Health-Relevant Phenotypes in the Offspring of Mice Given CAR Activators Prior to Pregnancy[S]

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ABSTRACT

Hepatic induction in response to drugs and environmental chemicals affects drug therapies and energy metabolism. We investigated whether the induction is transmitted to the offspring. We injected 3-day- and 6-week-old F0 female mice with TCPOBOP, an activator of the nuclear receptor constitutive androstane receptor (CAR, NR1I3), and mated them 1–6 weeks afterward. We detected in the offspring long-lasting alterations of CAR-mediated drug disposition, energy metabolism, and lipid profile. The transmission to the first filial generation (F1) was mediated by TCPOBOP transfer from the F0 adipose tissue via milk, as revealed by embryo transfer, crossfostering experiments, and liquid chromatography–mass spectrometry analyses. The important environmental pollutant PCB153 activated CAR in the F1 generation in a manner similar to TCPOBOP. Our findings indicate that chemicals accumulating and persisting in adipose tissue may exert liver-mediated, health-relevant effects on F1 offspring simply via physical transmission in milk. Such effects may occur even if treatment has been terminated far ahead of conception. This should be considered in assessing developmental toxicity and in the long-term follow-up of offspring of mothers exposed to both approved and investigational drugs, and to chemicals with known or suspected accumulation in adipose tissue.

Introduction

Activation of the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) by drugs and environmental pollutants induces the hepatic expression of genes involved in drug and energy metabolism (Prakash et al., 2015). This accelerates the disposition, and thereby reduces the efficacy, of numerous drugs, such as oral antiretrovirals, contraceptives, and immunosuppressants. Alterations in the metabolism of such endobiotics as steroids, bile acids, bilirubin, retinoids, carbohydrates, and lipids (Moreau et al., 2008) affect, among others, the risks of diabetes, obesity (Yang and Wang, 2014), and fatty liver disease (Yamazaki et al., 2007).

CAR target genes have been reported recently to undergo epigenetic imprinting following CAR activation (Chen et al., 2012; Tien et al., 2015; Li et al., 2016). This prompted us to assess the duration of

CAR-mediated induction. Specifically, we investigated whether CAR-mediated hepatic induction is transmitted to the next generation. Such a transmission is conceivable, since it would provide the next generation with an immediate protection against the momentary chemical environment.

We were also motivated by the gaps in developmental toxicity studies for medicinal drugs and for chemicals to be released into the environment. Currently, such studies focus on teratogenic effects. Drugs proven as teratogenic, exemplified by coumarins and valproic acid, are discontinued for the duration of the sensitive stages of pregnancy and replaced by safer alternatives (Schaefer, 2011). Treating pregnant women with drugs with unknown teratogenicity is prohibited or at least discouraged until teratogenicity has been excluded by analyses of sufficient numbers of unintended exposures. Well known teratogenic environmental chemicals that should be avoided during pregnancy include TCDD (Couture et al., 1990) and DEHP (Abdul-Ghani et al., 2012).

Less is known about the extent of postnatal and nonteratogenic toxicity, as has been described following vinclozolin (Anway et al., 2005), bisphenol A (Salian et al., 2009), or cocaine (Vassoler et al., 2013) exposures. It is also unclear how these toxicities arise. Several mechanisms of "soft inheritance" have been suggested for the transmission of substance-induced phenotypes from one generation to the next, including stable epigenetic mutations and transfer of RNA or substances through seminal fluid, the placenta, or via lactation

ABBREVIATIONS: BPA, 4,4′-(Propane-2,2-diyl)diphenol; CAR, constitutive androstane receptor (NR1I3); DEHP, *Bis*(2-ethylhexyl) benzene-1,2-dicarboxylate; F1, first filial generation; HDL, high-density lipoprotein; HFD, high-fat diet; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; LDL, low-density lipoprotein; logP, logarithmic partition coefficient; PCB153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; SD, standard diet; TCDD, 2,3,7,8-tetrachlorooxanthrene; TCPOBOP, 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene; VLDL, very low-density lipoprotein.

(Hughes, 2014). The extent or even the mere existence of such transmission in mammals, including humans, is still contentious (Heard and Martienssen, 2014).

In the following, we report and describe the mechanism of health-relevant phenotypes in the offspring of mice treated well ahead of pregnancy with CAR activators.

Material and Methods

Animals

All animal experiments have been carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the US National Institutes of Health, and were approved by the responsible local ethics committee (Landesuntersuchungsamt Rheinland-Pfalz, reference number 23 177-07/G 13-1-078). All animals were treated humanely with regard to the alleviation of suffering. C57BL/6J were obtained from Envigo (Venray, Netherlands) and CD1 from Janvier (Saint Berthevin, France). A maximum of five animals per cage were kept on a 12-hour light cycle at a temperature of 20–24°C and 45%–65% humidity. Females were housed individually beginning with the last 3 days before giving birth.

Induction of Mice. Newborn mice were injected with a volume of 10 μl/g using 30-guage needles, adult mice with 7 or 10 μl/g using 27-guage needles. CAR inducers were injected as follows, except if stated otherwise: TCPOBOP (1,4-*Bis* [2-(3,5-dichloropyridyloxy)]benzene; Sigma-Aldrich, Munich, Germany), 3 mg/kg in corn oil, single intraperitoneal injection to adult and newborn mice; PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl), 100 mg/kg in corn oil, single intraperitoneal injection to adult mice; phenobarbital sodium salt (Sigma-Aldrich), 100 mg/kg in 0.9% NaCl, i.p. on three consecutive days. Phenobarbital injections to newborn mice were given subcutaneously and only once 5 days after birth.

Female mice used to obtain the first filial generation (F1) offspring were induced 1 week prior to mating with an uninduced male. Adult males were depleted of spermatozoa by housing with females for 1 day and injected the following day with TCPOBOP or corn oil. Three, 19, and 31 days after TCPOBOP injection, each male was mated for 4 days with three age-matched, untreated, virgin females. The induction was confirmed in all experiments by measuring the expression of the CAR target *Cyp2b10* using a TaqMan assay.

Diets. Animals were fed R/M-H Extrudat (cat. no. 1536; Ssniff, Soest, Germany). For experiments with mice on a high-fat diet [HFD; DIO–60 kJ% fat (Lard), cat. no. E15742-34; Ssniff] the control group was fed an appropriate standard diet (SD; DIO LS–13 kJ% fat, 11% sucrose, cat. no. E15748-04; Ssniff). Food and water were provided ad libitum to all animals. In experiments with HFD, the diet was changed from the R/M-H Extrudat to HFD or SD simultaneously with the injection of the inducer in F0 animals.

Embryo Transfer. Seven-day-old female C57BL/6J mice were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone. Superovulation with pregnant mare serum gonadotropin (PMSG; 2.5–5 IU; i.p.) and human chorionic gonadotropin (hCG; 2.5 IU; i.p.) was started 3 weeks later with subsequent mating with untreated males for 12 hours. Zygotes were removed 24 hours later, cultured to two-cell embryos, and transferred to the oviduct of pseudopregnant, narcotized [medetomidin (0.5 μg/g), midazolam (5 μg/g), and fentanyl (0.05 μg/g)] 9-week-old CD1 mice pretreated with TCPOBOP or corn oil 1 week in advance. Postsurgery analgesia was conducted using metamizol (100 μg/g, by mouth) and carprofen (4.5 μg/g, s.c.).

Crossfostering. Female C57BL/6J and CD1 mice were treated with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone 1 week prior to 12-hour mating with untreated males of the same strain. E19.5 C57BL/6J F1 mice were delivered by cesarian section and substituted for up to 50% of CD1 litters. All F1 animals were analyzed 6 days after birth.

Zoxazolamine Paralysis Assay. Paralysis was induced with a single dose of the skeletal muscle relaxant zoxazolamine (175 mg/kg, i.p.; Sigma-Aldrich) in 10% dimethyl sulfoxide in corn oil. Mice were then placed on their backs at a constant temperature of 24°C. Paralysis time was defined and measured between the initiation of paralysis and the time point when the animal was able to right itself.

Glucose Tolerance Test. Mice were fasted for 16 hours prior to baseline glucose measurement with subsequent glucose injection (10% glucose, i.p.). All mice of one sex received the same amount of glucose irrespective of body weight. The amount of glucose per mouse was calculated as a dose of 2 g/kg of the mean body weight of the mice on standard diet of the respective sex (Ayala et al., 2010).

Glucose concentrations were measured using a CONTOUR XT glucometer (Bayer AG, Leverkusen, Germany) in blood samples collected from the tail 15, 30, 60, and 120 minutes following the injection.

Serum Lipoprotein Profile

Serum cholesterol lipoprotein profiles were determined by size-exclusion chromatography as described earlier (Parini et al., 2006; Gil-Pulido et al., 2017). In brief, 5 μl of serum was fractionated using a Superose 6 3.2/300 gel filtration column from GE Healthcare (Uppsala, Sweden) and phosphate-buffered saline, pH 7.4, as elution buffer, delivered by a first pump (1525 binary pump; Waters, Eschborn, Germany) at a flow rate of 50 μl/min. The separated lipoproteins were mixed in a T-tube with 50 μl/min of cholesterol reagent Chol (Roche, Mannheim, Germany) delivered by a second pump (1515 pump; Waters). Thereafter, the mixture went through a 500-μl reaction coil peep tubing (internal diameter 0.75 mm) at 37°C in a post–column reaction oven (Temperature Control Module II; Waters). Finally, absorption was measured with an UV-VIS detector at 500 nm (2489 UV/Visible Detector; Waters). Total run time for each sample was 60 minutes. Chromatograms were integrated by Waters Empower 3 software. Very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations were calculated by dividing the area under each pertinent peak through the area of all cholesterol peaks. Chromatograms for serum lipoprotein separation are shown in Supplemental Fig. 6.

Western Blot

Total protein (20–50 μg) lysed in a sample buffer was loaded onto each lane, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membrane by tank blotting. The antibodies used were as follows: anti-CYP2B10 (1:3000; cat. no. AB9916; Millipore, Darmstadt, Germany) and anti-GAPDH (1:20000; cat. no. sc-32233; Santa Cruz Biothechnology, Dallas, TX) with anti-rabbit (1:5000; cat. no. A0545; Sigma-Aldrich) and anti-mouse (1:10000; cat. no. A6782; Sigma-Aldrich), respectively. Bands were visualized by ECL+ detection method (FemtoMax, Rockland, Limerik, PA) using ChemiDoc XRS System with Quantity One (V 4.6.9; Bio-Rad, Munich, Germany) software. Densitometric analysis of the protein bands was done using Image Laboratory (V 4.1; Bio-Rad).

