP at 0, 66.7 µg/kg (0.33x ED50), 133 µg/kg (0.67x ED50), or 200 µg/kg (ED50) and

621   euthanized after 3 h (on PND4), after 27 h (on PND5), or after 17 d (on PND21). Shown are gene

622   expression data for the mature and primary RNA transcripts of *Cyp2b10* and *Cyp2c55* for n = 4 to n = 8

623   livers per group. Data presentation as in Fig. 1, with each experimental condition compared to its age-

624   matched control (first bar at each time point): *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. All Y-axis

625   values are relative to 0.2 mg/kg TCPOBOP, which was set = 100.
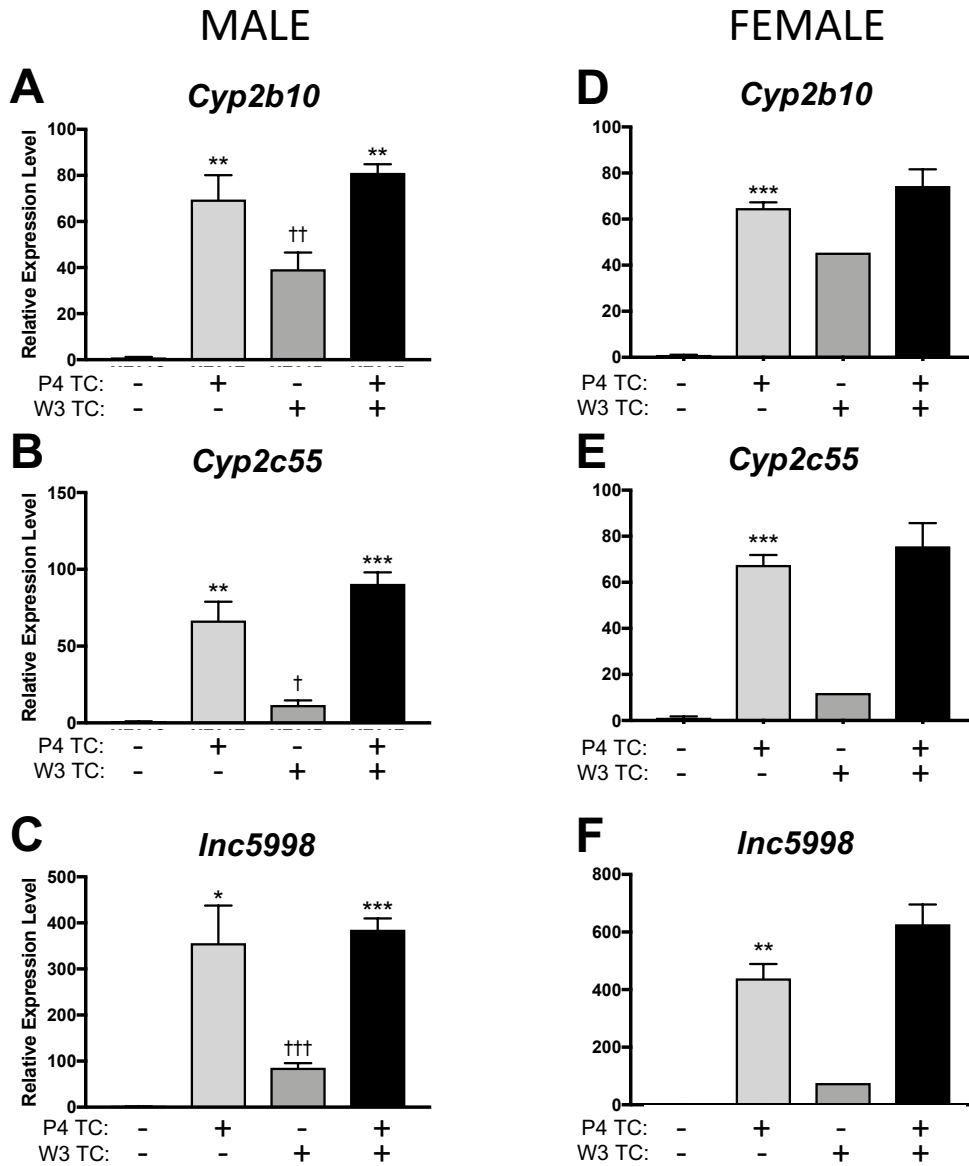
626

627   **Fig. 3**. **Neonatal TCPOBOP exposure followed by second TCPOBOP exposure in week 7.** Male and

628   female PND4 pups were injected with TCPOBOP at 133 µg/kg (0.67x ED50), or vehicle (control), and in

629   week 7 were re-challenged with a second dose of TCPOBOP at 40 µg/kg (0.2x ED50) or with vehicle

630   (control). All mice were euthanized in week 7, 51 h after the last injection (**Study design B**). Shown are

631   the expression levels of the mature and primary transcripts of *Cyp2b10* and *Cyp2c55*, as indicated, for n

632   = 5-7 livers per group. Data presentation as in Fig. 1, comparing the effects of TCPOBOP exposure in

633   week 7 alone (bar 3 vs bar 1) or in combination with TCPOBOP exposure on PND4 (bar 4 vs bar 2): †, $p <$

634   0.05; ††, $p < 0.01$; and †††, $p < 0.001$. The effects of TCPOBOP exposure on PND4 were not significant

635 by week7 for any of the genes (bar 2 vs bar 1, and bar 4 vs bar 3). Y-axis values are expressed relative to

636 the vehicle-treated control male group for each gene.