RNA-Isolation and Real-Time Polymerase Chain Reaction

Total hepatic RNA was isolated using TriFast (PeqLab, Erlangen, Germany) and 1 μg was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific, Darmstadt, Germany). *Cyp2b10* expression was quantified using TaqMan assays [primers (Chen et al., 2012), probe: 5′-[FAM]ACGTTCCTCTTCCAGTGCAT-CAC[BHQ1]-3′] on a StepOnePlus cycler (Applied Biosystems/Thermo Fisher Scientific). 18S rRNA (Mm03928990_g1; Applied Biosystems) was used as an internal control. The expression of *Cyp2b10* was calculated using the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

RNA Seq Data Analysis

Three- to four-day-old C57BL/6J mice were injected with TCPOBOP (3 mg/kg in corn oil) or corn oil. The hepatic RNA from newborn F0 and F1 mice (5–6 days old) was isolated with RNeasyKit (Qiagen, Hilden, Germany) to meet higher quality needs, and the hepatic RNA from adult F0 and F1 mice (3 months old) was isolated as described in the preceding section. The RNA of two to six newborn or adult male mice was pooled for the F0 and F1 generations. Hepatic RNA samples were sequenced on Illumina HiSEq 2000 using paired-end library protocol (TruSeq RNA Library Prep Kit; Illumina, San Diego, CA), at the StarSEQ GmbH, Mainz, Germany. We aimed for a sequencing depth of 25–50 million reads. Quality of raw sequencing reads was assessed using FastQC (Babraham Bioinformatics, Cambridge, UK) and reads were mapped to the mouse reference genome (gencode release M12 GRCm38.p5) using STAR aligner [v2.5.3a; (Dobin et al., 2013)], with the option "–quantMode GeneCounts" to count the number of reads per gene. EdgeR [v3.20.1; (Robinson et al., 2010)] was used for differential gene expression analysis. Genes with fold changes higher than two adjusted by *P* value below 0.05 were considered differentially expressed. Differentially expressed genes obtained from edgeR were uploaded to Ingenuity Pathway Analysis (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis; Qiagen). The core analysis was performed using Ingenuity

Knowledge Base as the reference set (accessible via the afore mentioned link). The analysis was restricted to human and mouse species and to the differentially expressed genes with fold change of at least two. Pathways and upstream regulators were compared among the experimental groups. The sequences and processed files containing the counts in TPM (Transcripts per Kilobase Million) have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002; Barrett et al., 2013) and are accessible through GEO Series accession number GSE109630 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109630).

TCPOBOP Extraction from Adipose Tissue and High-Performance Liquid Chromatography–Tandem Mass Spectrometry Analysis

Epididymal fat pads were excised and melted at 60°C. One hundred, twenty microliters of each sample were heated to 50°C in 5 ml of n-Hexan and mixed. Samples were kept at −20°C overnight and centrifuged at 2000g for 5 minutes. Supernatant was kept at −20°C overnight and centrifuged again. One milliliter of the supernatant was dried for 1.5 hours in a vacuum concentrator with vapor trap (SC 110 and RVT 100 Savant; Thermo Fisher Scientific, Waltham, MA), dissolved in 100 μl acetonitrile/water (1:1) and centrifuged at 13,000g for 2 minutes. Samples were kept at −20°C. Five microliters were taken from the liquid phase and analyzed by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) analysis. The separation was performed on an Agilent 1290 UHPLC system equipped with a ZORBAX RRHD SB-C18 column (1.8-μm 2.1 × 50 mm; Agilent Technologies, Santa Clara, CA) maintained at 30°C. The running buffer and the solvent were (A) 5 mM ammonium acetate at pH 6.9 and (B) acetonitrile. The separations were performed at a flow rate of 0.4 ml/min using the following gradients: 50% of solvent B from 0 to 2 minutes, linear increase to 95% of solvent B for next 3 minutes, and kept at 95% of solvent B for another 2 minutes. To recondition the column, the flow rate was then switched to 0.5 ml/min and washed with 50% of solvent B for 3 minutes. During the last minute, the flow rate was linearly decreased to the initial value of 0.4 ml/min. The detection was performed on Agilent 6090 triple quadruple mass spectrometer operated in the positive ion mode, using the settings listed in Supplemental Table 4 and Supplemental Table 5.

Statistics

Data were analyzed with GraphPad Prism version 7.00 for Windows. Parametric or nonparametric tests were used according to the results of the Shapiro-Wilk test. Further statistical analysis and numbers of replicates are provided in the figure legends. Mixed models were fit to the mouse body-weight time-course data. In detail, the subsets split by diet (SD vs. HFD) were fit separately with modeling time and treatment as fixed-effects, and animal as random-effect. Data at time point 0 were excluded for this purpose, as the diet and treatment were applied after this initial measurement. For this purpose, the R statistical software (R Core Team, 2017) (https://www.R-project.org) and the lme4 package (Bates et al., 2015) were used. Throughout the analysis, a significance level $\alpha = 0.05$ was chosen. Since no estimates of effect sizes were available from literature, the target group size was set to five animals. With this group size, the power to detect a standardized difference in means of 2 (Cohen's d) is 79% given an α-level of 5%, as computed with the function power.t.test in the statistics software R. We expected higher intragroup variance for bodyweight, glucose measurements, and serum lipids, and therefore strove to double the group size. The presented group sizes reflect the additional effect of variabilities in breeding success, survival rates, and sex distributions of litters.

Results

Transmission of *Cyp2b10* Induction to F1 and Its Effects on Drug Metabolism. The transmission of drug effects to the next generation was first assessed for the prototypical murine CAR-ligand 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and the CAR target gene *Cyp2b10*. Six-day-old F1 offspring of F0 females injected once with 3 mg/kg TCPOBOP 1 week prior to mating at the age of 8 weeks exhibited 747-fold (females) and 517-fold (males) increased *Cyp2b10* mRNA expression compared with the female or male offspring of corn oil (vehicle)-injected females (Fig. 1A, left panel), respectively. The induction of the CYP2B10 protein was confirmed by Western blot

(Fig. 1A, right panel). In 8-week-old F1 offspring, the *Cyp2b10* mRNA expression was still increased 3.8-fold in females and 37-fold in males (Fig. 1B). To extend further the interval between the TCPOBOP treatment and the time point of the F1 induction measurement, we injected 3-day-old female F0 mice with the same TCPOBOP dose as above and mated them 6 weeks later with untreated males. The *Cyp2b10* mRNA expression was induced 2.3-fold in the female F1 offspring and 3.7-fold in the male F1 offspring 12 weeks after birth (Fig. 1C). In agreement with previous studies (Chen et al., 2012; Li et al., 2016), we found a robust and long-lasting *Cyp2b10* induction also in F0 females injected with TCPOBOP either as newborns or as adults (Supplemental Fig. 1). Among other *Cyp* genes, we found a 30-fold induction of *Cyp2c55* in newborn but not in adult F1 offspring of TCPOBOP-treated females.

To determine the induction transmission through the paternal line, males were treated 3, 19, and 31 days prior to mating, to expose to TCPOBOP spermatozoa/spermatids, spermatocytes, and spermatogonia, respectively. There was a statistically significant induction in offspring generated with TCPOBOP-treated spermatocytes, but its 2-fold magnitude was negligible (Supplemental Fig. 2).

The impact of the transmitted CYP2B10 induction on F1 drug metabolism was assessed as the duration of paralysis upon injection of the muscle relaxant and CYP2B10 substrate zoxazolamine. The paralysis time was defined as the time required by the F1 mice to consciously right themselves. The paralysis time was 55% shorter in the female F1 offspring of F0 females treated with TCPOBOP 1 week prior to mating compared with the female F1 offspring of control F0 females. An effect of similar magnitude (40%) in the male F1 offspring failed to reach the preset significance level (Fig. 2).

TCPOBOP Effects on Energy Metabolism in the F1 Offspring. The 12-week-old F1 offspring of TCPOBOP-treated F0 females gained less weight when set on a standard diet at the age of 6 weeks, but there was no difference between offspring of treated and untreated females when set on a high-fat diet (males Fig. 3A, females Supplemental Fig. 3A). The food intake was similar in the F1 offspring of TCPOBOP- and corn oil-treated females (Supplemental Fig. 4). Glucose tolerance was improved only in the male F1 offspring of TCPOBOP-treated F0 females, when the offspring were kept on HFD diet and only at one time point (males Fig. 3B, females Supplemental Fig. 3B). The F1 offspring of TCPOBOP-treated F0 females also displayed decreased total cholesterol and HDL levels, whereas LDL and VLDL levels were unchanged (Fig. 3C). These phenotypes mostly resembled the TCPOBOP effects in male F0 mice 3 days after the injection (Supplemental Fig. 5). (F0 females did not tolerate diet change following TCPOBOP injection.) The only exception was that TCPO-BOP reduced weight gain irrespective of the diet and that it additionally reduced LDL (Supplemental Fig. 5).

We compared these phenotypes with gene expression changes in livers of male mice induced with TCPOBOP as newborns and analyzed 8 weeks later, as well as in 6-day- and 8-week-old male F1 offspring of TCPOBOP-treated females. Ingenuity Pathway Analysis revealed changes in pathways related to energy metabolism in all TCPOBOP groups, including CAR itself and known CAR signaling partners peroxisome proliferator-activated receptor α (PPARα), liver X receptor (LXR), nuclear factor (erythroid-derived 2)-like 2 (NRF2), farnesoid X receptor (FXR), interleukin 6 (IL6), hepatocyte nuclear factor 4 α (HNF4A) (Supplemental Tables 1 and 2). Among the five categories of functions and diseases affected by TCPOBOP were lipid and drug metabolism (Supplemental Table 3).

Long-Term CAR Activation in F1 by Transmission of the Lipophilic CAR Ligand via Milk. The transmission of TCPOBOP-driven induction to F1 could result from epigenetic modifications in maturating oocytes that happen as early as the injection of newborn
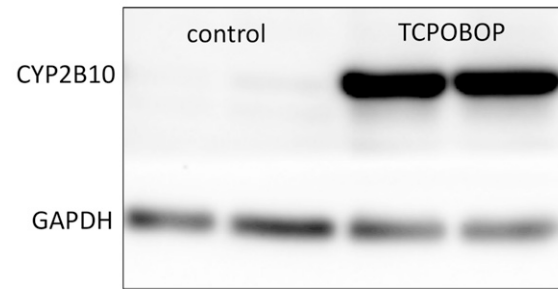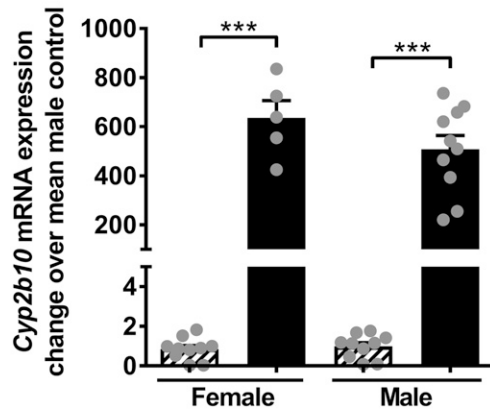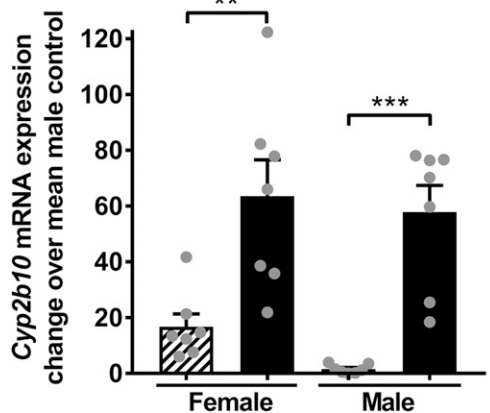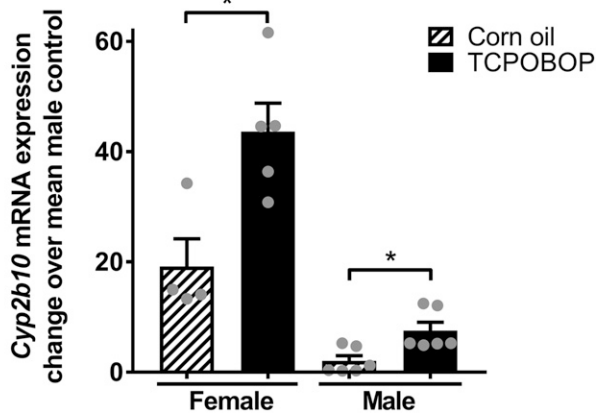
A 6-day-old F1 (F0 adults injected)

B 8-week-old F1 (F0 adults injected)

C 12-week-old F1 (F0 newborns injected)

Fig. 1. *Cyp2b10* induction in the F1 offspring driven by a single maternal, preconceptional TCPOBOP injection. Hepatic *Cyp2b10* mRNA expression was determined by real-time polymerase chain reaction and ΔΔCt-method. Adult (A and B) or 3-day-old (C) female F0 mice were injected intraperitoneally with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone. *Cyp2b10* expression in F1 mice was assessed 6 days (A), 8 (B), or 12-weeks (C) after birth. Data are presented as mean fold-expression changes ± S.E.M. over the mean male corn oil group ($n = 4$–10), which were analyzed by t test or Mann-Whitney test. Statistically significant differences are indicated by asterisks ($***P < 0.001$; $*P < 0.05$). (A) (right panel) Western blot of hepatic CYP2B10 expression of four male 6-day-old F1 mice born from females injected with TCPOBOP or with corn oil 1 week prior to mating. GAPDH expression served as a loading control.

F0 females. Alternatively, it could occur post-conceptionally, either by placenta- or feeding-mediated (via milk) TCPOBOP transfer to the F1 offspring. To distinguish between these possibilities, we conducted embryo transfer experiments. F1 mice conceived by untreated females,
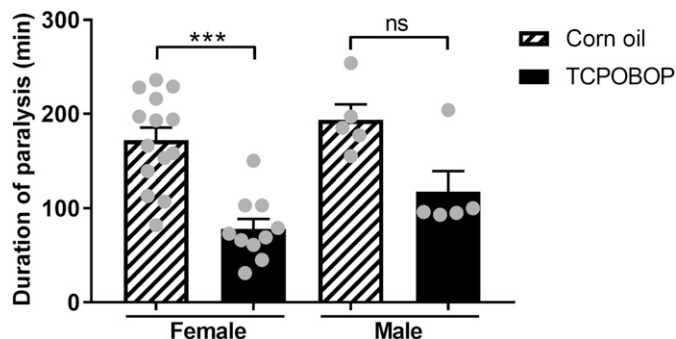

Fig. 2. Effects of F0 TCPOBOP treatment on the zoxazolamine-induced paralysis in F1 mice. Female F0 mice were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone 1 week prior to mating with untreated males. Shown is the duration of zoxazolamine (175 mg/kg, i.p.)-induced paralysis time in 8-week-old F1 offspring. Data are presented as mean ± S.E.M. ($n = 5$–14) and were analyzed by t test. Statistically significant differences are indicated by asterisks ($***P < 0.001$).

but carried to term by foster mothers pretreated with TCPOBOP, exhibited increased *Cyp2b10* expression. In contrast, F1 mice conceived by TCPOBOP-pretreated donor mice, but carried to term by untreated foster mothers, showed no increase in *Cyp2b10* expression (Fig. 4A). Both of these results suggested a post- rather than preconceptional mechanism of induction transmission.

To differentiate between a transplacental (in utero) and a milk-mediated (postnatal) TCPOBOP transfer, we conducted crossfostering experiments (Fig. 4B). E19.5 F1 offspring of corn oil-treated mice transferred to TCPOBOP-pretreated foster mothers showed 82% of the *Cyp2b10* induction measured in E19.5 F1 offspring of TCPOBOP-treated mice transferred to TCPOBOP-pretreated foster mothers. Conversely, E19.5 F1 offspring of TCPOBOP-treated mice transferred to corn oil-injected foster mothers showed 1% of the *Cyp2b10* induction measured in E19.5 F1 offspring of TCPOBOP-treated mice transferred to TCPOBOP-pretreated foster mothers. These results indicated that a vast majority of the induction occurs via postnatal TCPOBOP transmission. This was confirmed additionally by injecting lactating females with TCPOBOP at different time points (5, 10, or 15 days) after they had given birth. *Cyp2b10* expression strongly increased in the F1 mice suckling for 3 days after the injection of F0 mothers, as well as in 60-day-old F1 mice, i.e., 6 weeks after weaning
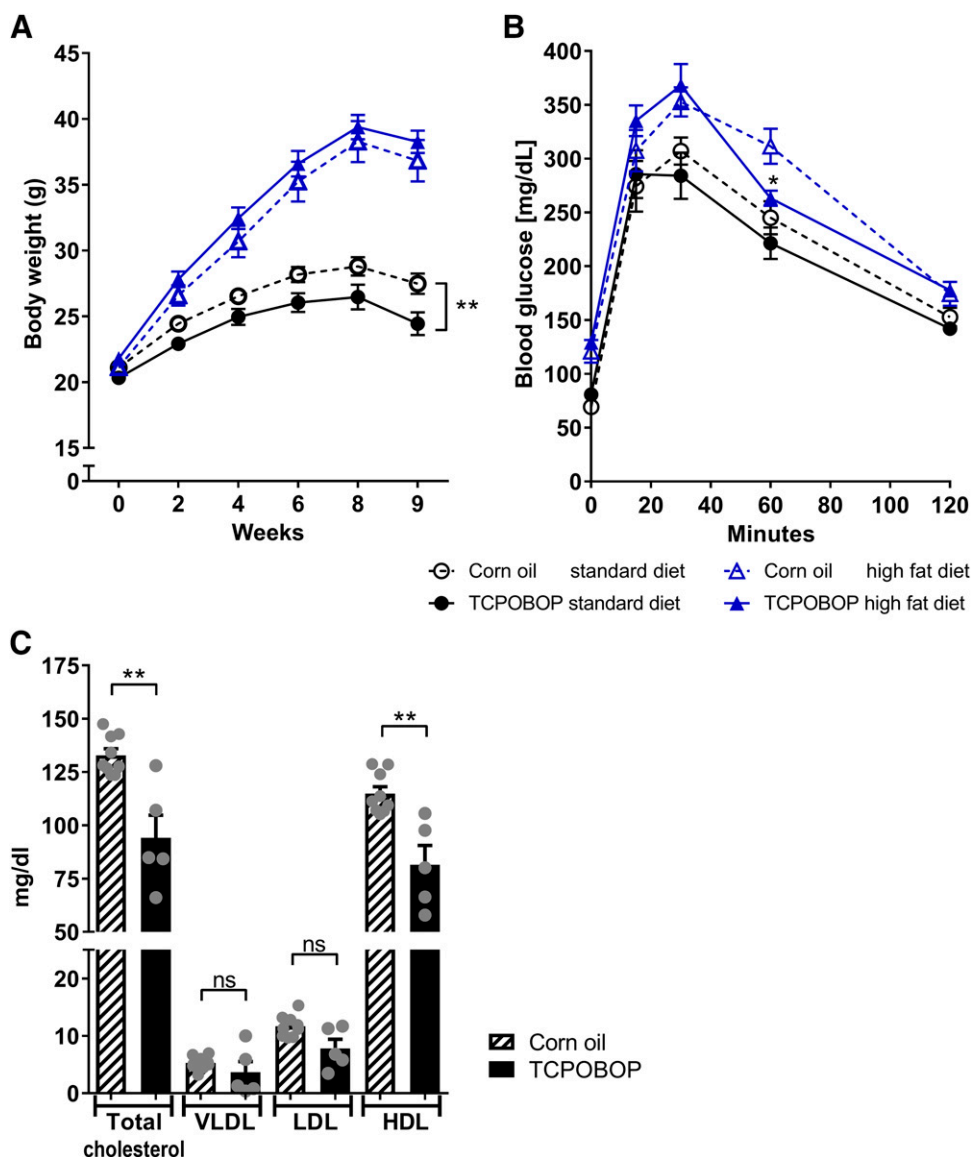
Fig. 3. Body weight, glucose tolerance, and serum lipids in the F1 offspring following a single, preconceptional TCPOBOP injection. (A) Six-week-old male offspring of females injected with TCPOBOP (3 mg/kg) in corn oil (solid line) or with corn oil alone (dotted line) 1 week prior to mating with untreated males were set on HFD (blue) or (SD) (black) for 9 weeks. Statistically significant differences in body weight were analyzed using a mixed model and treatment-related differences are indicated by asterisks (***$P < 0.001$). Data are presented as mean $\pm$ S.E.M., $n = 6$–11. (B) Following week nine, all mice were fasted for 16 hours and challenged with glucose (2 g/kg) according to the mean body weight of male animals on standard diet (same dose for each animal). Shown are mean concentrations $\pm$ S.E.M. in male offspring. Blood glucose was analyzed by t test or Mann-Whitney test with Bonferroni correction for four time points (0, 15, 30, 60 minutes). Statistically significant differences are indicated by asterisks (*$P < 0.05$), $n = 6$–9. (C) Total cholesterol, VLDL, LDL, and high-density lipoprotein (HDL) were analyzed using HPLC in 12-week-old offspring on normal diet. Mice were fasted for 16 hours before the analysis. Serum lipid concentrations were analyzed by t test with Bonferroni correction for four parameters, statistically significant differences are indicated by asterisks (**$P < 0.01$; *$P < 0.05$). Data are presented as mean $\pm$ S.E.M., $n = 5$–9. Example serum lipid profiles are shown in Supplemental Fig. 6.

(females Fig. 5, males Supplemental Fig. 7). After weaning of F1 offspring, the *Cyp2b10* expression in TCPOBOP-pretreated mothers returned to the level of mothers pretreated with corn oil (Supplemental Fig. 8).