637

638 **Fig. 4**. **Neonatal exposure to TCPOBOP (3 mg/kg) results in significant residual TCPOBOP and**

639 **persistent *Cyp2* gene expression after 7 and 12 weeks**. **A**: Time course and experimental design. Green

640 arrows, TCPOBOP treatment; red arrows, collection of liver for analysis. Male and female PND4 pups

641 were injected with 3 mg/kg of TCPOBOP (15x ED50 dose) and were euthanized in week 3, 7 or 12

642 (groups E-J, green and red arrows). For comparison, 7-week-old male mice were injected with TCPOBOP

643 at 0, 0.2x, 1x, and 15x ED50 doses (0, 0.04, 0.2, or 3 mg/kg, respectively) and were euthanized 51 h later

644 (groups A-D; gray and black bars in panels B-D). Further details about each group are shown along the x-

645 axis of panels B-D. **B, C**: RT-qPCR analysis of liver RNA, with expression levels of *Cyp2b10* and *Cyp2c55*

646 normalized to group A (vehicle control). **D**: TCPOBOP was extracted from 0.5 g of each liver with hexane

647 and quantified by LC/MS. In B-D, data shown are mean and SEM, n = 4 per group. Values above the

648 tallest bars indicate the actual y-axis values. Significance compared to vehicle control (group A): *, p <

649 0.05; **, p < 0.01; ***, p < 0.001.

650

651 **Fig. 5**. Neonatal phenobarbital exposure (0.05% (w/v) in drinking water fed to the dams) from PND2 to

652 PND7, with a second phenobarbital exposure (10 mg/kg, daily for 3 days), or vehicle control, given in

653 week 7. All mice were euthanized in week 7, 3 h after the last injection (**Study design C**). Shown are RT-

654 qPCR expression data for mature and primary transcripts for *Cyp2b10* and *Cyp2c55*, normalized to the

655 vehicle control (first bar in each set), mean +/- SEM (n = 21, 12, 5, 12 individual males, for bars 1-4,

656 respectively, and n = 23, 8, 8, 14 individual females, for bars 1-4, respectively). Data presentation as in

657 Fig. 1, comparing the effects of phenobarbital exposure in week 7 alone (bar 3 vs bar 1) or in week 7 in

658 combination with PND2-PND7 phenobarbital exposure (bar 4 vs bar 2): *, comparison of bar 2 vs bar 1,

659 and of bar 4 vs bar 3; and †, comparison of bar 3 vs bar 1, and of bar 4 vs bar 2. Significance: * or †, p <

660 0.05; ** or ††, p < 0.01; *** or †††, p < 0.001.

Fig. 1

Fig. 2

A *Cyp2b10* (mature)

C *Cyp2b10* (primary)

Legend:
- □ Vehicle
- ▨ TCPOBOP (0.067 mg/kg, 0.33x ED50)
- ▩ TCPOBOP (0.133 mg/kg, 0.67x ED50)
- ■ TCPOBOP (0.2 mg/kg, ED50)

B *Cyp2c55* (mature)

D *Cyp2c55* (primary)