These results suggested a lactation-mediated transfer of TCPOBOP to F1 offspring, which was confirmed by HPLC-MS/MS analysis of F0 and F1 adipose tissue. The concentration of TCPOBOP, injected in adult F0 females at a dose of 3 mg/kg, was stable in F0 adipose tissue over a period of at least 12 weeks (Fig. 6A, lanes 2 and 9). The TCPOBOP concentration in the adipose tissue of 12-week-old F1 offspring of females induced 1 week prior to mating was 160-fold lower (Fig. 6A, lane 11). The TCPOBOP concentration in the adipose tissue of F1 offspring was thus like that observed in F0 females injected with 100- to 500-times smaller amounts of TCPOBOP (Fig. 6A, lanes 5 and 6). The magnitude of *Cyp2b10* induction in the F1 generation corresponded to that following 1/10th to 1/100th of the original dose given to F0 (Fig. 6B, lane 4, 5 and 11). Taken together, these results demonstrated that TCPOBOP is transmitted to F1 and that its amount determines the amplitude of *Cyp2b10* induction.

Transmission to F1 May Require Lipophilicity. Since TCPOBOP is lipophilic, we hypothesized that induction transmission would be a general property of lipophilic compounds. To provide a first verification,

we used the lipophilic environmental pollutant and CAR inducer PCB153 (Gahrs et al., 2013; Wahlang et al., 2014). One week after treatment with 100 mg/kg PCB153, adult female mice were mated with untreated males. *Cyp2b10* expression was induced 820-fold in female and 700-fold in male 6-day-old F1 offspring compared with the respective corn oil controls (Fig. 7). We then compared PCB153 with the hydrophilic CAR inducer phenobarbital. Newborn female F0 mice injected once intraperitoneally with 100 mg/kg phenobarbital showed a 70- and 200-fold *Cyp2b10* induction after 24 hours in females and males, respectively. Six weeks later, the induction declined to 3.3-fold in females and 1.6-fold in males (Supplemental Fig. 9A). Newborn F1 offspring of these phenobarbital-treated mice showed no *Cyp2b10* induction (Supplemental Fig. 9B).

Discussion

We demonstrate that long lasting, lipophilic chemicals applied far ahead of pregnancy are transmitted via milk and evoke health-relevant phenotypes in the adult F1 offspring. We show it for two ligands of the xenosensing nuclear receptor CAR, but the mechanism probably applies to any other receptor modifiable by lipophilic chemicals. The resulting
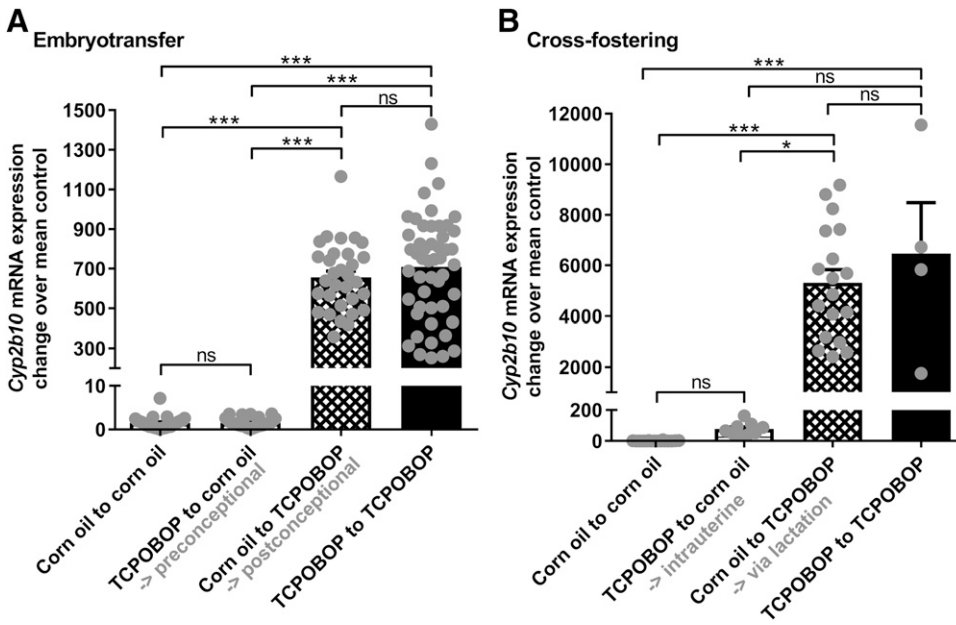
Fig. 4. *Cyp2b10* mRNA expression in the embryo-transferred or crossfostered F1 offspring. (A) Embryo transfer: Donor C57BL/6J females were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone and super-ovulated and mated with untreated males 3 weeks later. Two-cell embryos were transferred to CD1 recipients pretreated 1 week earlier with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone, and carried to term ($n = 29$–50). (B) Crossfostering: C57BL/6J and CD1 females were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone, and mated with untreated males 1 week later. Following cesarian section on gestational day 19.5, F1 C57BL/6J offspring mice ($n = 4$–18) were substituted for up to 50% of litters nursed by CD1 mice. The hepatic *Cyp2b10* mRNA expression was determined in 6-day-old F1 animals by real-time polymerase chain reaction and $\Delta\Delta$Ct-method and was analyzed by Kruskal-Wallis test and Dunn's post hoc-test. Statistically significant differences over the control "corn oil to corn oil" are indicated by asterisks (***$P < 0.001$; *$P < 0.05$). Data are presented as mean $\pm$ S.E.M.

F1 phenotypes include subtle changes of energy and drug metabolism, which may manifest clinically only later in life, or under specific conditions such as drug treatments. Owing to long-term accumulation in adipose tissue, the ligand transmission to F1, exemplified by TCPOBOP, takes place even if the treatment has been terminated far ahead of conception. These findings are of importance for the management of chemicals released into the environment, for the development of new drugs, and for planning and management of pregnancies.

Mechanism. The latest literature suggests that persistent epigenetic changes in the promoters of CAR target genes, including *Cyp2b10,* are responsible for prolonged induction within the F0 generation in the liver (Chen et al., 2012; Peng and Zhong, 2015; Tien et al., 2015, 2017; Li et al., 2016; Piekos et al., 2017; Lodato et al., 2018). Our work indicates that at least some of these effects may have been driven by activator accumulation rather than by stable DNA modifications.

The involvement of a physical ligand transfer in the induction transmission is supported by the detection in F1 of TCPOBOP (this study) and of PCB153 (Gallenberg and Vodicnik, 1987; Ring et al., 1990). It is also consistent with the long-term accumulation of these compounds in F0 adipose tissue (Poland et al., 1980; Gallenberg and Vodicnik, 1987; Gallenberg et al., 1990; Ring et al., 1990; Smith et al., 1993), confirmed in this study. Adipose tissue undergoes mobilization upon lactation

(Lederman, 2004). The resulting TCPOBOP transfer to F1 is in accordance with the absence of *Cyp2b10* induction in mothers after weaning. The mobilization also reduces the PCB153 burden in F0 mothers, just as the chemical becomes detectable in the F1 offspring (Gallenberg and Vodicnik, 1987). Milk is the main vehicle of transmission, as demonstrated previously for PCB153 (Gallenberg and Vodicnik, 1987) and in this study for TCPOBOP. Accordingly, we found a nearly maximal *Cyp2b10* induction in cesarian section-delivered F1 offspring of TCPOBOP-naïve mothers transferred to TCPOBOP pretreated foster (i.e., lactating) mothers. Induction also occurred upon injection of lactating females with TCPOBOP. Significantly, PCB153 has been found in human breast milk samples (Lignell et al., 2009).

In comparison with the amounts of TCPOBOP transferred via milk, the transplacental transmission was minimal, judging from the negligible induction in F1 offspring of TCPOBOP-treated females transferred to TCPOBOP-naïve foster mothers for lactation. Likewise, PCB153 does not cross the placenta, which has been attributed to the strong binding to VLDL (Gallenberg and Vodicnik, 1987). TCPOBOP transfer via fur licking could not have contributed to induction transmission, since allo-grooming starts only several days after our time point of F1 induction analysis (Williams and Scott, 1953; Fox, 1965). We excluded the possibility of preconceptional "imprinting" of induction by TCPOBOP
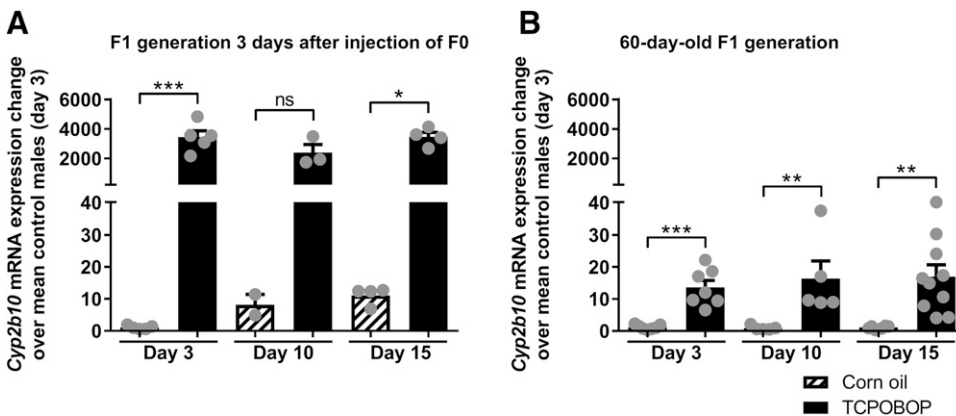

Fig. 5. TCPOBOP transfer via lactation initiated at three time points after birth. Lactating F0 females were treated with TCPOBOP on day 3, 10, or 15 after giving birth. Hepatic *Cyp2b10* mRNA expression was analyzed in female F1 newborns 3 days after maternal TCPOBOP treatment, $n = 2$–5 (A) or in 60-day-old female F1 mice, $n = 5$–10 (B). Shown are mean expression changes over mean male controls $\pm$ S.E.M. determined by real-time polymerase chain reaction and $\Delta\Delta$Ct-method, which were analyzed by t test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$). Similar results were obtained for male offspring (Supplemental Fig. 7).