Time after PND4 injection

Relative Expression Level

3 h   27 h   17 d
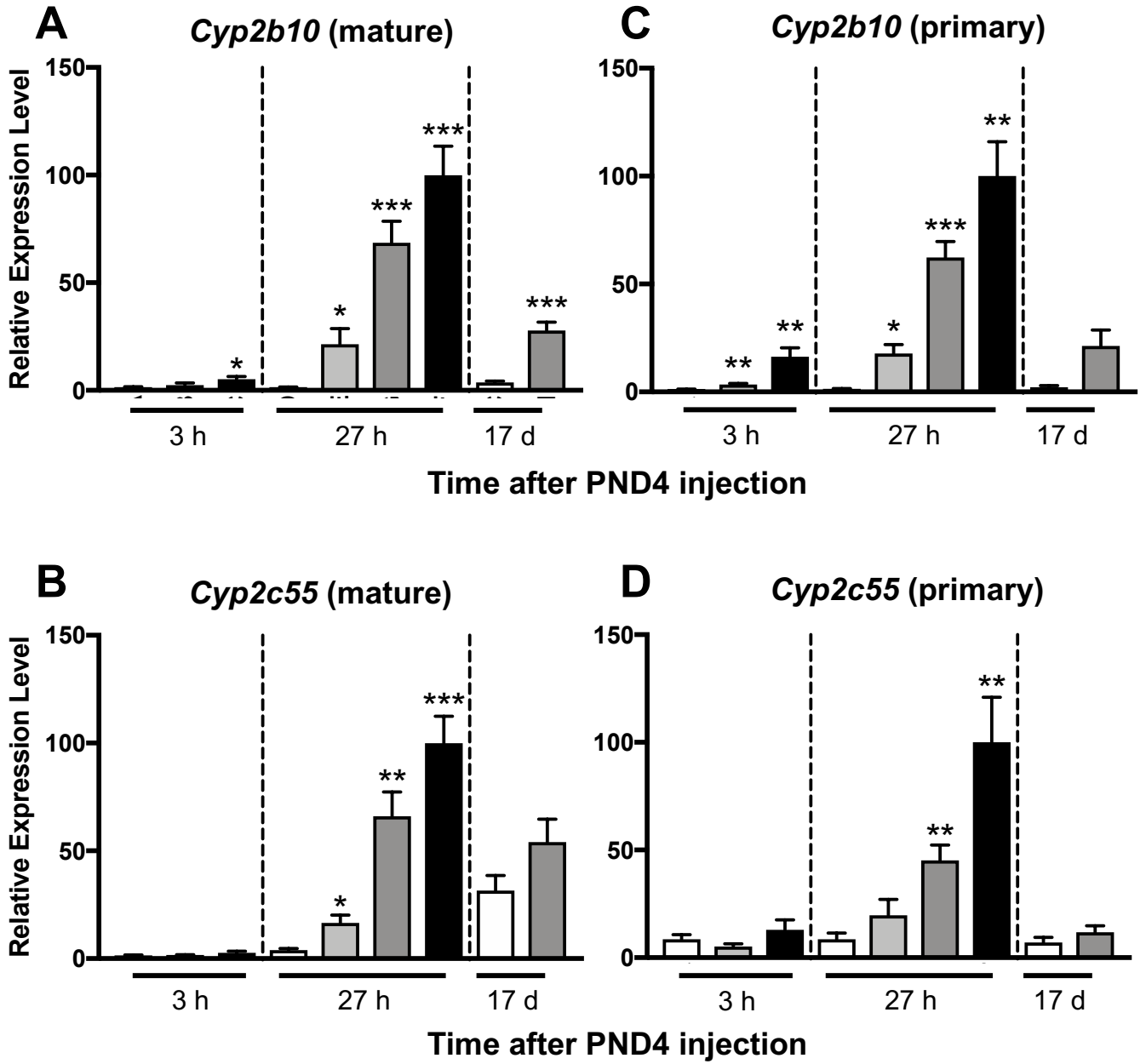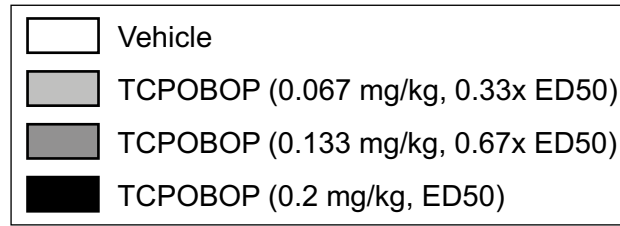
Fig. 3