A TCPOBOP concentration in adipose tissue
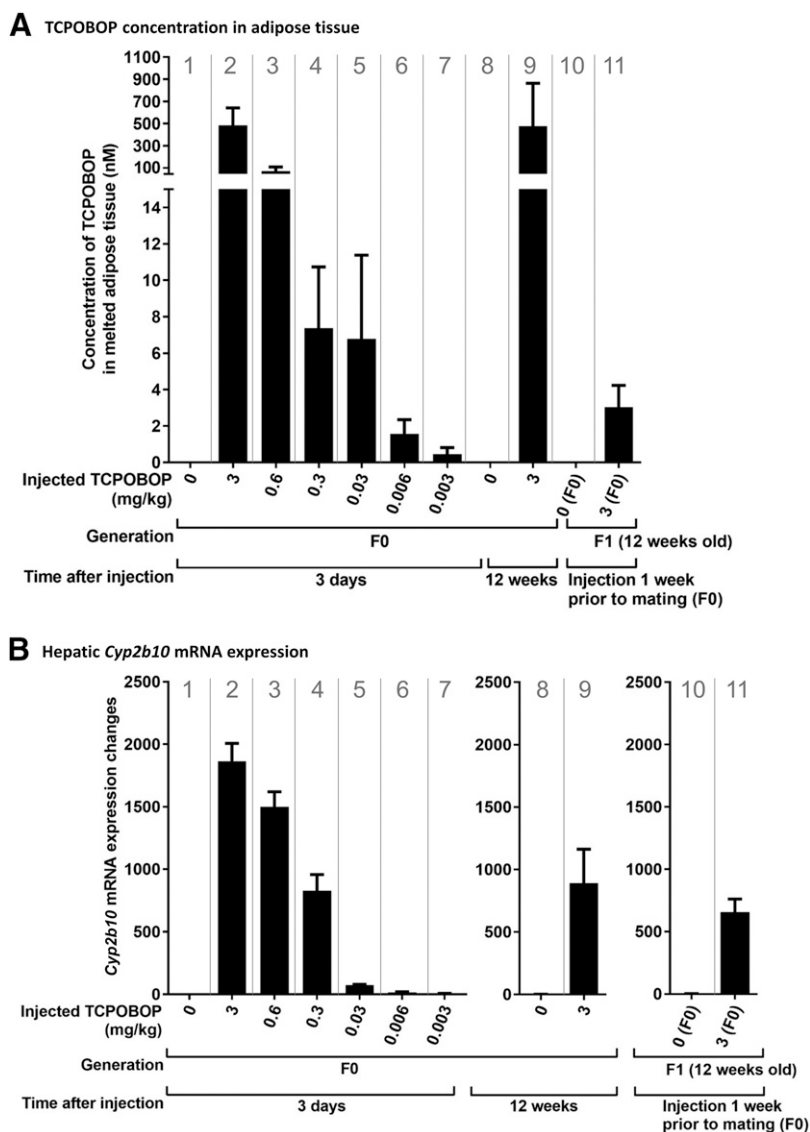

B Hepatic *Cyp2b10* mRNA expression

Fig. 6. TCPOBOP concentrations in the adipose tissue and the corresponding hepatic *Cyp2b10* induction. (A) TCPOBOP was detected by HPLC-MS/MS. TCPOBOP was extracted from 120 μl of melted epididymal adipose tissue of mice treated at the age of 11 weeks with different TCPOBOP concentrations (0, 3, 0.6, 0.3, 0.03, 0.006, 0.003 mg/kg) 3 days prior to analysis, and with 3 mg/kg 12 weeks prior to analysis. F1 offspring of F0 female mice injected with TCPOBOP (3 mg/kg) and mated 1 week later were analyzed 12 weeks after birth. Shown are mean TCPOBOP concentrations $\pm$ S.E.M. (n = 4). (B) *Cyp2b10* mRNA expression changes over the respective time point- and generation-mated, corn oil-injected controls in livers of mice shown in (A) was determined by real-time polymerase chain reaction and $\Delta\Delta$Ct-method. Data are presented as mean expression fold-changes $\pm$ S.E.M.

by embryo transfer experiments, and we did not detect any effects in F1 following the less lipophilic phenobarbital.

Nongenetic transfer of phenotypes originating from a treatment *prior to* pregnancy has been attributed to epigenetic marks in the gametes (Anway et al., 2005), to somatic reprogramming following in utero exposure (Iqbal et al., 2015), or to RNA transfer via sperm cells (Rodgers et al., 2015). Our work demonstrates that such phenotypes may arise via accumulation of chemicals and their transfer to the next generation. The possible routes are placenta (Jeong et al., 2018), seminal fluid (Bromfield et al., 2014), and milk, as demonstrated here using a specific ligand-receptor pair.

Metabolic Effects. The CYP2B10 induction in the F1 generation decreased efficacy of the muscle relaxant zoxazolamine. The transcriptional targets of human CAR, *CYP2B6*, *CYP3A4*, and *UGT1A1,* (Park et al., 2016) metabolize a majority of clinically used drugs, including antineoplastics, anesthetics, opioids, and antiretrovirals (Wang and Tompkins, 2008; Hedrich et al., 2016). It is tempting to speculate that long-lasting CAR ligands are responsible for interindividual expression variability of these targets that cannot be explained by genetic variants (Zanger et al., 2001; Peng and Zhong, 2015; Hedrich et al., 2016), although further experiments are needed to validate this hypothesis.

This variability further complicates drug dose finding, especially in newborns, or may cause toxicities (Tayman et al., 2011; Pauwels and Allegaert, 2016).

TCPOBOP-mediated effects on F1 energy metabolism included a reduction in body weight gain, improved glucose tolerance during high-fat diet, and reduced serum lipids. They are consistent with a protective role of CAR induction against metabolic syndrome-like phenotypes in F0 (Gao et al., 2009; Kobayashi et al., 2015). The reduction of body weight gain in F0 and F1 was independent of food intake, supporting the previously reported higher energy expenditure upon CAR activation in brown adipose tissue (Gao et al., 2009). The observed F1 phenotypes match those in F0 animals in terms of directionality. The subtle differences most probably result from lower exposure of the F1 to TCPOBOP compared with the F0.

The phenotypes were in line with transcriptomic changes. Besides drug metabolism, TCPOBOP affected regulators of pathways involved in the energy homeostasis of newborn and adult F0 mice as well as newborn and adult F1 mice. PPARα, FXR, and LXR have an impact on energy metabolism and the pathogenesis of fatty liver disease (Kersten and Stienstra, 2017; Li et al., 2017; Tanaka et al., 2017). IL6 plays a role in type 2 diabetes (Phosat et al., 2017) and HNF4A in hereditary diabetes
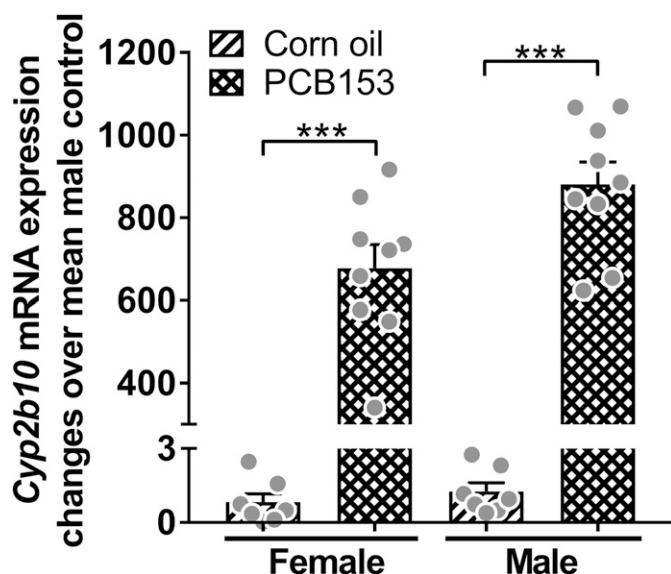
Fig. 7. *Cyp2b10* induction in the F1-generation by PCB153. Seven-week-old adult F0 female mice were injected once with PCB153 (100 mg/kg) or vehicle (corn oil). After 1 week, they were mated with untreated males. *Cyp2b10* expression was assessed in 6-day-old F1 offspring. Shown are mean expression changes over the mean of male controls ± S.E.M. determined by real-time polymerase chain reaction and ΔΔCt-method and analyzed by *t* test. Statistically significant differences are indicated by asterisks (***$P < 0.001$).

(Yamagata et al., 1996). Nrf2 is involved in glucose resistance and dyslipidemia (Xu et al., 2015; Zhang et al., 2015b). Lipid metabolism was one of the top three regulated pathways 60 days after TCPOBOP treatment of F0 animals in a previous study (Li et al., 2016).

Significance for Chemical Safety and Pregnancy Management. Termination of therapy 6–24 months ahead of pregnancy is presently recommended only for a handful of long-term accumulating drugs, including cytostatics, radioiodine, vitamin K antagonists, amiodarone, leflunomide, methotrexate, and retinoids. These drugs are either teratogenic or they cause adverse effects manifesting immediately after birth, exemplified by hypothyroidism after amiodarone (Magee et al., 1995).

Our work strongly suggests that these recommendations should be expanded to other drugs and extended to the avoidance of certain environmental chemicals. Indeed, long-term accumulating chemicals, exemplified here by TCPOBOP and PCB153 (Schecter et al., 2010; Whitehead et al., 2013; Pozo et al., 2017), may be transmitted to F1 and induce health-relevant phenotypes even if treatment has been terminated far ahead of conception. Such phenotypes may remain undetected, since developmental toxicology currently focuses on teratogenic effects. These phenotypes are more subtle and manifest later in life. It is probable that such unapparent phenotypes arise in F1 after the transmission of lipophilic ligands of other receptors. This could and should be assessed for both approved and investigational drugs and chemicals known or suspected of accumulation in adipose tissue.

Such drugs and chemicals can be identified by a combination of small molecular weight, high logarithmic partition coefficient (logP), and logarithmic acid dissociation constant (pKa) <7 (Moor et al., 1992), which also correlate with the probability of transfer via milk (Howard and Lawrence, 2001; Kar and Roy, 2013). Highly lipophilc drugs (logP > 6) include the CAR ligand atorvastatin (Režen et al., 2017), as well as montelukast, raloxifene, cinacalcet, fexofenadine, repaglinide, telmisartan, adapalene, terbinafine, dutasteride, and levothyroxine (Zhong et al., 2013). Many common environmental pollutants are lipophilic (logP > 3) CAR ligands [e.g., DDT (Kamata et al., 2018), permethrin

(Kublbeck et al., 2011), chlordane (Kamata et al., 2018), dibutylphthalate (Wyde et al., 2005), dieldrin (Wei et al., 2002), methoxychlor (Kamata et al., 2018), pyren (Zhang et al., 2015a), and toxaphen (Wei et al., 2002)], which cross the placenta or are transferred via milk (Campoy et al., 2001; Skopp et al., 2002; Cok et al., 2012; Corcellas et al., 2012; Limon-Miro et al., 2017; Muller et al., 2017; Wang et al., 2017; Yasmeen et al., 2017). The lipophilic pollutants and CAR modulators BPA and DEHP (DeKeyser et al., 2011) exhibit not only multi- but even transgenerational (beyond F1) effects (Ziv-Gal et al., 2015; Pocar et al., 2017). The assessment of F1 effects could start with transcriptional profiling in appropriate animal models.

Authorship Contributions

Participated in research design: Dietrich, Baumgart, Eshkind, Butt, Musheev, Niehrs, Wojnowski, Mathäs.

Conducted experiments: Dietrich, Baumgart, Eshkind, Reuter, Gödtel-Armbrust, Butt, Musheev, Grosser, Mathäs.

Contributed new reagents or analytic tools: Butt, Musheev, Niehrs.

Performed data analysis: Dietrich, Butt, Musheev, Marini, More, Mathäs.

Wrote or contributed to the writing of the manuscript: Wojnowski, Mathäs.

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DRUG METABOLISM AND DISPOSITION

Supplemental Data

Health-relevant phenotypes in the offspring of mice given CAR activators prior to pregnancy

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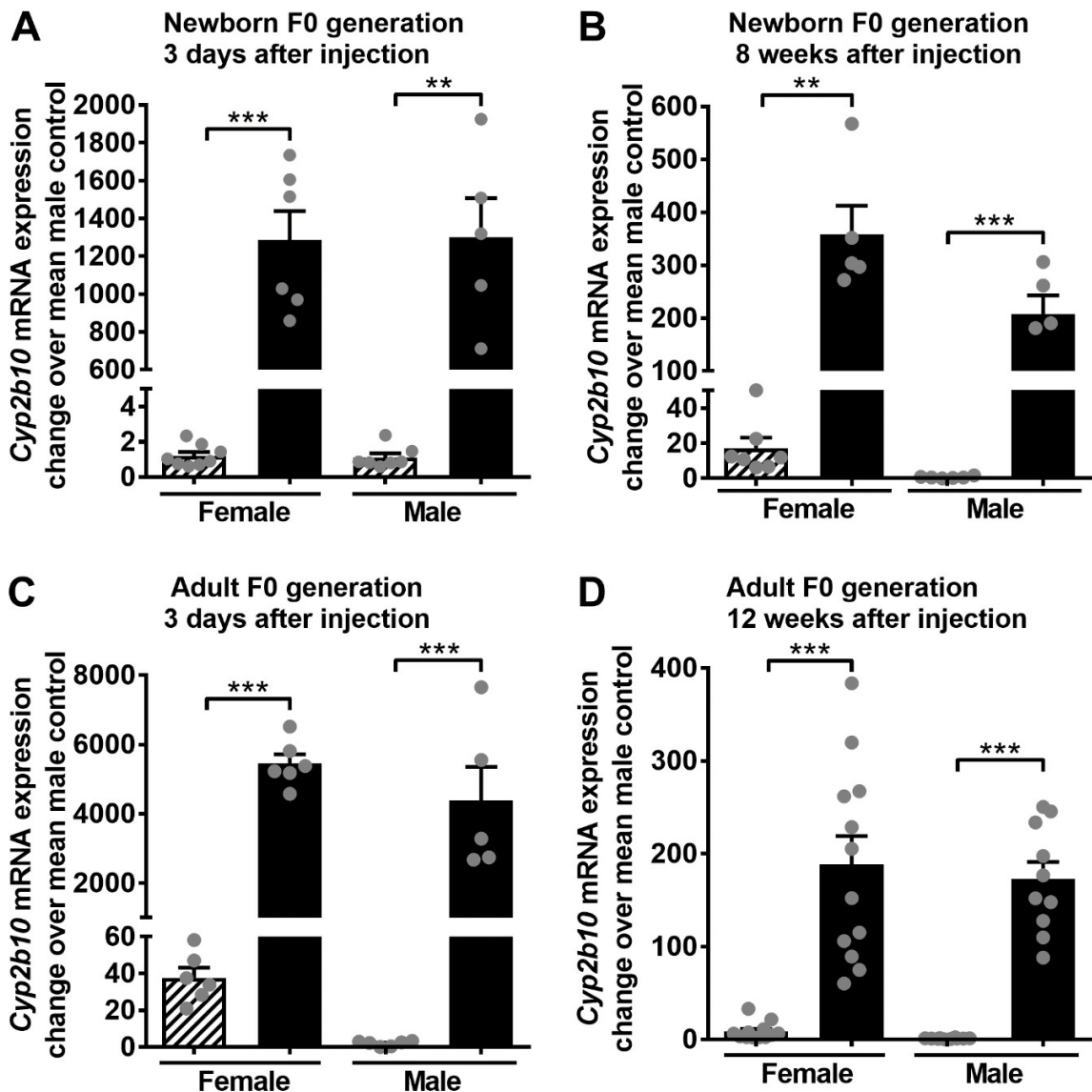
Supplementary Fig. 1 TCPOBOP mediated *Cyp2b10* induction in the F0 generation
Hepatic *Cyp2b10* mRNA expression was determined by q-PCR and ΔΔct-method. Three-day-old newborns (A, B) or 8-week-old adults (B, C) F0 mice were injected i.p. with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone. *Cyp2b10* mRNA expression was assessed 3 days (A, C), 8 weeks (B) or 12 weeks (D) after the TCPOBOP injection. Data are presented as mean fold-expression changes ± SEM over the mean male control group (n = 5-12). Expression changes were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***,P < 0.001; **,P < 0.01).
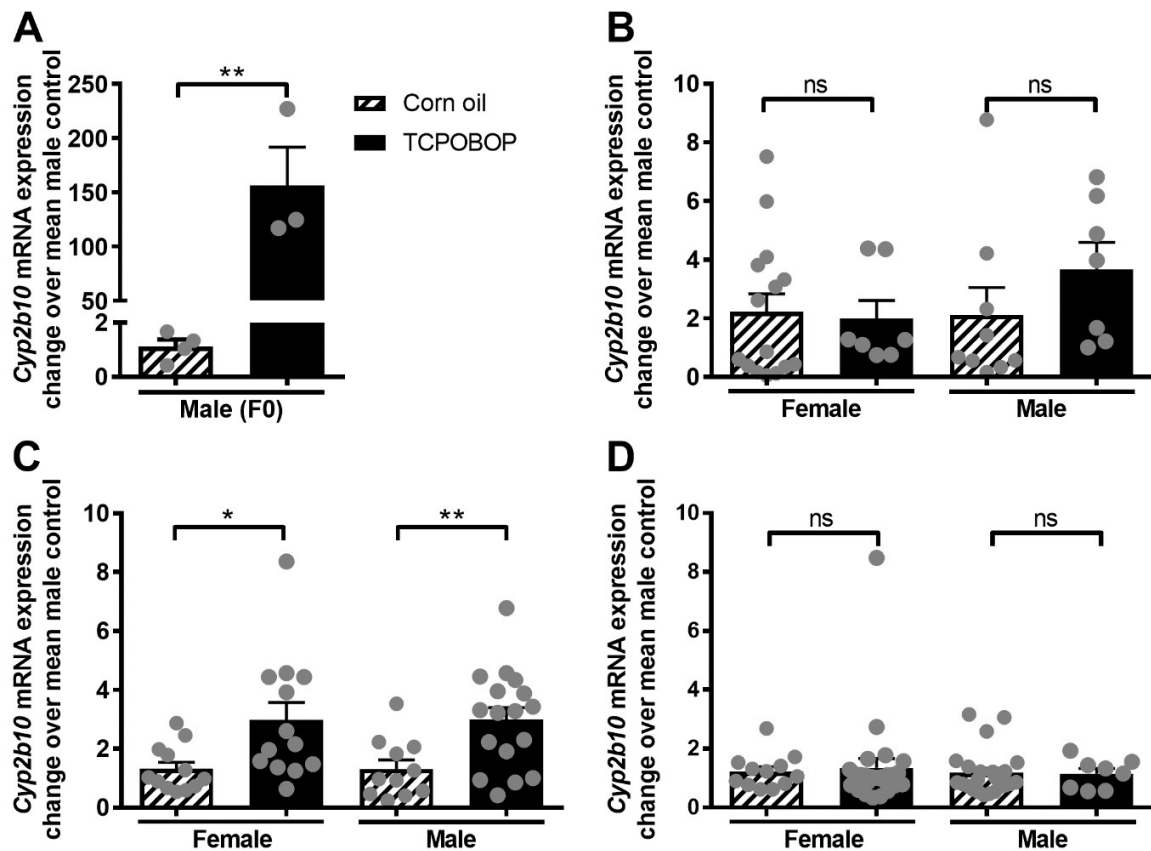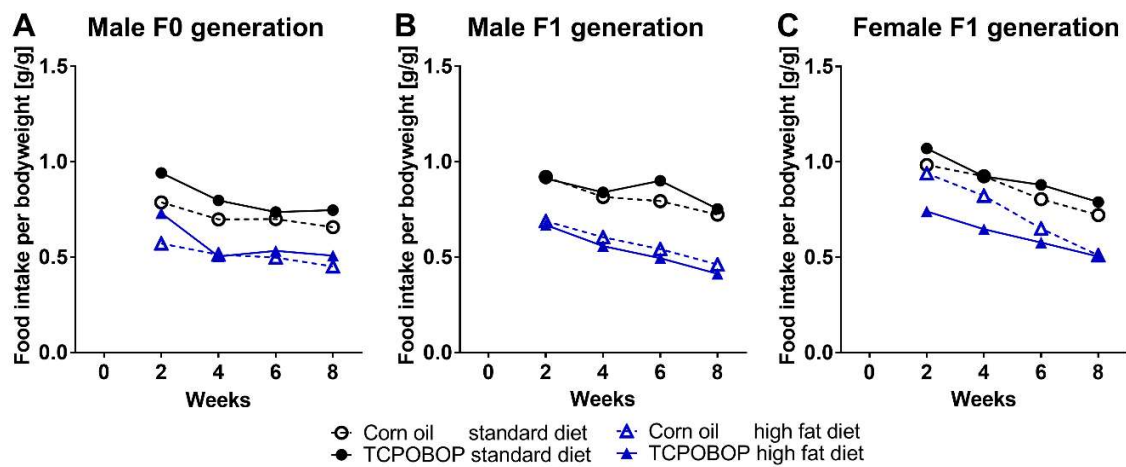
Supplementary Fig. 2 *Cyp2b10* induction in the F1 generation following paternal TCPOBOP exposure