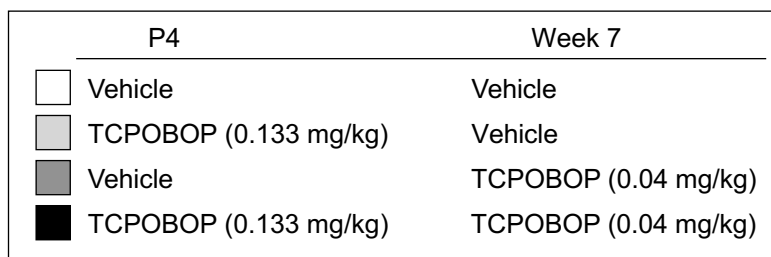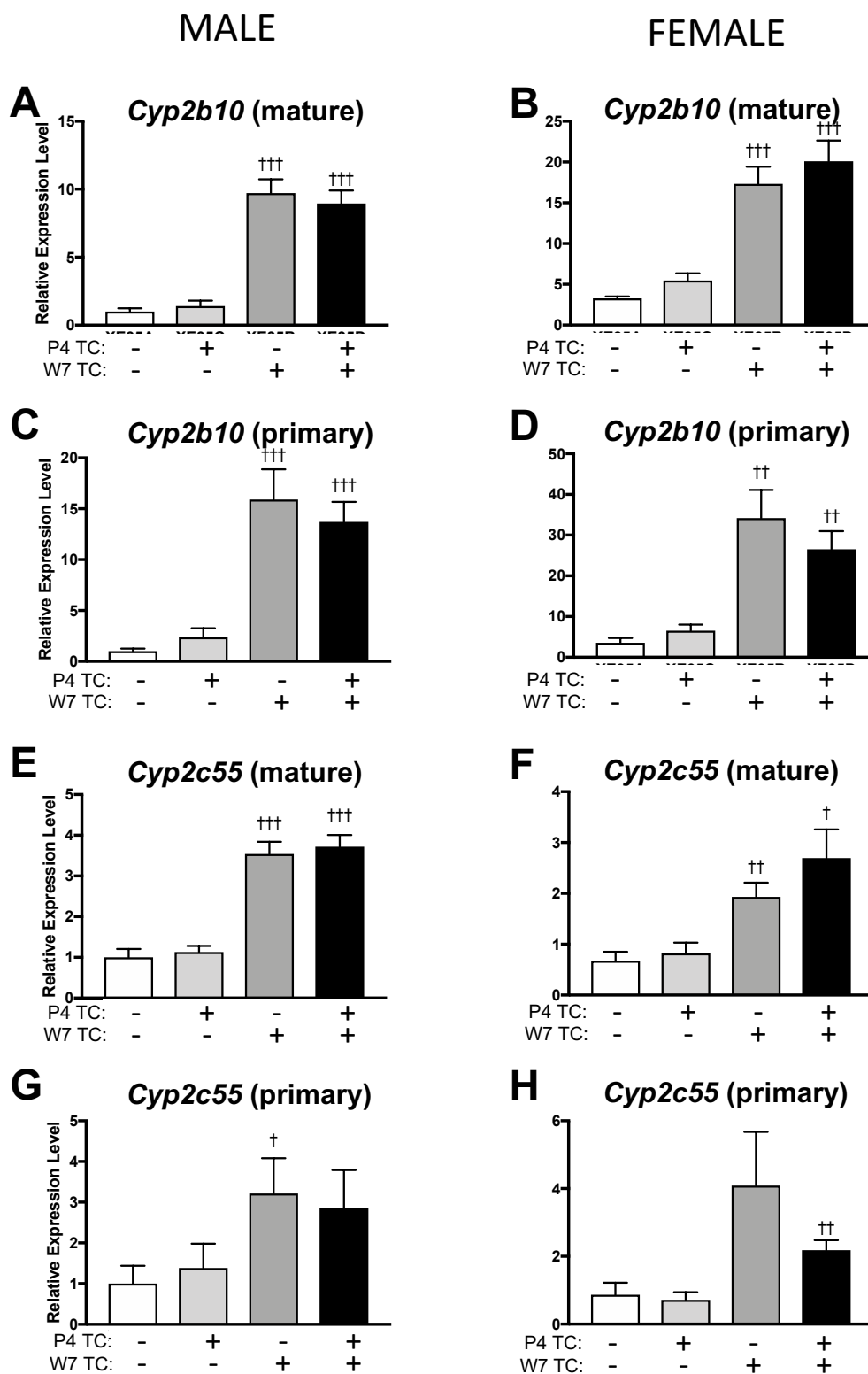
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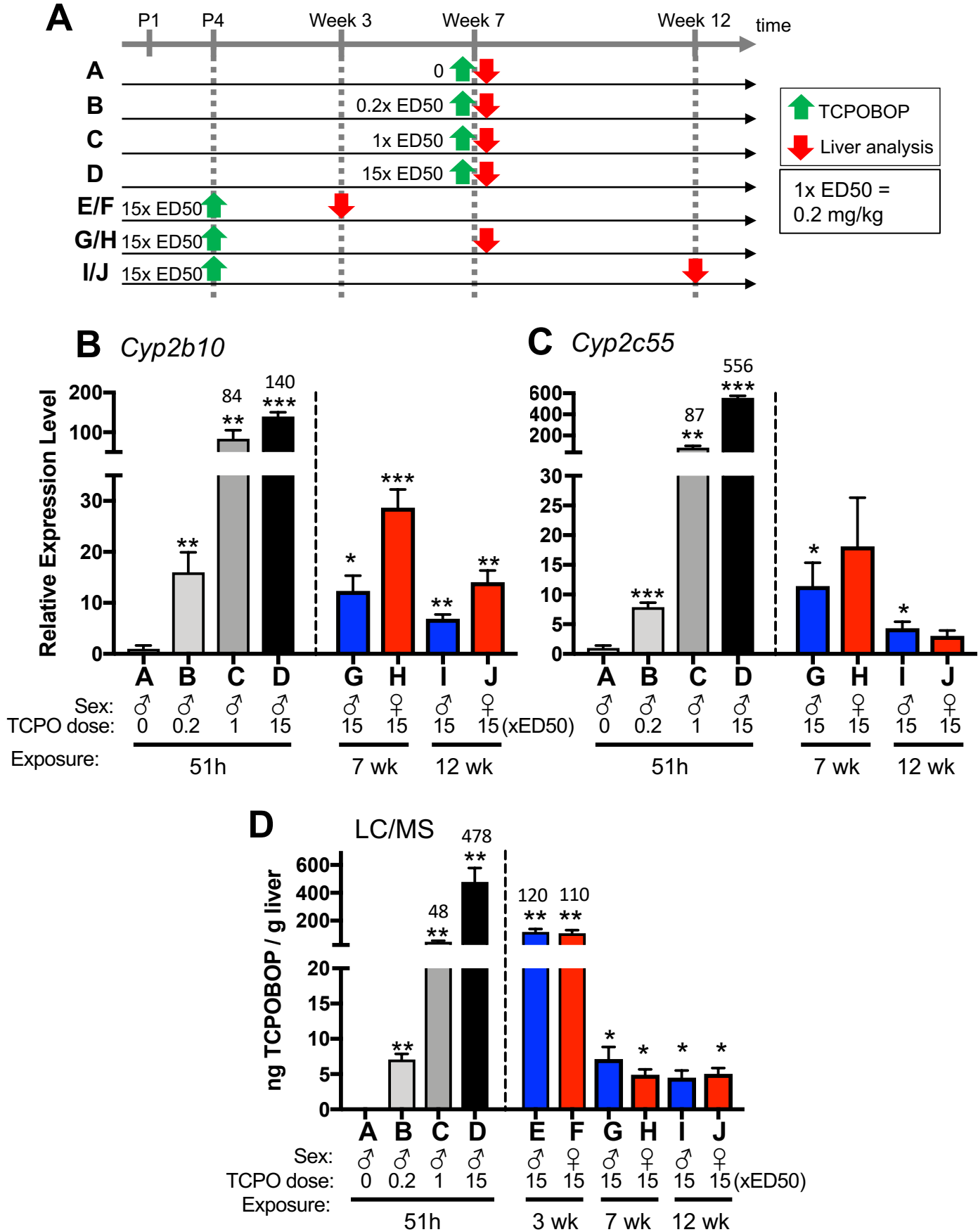
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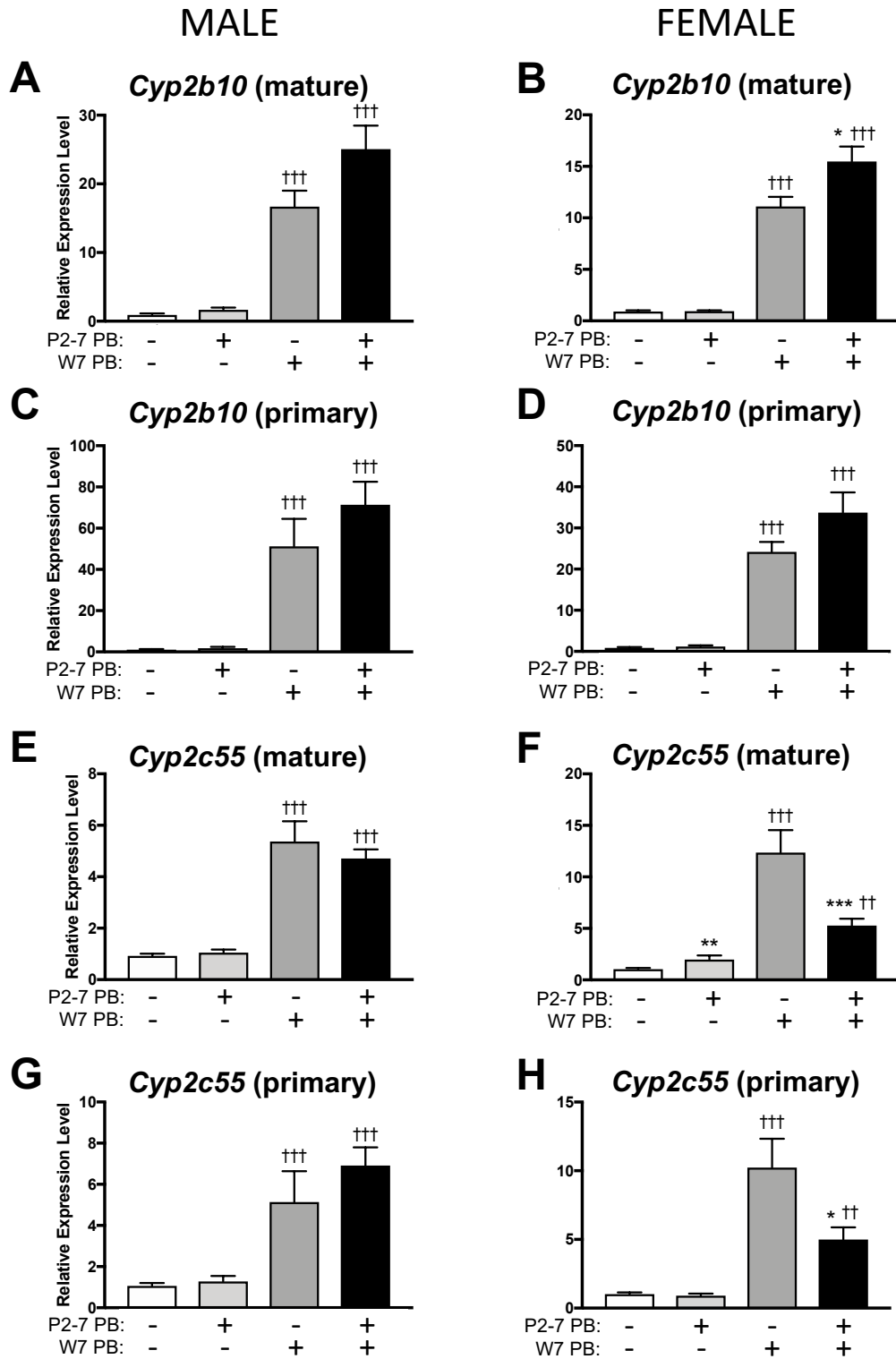
Impact of neonatal activation of nuclear receptor CAR (NR1I3) on Cyp2 gene expression in adult mouse liver