Males were treated with TCPOBOP (3 mg/kg, i.p.) in corn oil or with corn oil alone 3 days (B), 19 days (C), and 31 days (D) prior to mating, to achieve an TCPOBOP effect on spermatids/spermatozoa, spermatocytes or spermatogonia, respectively. Hepatic *Cyp2b10* induction was determined by q-PCR and $\Delta\Delta$ct-method 6 days after birth in the F1 offspring, n=7-25. (A) depicts the hepatic expression of *Cyp2b10* in F0 males immediately after mating (n=3-4). Data are presented as mean fold-expression changes ± SEM over the mean male control group. Expression changes were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (**,P < 0.01; *,P < 0.05).
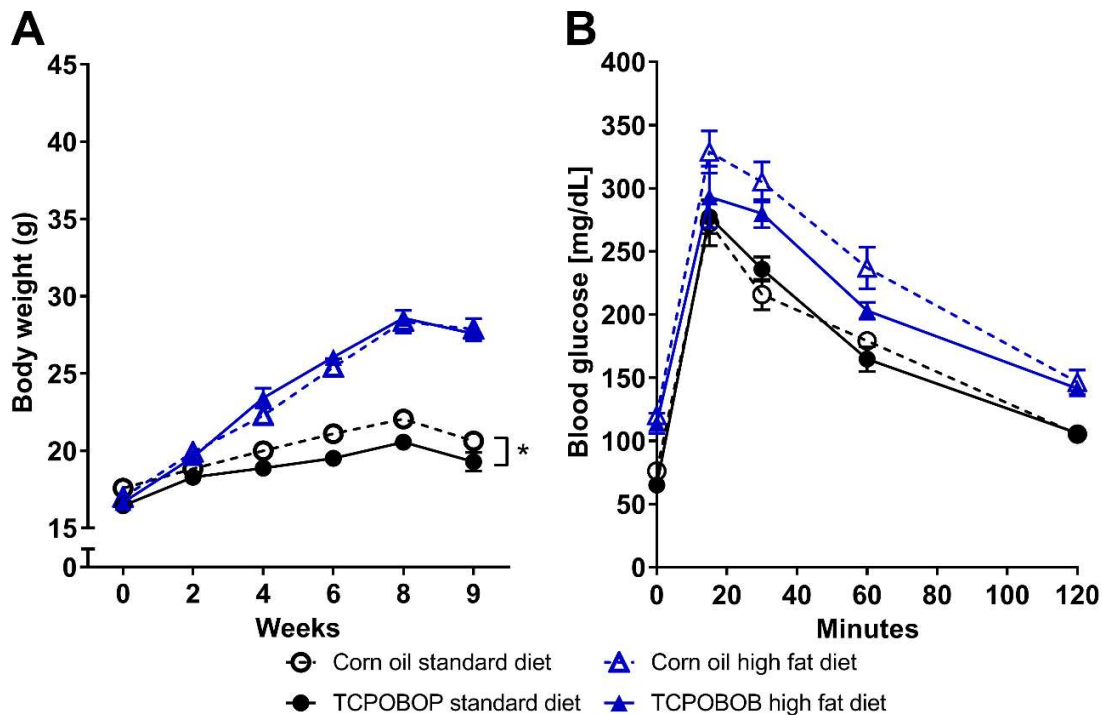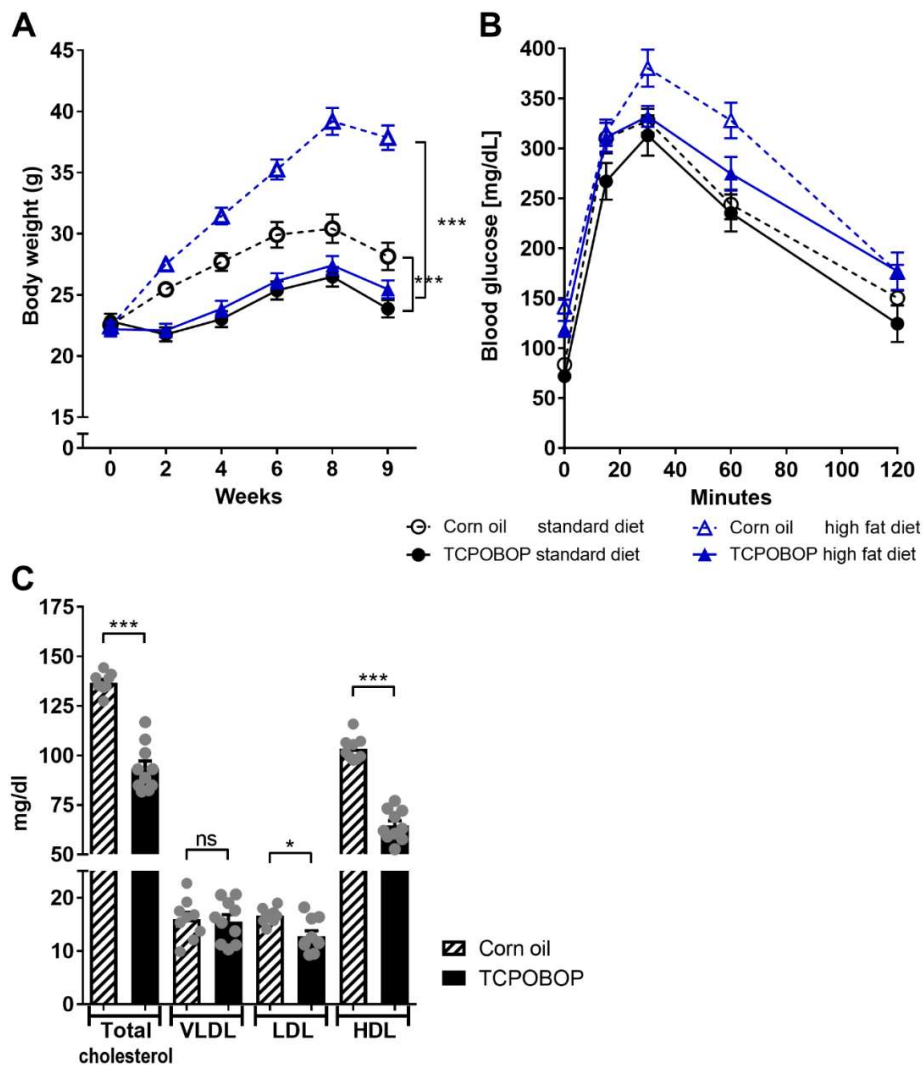
Supplementary Fig. 3 Body weight and glucose tolerance of female F1 mice following a single pre-conceptional TCPOBOP injection

A. 6-week-old female offspring of females injected with TCPOBOP (3 mg/kg) in corn oil (solid line) or with corn oil alone (dotted line) one week prior to mating with untreated males and were set on high fat diet (blue) or standard diet (black) for nine weeks. Statistically significant differences in body weight were analyzed using a mixed model and treatment related differences are indicated by asterisks (*, $P < 0.05$). Data are presented as mean ± SEM. n = 6 B. Following week nine, all mice were fasted for 16 h and challenged with glucose (2 g/kg) according to the mean body weight of female animals on standard diet (same dose for each animal). Shown are mean concentrations ± SEM in female offspring. Blood glucose was analyzed by t-test or Mann-Whitney test with Bonferroni correction for four time points (0 min, 15 min, 30 min, 60 min). The differences did not reach the preset significance level ($P < 0.05$). n = 6

A   Male F0 generation   **B**   Male F1 generation   **C**   Female F1 generation

Corn oil   standard diet
TCPOBOP standard diet
Corn oil   high fat diet
TCPOBOP high fat diet

Supplementary Fig. 4 Food intake per body weight
Food intake per body weight of mice shown in Fig. 3A was calculated from total food intake and body weight per group as a mean value every two weeks.

Supplementary Fig. 5 The effect of TCPOBOP on body weight, glucose tolerance and serum lipids in F0 generation
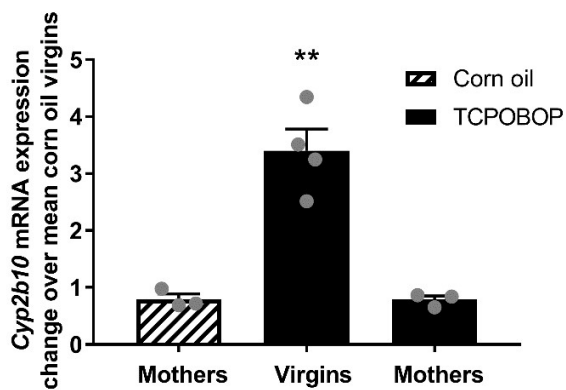
A. Six-week-old F0 male mice were injected with a single dose of TCPOBOP (3 mg/kg) in corn oil (solid line) or with corn oil alone (dotted line) and set on high fat diet (blue) or standard diet (black) on the same day for nine weeks. Body weight was analyzed using a mixed model and statistically significant differences are indicated by asterisks (***, $P < 0.001$). Data are presented as mean ± SEM. n = 7-8 **B.** Following week nine, all mice were fasted for 16 h and challenged with glucose (2 g/kg) according to the mean body weight of male animals on standard diet (same dose for each animal). Shown are mean concentrations ± SEM of males. Blood glucose was analyzed by t-test or Mann-Whitney test with Bonferroni correction for four time points (0 min, 15 min, 30 min, 60 min). Statistically significant differences are indicated by asterisks (*,$P < 0.05$). n = 6-9 **C.** Total cholesterol, VLDL, LDL and HDL were analyzed using HPLC in blood serum of 9-week-old male mice one week after injection. Mice were fasted 16 h before the analysis. Serum lipid concentrations were analyzed by t-test with Bonferroni correction for four parameters, statistically significant differences are indicated by asterisks (***,$P < 0.001$; *,$P < 0.05$). Data are presented as mean ± SEM. n = 9-10
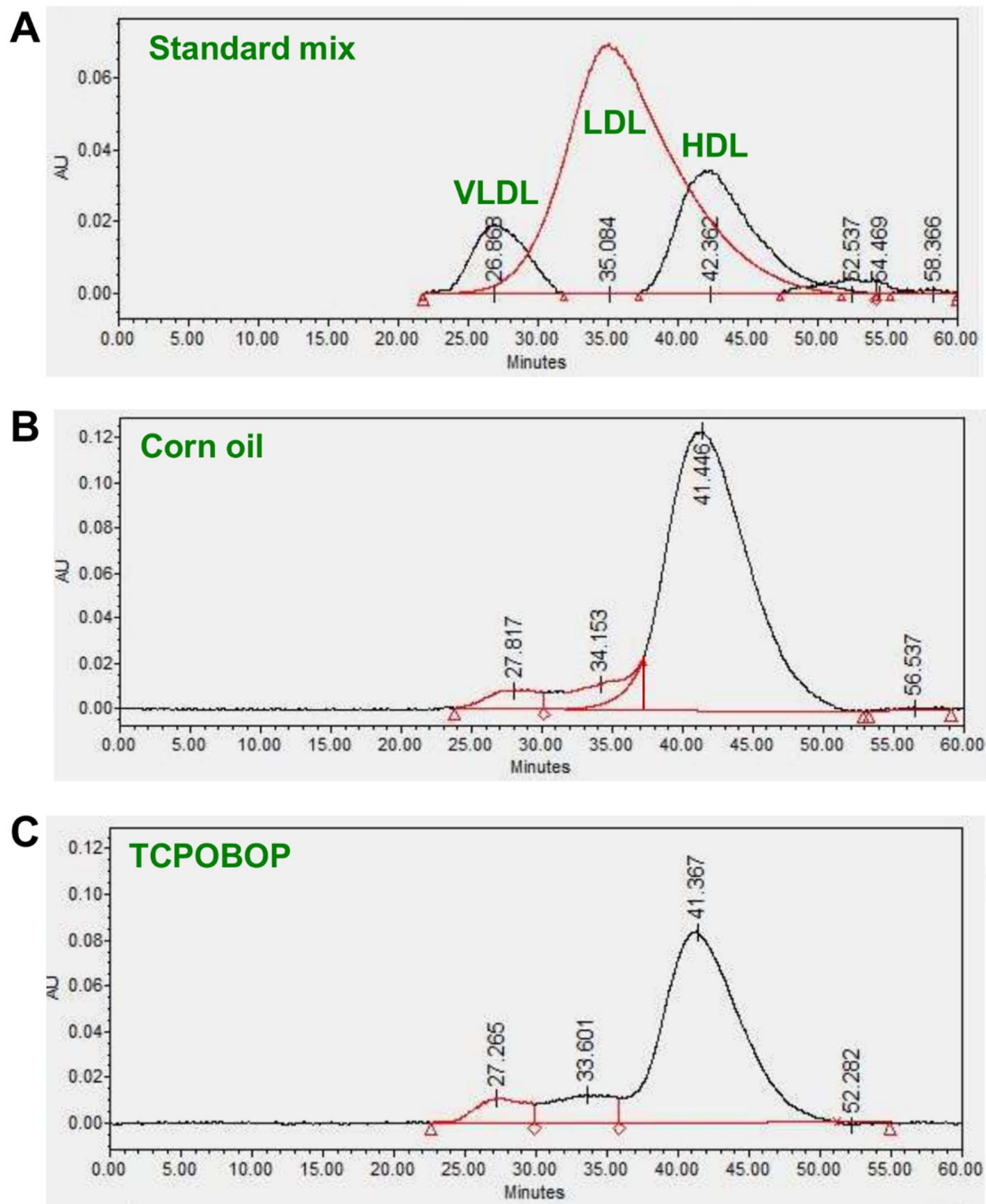
Supplementary Fig. 6 Exemplary lipid profiles of F1 mice depicted in Fig. 3
Serum lipid profile upon size-exclusion chromatography of 12-week-old F1 males fasted for 16h.
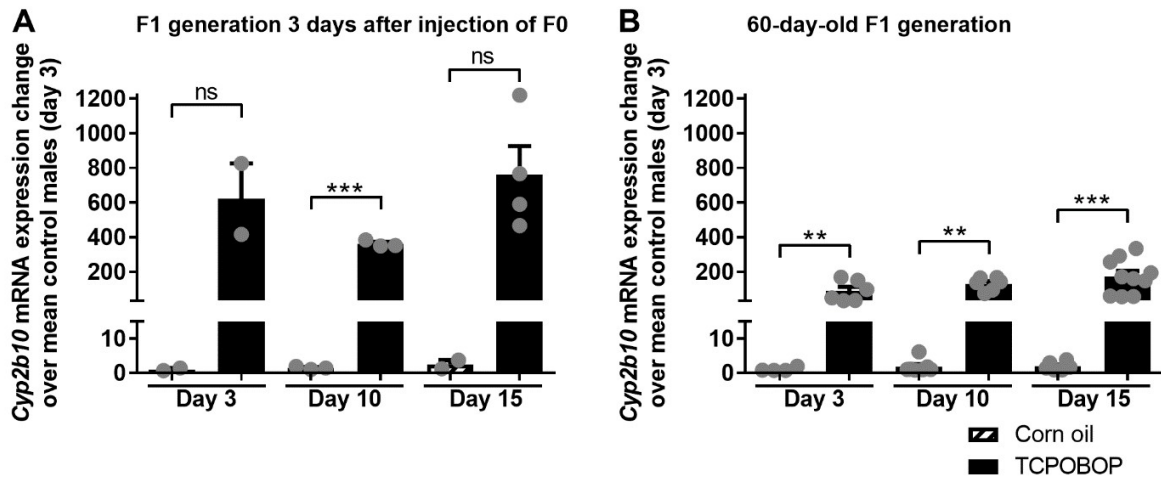
Supplementary Fig. 7 TCPOBOP transfer via lactation initiated at 3 time points after birth
Lactating females were treated with TCPOBOP on day 3, 10 or 15 after having given birth. Hepatic *Cyp2b10* mRNA expression was analyzed in male newborns 3 days after maternal TCPOBOP treatment, n=2-4 (A) or in 60-day-old male F1 mice, n = 4-10 (B). Shown are mean expression changes over mean male controls ± SEM determined by q-PCR and ΔΔct-method, which were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***,P < 0.001; **,P < 0.01; *,P < 0.05). Similar results were obtained for female offspring (Fig.5).