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Supplemental Figures S1-S3

Table S1

Supplementary Figures

Fig. S1. TCPOBOP dose-response in 7-week-old male mice. Mice were injected with TCPOBOP intraperitoneally at 0, 10 µg/kg (0.05x ED50), 40 µg/kg (0.2x ED50), or 200 µg/kg (1x ED50), and euthanized at 51 h later. Total liver RNA was extracted and analyzed by RT-qPCR. Expression levels were normalized to that of the vehicle control group. Shown are the expression levels of the mature and primary transcripts of Cyp2b10 and Cyp2c55, mean and SEM error bar for n = 5 livers per group. Values above the 0.05x ED50 and the 0.2x ED50 bars indicate how many fold-lower gene expression was at the indicated dose as compared to the 1x ED50 dose. Thus, Cyp2b10 levels were 5.4-5.6 fold lower at 0.2x ED50 than at 1x ED50 (mature and primary RNA, respectively); and corresponding values were 8.5 and 3.4 fold higher for Cyp2c55 (mature and primary RNA, respectively). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$, pair-wise t-test for comparisons between each treatment and the vehicle control (first bar).

Fig. S2. Impact of neonatal phenobarbital on PND4 and PND5 (40 mg/kg on each day) with re-challenge in week 7 (10 mg/kg daily for 3 consecutive days) (Study design D). Mice were euthanized 3 h after the last phenobarbital injection. Shown are total liver RNA expression levels determined by RT-qPCR for the mature and primary transcripts of Cyp2b10 and Cyp2c55, mean and SEM error bar for n = 4-8 livers per group in both male and female mouse livers. Pair-wise t-tests comparing: 1) neonatal phenobarbital vs vehicle control (bar 2 vs bar 1, and bar 4 vs bar 3: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; and 2) week 7 phenobarbital vs vehicle control (bar 3 vs bar 1, and bar 4 vs bar 2: †: $p < 0.05$, ††: $p < 0.01$, †††: $p < 0.001$)