Supplementary Fig. 8 TCPOBOP-mediated long-term *Cyp2b10* induction in virgins and mothers.

Three-day-old F0 female mice were injected once with TCPOBOP (3 mg/kg) or vehicle (corn oil). Eight weeks later, they were mated with untreated males and hepatic *Cyp2b10* expression was assessed 4 weeks after having given birth in comparison to 3-month-old virgins. Shown are mean expression changes over the mean of corn oil treated virgins ± SEM determined by q-PCR and ΔΔct-method and analyzed by one sample t-test against 1 as a theoretical mean (n = 3-4). Statistically significant differences are indicated by asterisks (**,P < 0.01).
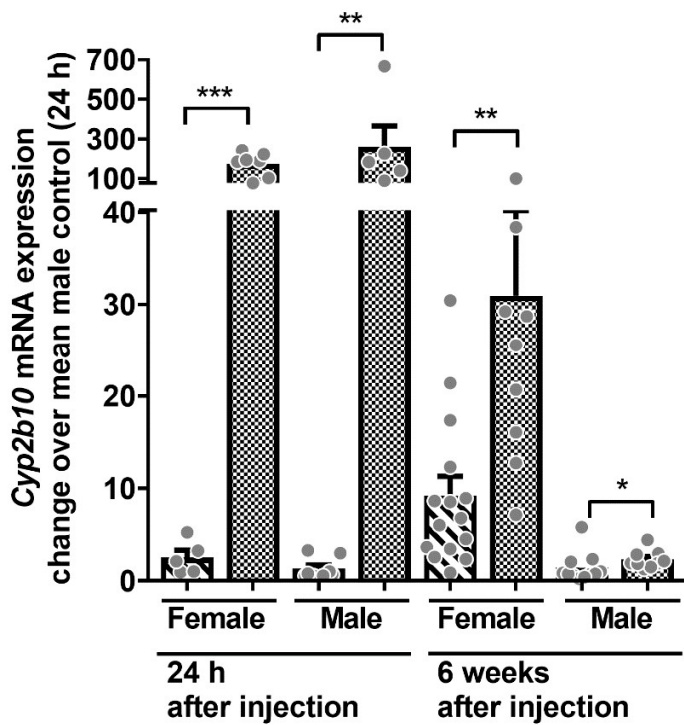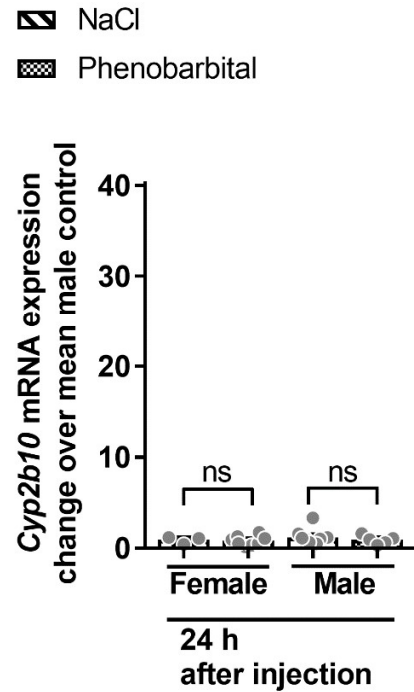
Supplementary Fig. 9 Immediate, but no F1 *Cyp2b10* induction after phenobarbital treatment.

F0 mice were injected once with phenobarbital (100 mg/kg) or 0.9% NaCl (vehicle) five days after birth. Hepatic *Cyp2b10* expression was assessed 24 h (n = 5-8) or six weeks after the injection (n = 9-15) (A). *Cyp2b10* expression in F1 offspring of females mated at the age of 8 weeks was assessed in 6-day-old mice (B). Shown are mean expression changes over the mean of male controls 24 h after the injection ± SEM determined by q-PCR and ΔΔct-method, which were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***,P < 0.001; **,P < 0.01; *,P < 0.05).

Supplementary Table 1 Pathways affected by TCPOBOP.

Pathways*
Acetone Degradation I (to Methylglyoxal)
Aryl Hydrocarbon Receptor Signaling
Basal Cell Carcinoma Signaling
Bupropion Degradation
Ceramide Signaling
Chondroitin Sulfate Biosynthesis
Dermatan Sulfate Biosynthesis
Estrogen Biosynthesis
FXR/RXR Activation
Heparan Sulfate Biosynthesis
IL-6 Signaling
LPS/IL-1 Mediated Inhibition of RXR Function
LXR/RXR Activation
Melatonin Degradation I
Neuroprotective Role of THOP1 in Alzheimer's Disease
Nicotine Degradation II
Nicotine Degradation III
NRF2-mediated Oxidative Stress Response
PPARα/RXRα Activation
PXR/RXR Activation

Superpathway of Melatonin Degradation
UVA-Induced MAPK Signaling
VDR/RXR Activation
Xenobiotic Metabolism Signaling

* Table represents pathways affected by TCPOBOP in all experimental groups, as identified by Ingenuity Pathway Analysis: Male F0 mice treated with TCPOBOP at the age of 3 days and assessed 3 days or 8 weeks later, as well as 6-day-old or 8-week-old F1 offspring of TCPOBOP-treated females.

Supplementary Table 2 Putative regulators of pathways affected by TCPOBOP.

Upstream regulators*
ABCC4
AHR
CREB3L3
FECH
GPD1
HNF4A
IL6
MED1
CAR (NR1I3)
NR5A2
PPARA
PRKAA2
RORA
RORC
SLC25A13
STAT3
ZBTB20
ZMPSTE24

* Table represents putative regulators of pathways affected by TCPOBOP in all experimental

groups, as identified by Ingenuity Pathway Analysis: Male F0 mice treated with TCPOBOP at the

age of 3 days and assessed 3 days or 8 weeks later, as well as 6-day-old or 8-week-old F1

offspring of TCPOBOP-treated females.

Supplementary Table 3 Physiological processes and diseases related to pathways affected by TCPOBOP

Categories of functions and diseases*
Cardiovascular Disease, Connective Tissue Disorders, Hematological Disease, Organismal Injury and Abnormalities
Digestive System Development and Function, Hepatic System Development and Function, Organ Morphology, Organismal Development
Drug Metabolism
Gastrointestinal Disease, Hepatic System Disease, Organismal Injury and Abnormalities
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry

* Table represents physiological processes and diseases related to pathways affected by TCPOBOP in all experimental groups, as identified by Ingenuity Pathway Analysis: Male F0 mice treated with TCPOBOP at the age of 3 days and assessed 3 days or 8 weeks later, as well as 6-day-old or 8-week-old F1 offspring of TCPOBOP-treated females.

Supplementary Table 4 Agilent 6490 triple quadruple mass spectrometer settings used to detect

TCPOBOP extracted from adipose tissue

Source parameters	
Ion Mode	positive
Gas temperature	110 °C
Gas flow	19 L/min
Nebuliser	25 psi
Sheath gas temperature	375 °C
Sheath gas flow	11 L/min
Capillary voltage	2000 V
Nozzle voltage	0 V
iFunnel parameters	
High pressure RF	150 V
Low pressure RF	60 V

Supplementary Table 5 Multiple reaction monitoring (MRM) transitions used for the detection of the extracted TCPOBOP.

Nucleoside	Precursor ion (m/z)	Fragment ion (m/z)	Collision energy	Cell accelerator voltage
TCPOBOP (4x (^{35}Cl))	400.7	237.9	30	5
TCPOBOP (3x (^{35}Cl)1x(^{37}Cl)	402.8	175.0	30	5