Fig. S3. Impact of neonatal phenobarbital and adult phenobarbital rechallenge on expression of CAR and two sex-specific genes. Male and female mouse pups were injected either with vehicle or with phenobarbital at 40 mg/kg/day of phenobarbital on PND4 and P5, and later in week 7, were re-challenged with either vehicle or 10 mg/kg/day of phenobarbital for 3 consecutive days. Mice were euthanized at 3 h after the last phenobarbital injection. Shown are total liver RNA expression levels determined by RT-qPCR and normalized to the vehicle control group for CAR, for the female-specific A1bg, and for the male-specific Cyp7b1. Data shown are mean and SEM error bar for each group, for n = 3-6. Pair-wise t-tests comparing: 1) neonatal phenobarbital vs vehicle control: nothing significant at $p < 0.05$; 2) week 7 phenobarbital vs vehicle control (bar 3 vs bar 1, and bar 4 vs bar 2: †: $p < 0.05$); and 3) males vs females for the indicated 4 sets of comparisons, where #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$.
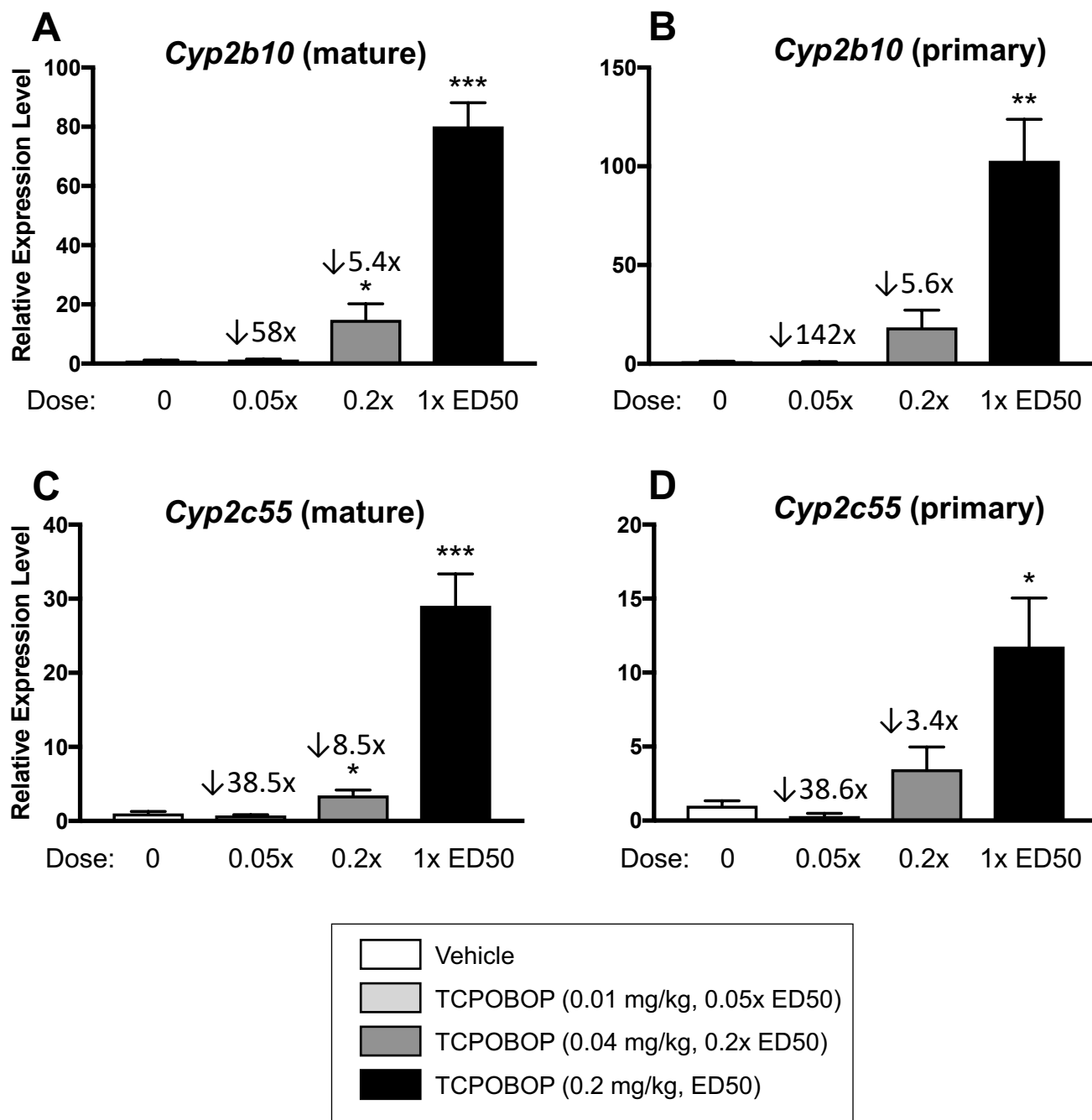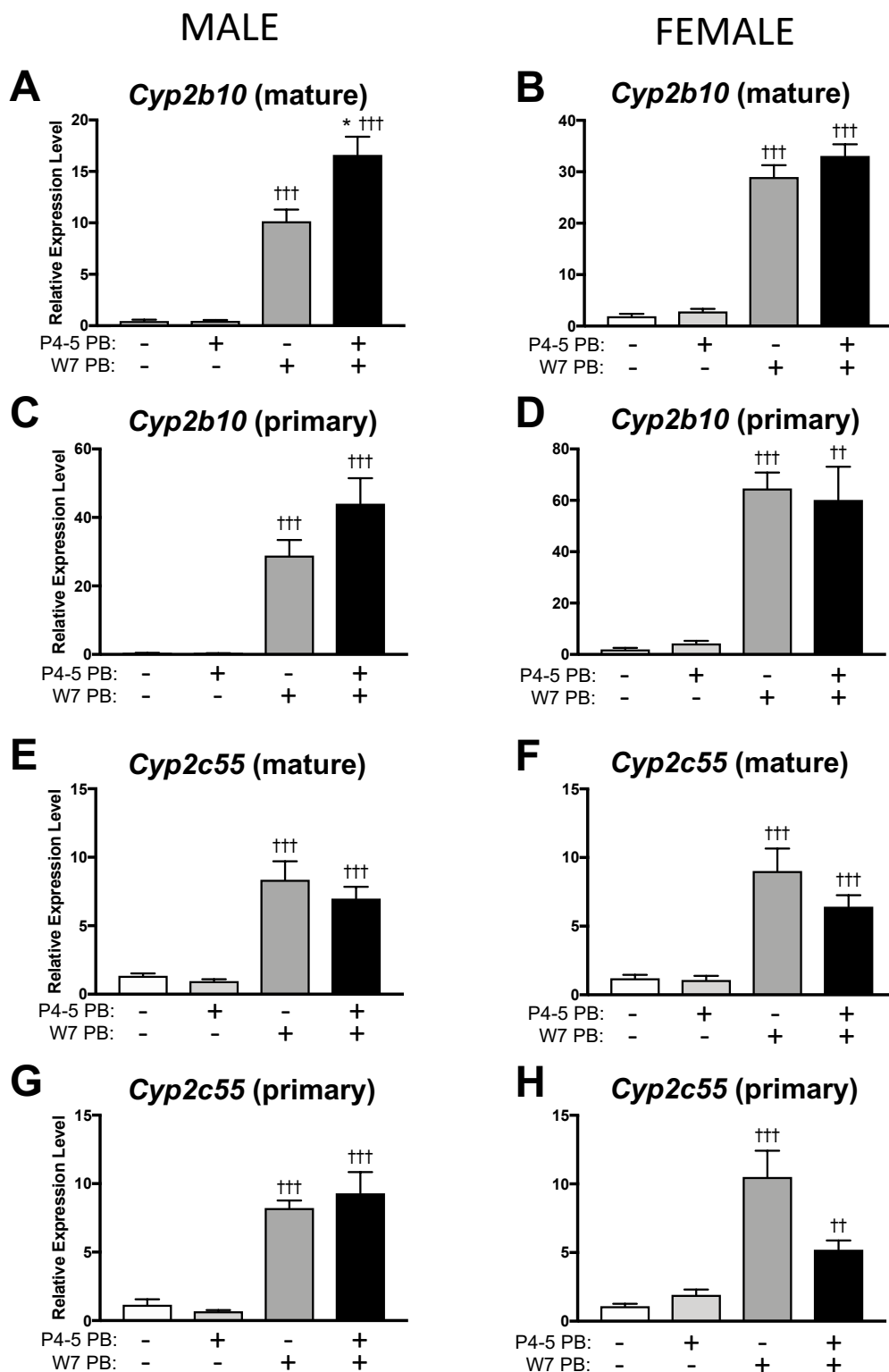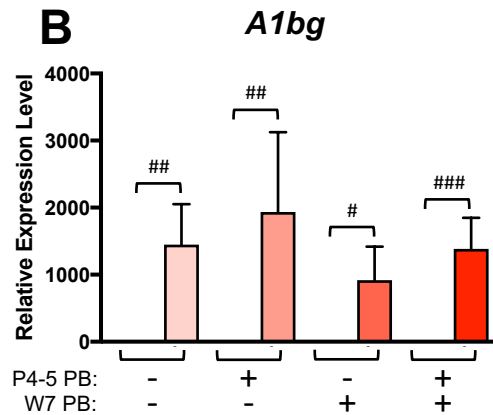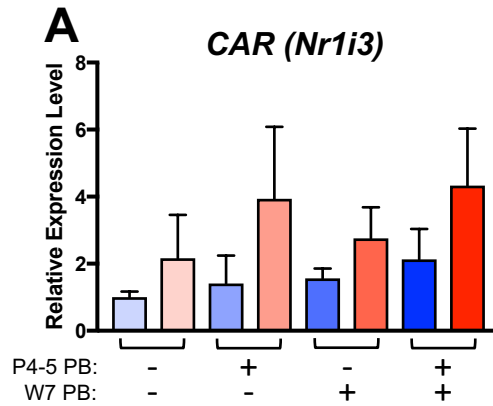
A *Cyp2b10* (mature)

B *Cyp2b10* (primary)

C *Cyp2c55* (mature)

D *Cyp2c55* (primary)

Legend:
- Vehicle
- TCPOBOP (0.01 mg/kg, 0.05x ED50)
- TCPOBOP (0.04 mg/kg, 0.2x ED50)
- TCPOBOP (0.2 mg/kg, ED50)

MALE

FEMALE

A *Cyp2b10* (mature)

B *Cyp2b10* (mature)

C *Cyp2b10* (primary)

D *Cyp2b10* (primary)

E *Cyp2c55* (mature)

F *Cyp2c55* (mature)

G *Cyp2c55* (primary)

H *Cyp2c55* (primary)

	P4-P5	Week 7
☐	Vehicle	Vehicle
☐	PB (40 mg/kg/day, 2 days)	Vehicle
☐	Vehicle	PB (10 mg/kg/day, 3 days)
■	PB (40 mg/kg/day, 2 days)	PB (10 mg/kg/day, 3 days)

A

CAR (Nr1i3)

Relative Expression Level

| P4-5 PB: | - | + | - | + |
| W7 PB: | - | - | + | + |

B

A1bg

Relative Expression Level

| ## | # |

| P4-5 PB: | - | + | - | + |
| W7 PB: | - | - | + | + |

Statistical significance

*: neonatal vehicle vs. PB
†: Week 7 vehicle vs. PB
#: male vs. female
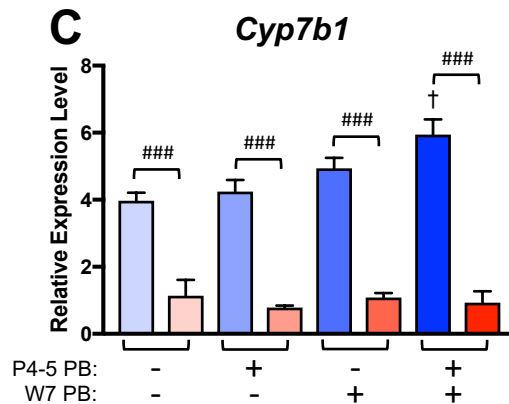
C

Cyp7b1

Relative Expression Level

| ### | ### |
†

| P4-5 PB: | - | + | - | + |
| W7 PB: | - | - | + | + |

Male	Female	P4-P5	Week 7
		Vehicle	Vehicle
		PB (40 mg/kg/day, 2 days)	Vehicle
		Vehicle	PB (10 mg/kg/day, 3 days)
		PB (40 mg/kg/day, 2 days)	PB (10 mg/kg/day, 3 days)

Table S1 - Primer sequences used for qPCR analysis.				
		Forward Primer		Reverse Primer
Mouse Gene Target	Number	Sequence	Number	Sequence
18S	ON842	CGCCGCTAGAGGTGAAATTC	ON843	CCAGTCGGCATCGTTTATGG
Cyp2b10 (mature transcript)	ON5932	GGAGGAACTGCGGAAATCCC	ON5933	ATAGAACAGCTCCAGCAGGC
Cyp2b10 (primary transcript)	ON5932	GGAGGAACTGCGGAAATCCC	ON6815	CCATCTTCCTTCATGCCCATT
Cyp2c55 (mature transcript)	ON5934	AAGAACATCAGCAAATCCTTCAAC	ON5935	CTTTGAACCAAAGTACAGAGTGAACA
Cyp2c55 (primary transcript)	ON5934	AAGAACATCAGCAAATCCTTCAACT	ON6816	GCAGAGAATAATTCTTACTTTTCAACCA
Lnc5998	ON6126	CAGCTAAACCAGGCTTCAGGAA	ON6127	TTGCCCCAGAATGAACACTAGTAGT
CAR	ON6660	CTCAACTCCTCCCACATTCAG	ON6661	GCAAACGGACAGATGGGAC
Cyp7b1	ON6596	ACCCGGAAATCTTCGATGCT	ON6597	CTATGAAGCGATCGAACCTAAATTC
A1bg	ON5723	GAACCCTCTGAGCCCAGTGA	ON5724	GAGTGGGTGGAGCCTGTGAG