A truncation in the Aryl Hydrocarbon Receptor of the CRL:WI(Han) rat does not affect the developmental toxicity of TCDD

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Short title: The Wistar(Han) AhR and toxicity

ABSTRACT

The Aryl Hydrocarbon Receptor (AhR) is required for the toxicity of TCDD, and so the AhR of CRL:WI and CRL:WI(Han) rats was characterised. Western blot showed AhR proteins of ~110 and ~97 kDa in individual rats from both strains. The AhR cDNA from a CRL:WI(Han) rat with the ~110kDa protein revealed a sequence that was identical to that of the CRL:WI and SD rat. However, cloning of the AhR from a rat with the ~97kDa protein revealed a point mutation, and five variants encoding two C-terminally truncated variants of the AhR protein, arising from a point mutation in the intron/exon junction and consequent differential splicing. These C-terminally truncated variants were expressed and shown to give rise to a protein of ~97kDa; the recombinant AhR bound TCDD with an affinity that was not statistically different from the full-length protein. A single-nucleotide polymorphism (SNP) assay was developed, and showed that both alleles were represented in a Hardy-Weinberg equilibrium in samples of CRL:WI and CRL:WI(Han) populations; both alleles are abundant. Rats from two studies of TCDD developmental toxicity were genotyped, and the association with toxicity investigated using statistical analysis. There was no plausible evidence that the AhR allele had a significant effect on the toxic endpoints examined. These data show that the two AhR alleles are common in two strains of Wistar rat, and that the AhR alleles had no effect on TCDD-induced developmental toxicity in two independent studies.

Keywords

Aryl hydrocarbon receptor; genetics; SNP; TCDD; developmental toxicity

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous toxin, and a prototypical representative of a series of chemicals which effect toxicity through a common mechanism, binding to the Aryl Hydrocarbon Receptor (AhR) (Poland et al., 1976a) (Poland and Knutson, 1982). Research has focussed on the toxicity of TCDD, on the basis that other chemical congeners will show the same toxicity as TCDD, but will merely have different potency (Van den Berg et al., 2006). Mouse genetic studies demonstrated that dioxin toxicity is mediated through the AhR locus (Poland and Knutson, 1982). The subsequent cloning of the AhR (Burbach et al., 1992) (Ema et al., 1992) characterised the AhR as a transcription factor. The creation of AhR knockout mice enabled the confirmation that AhR was required for dioxin toxicity (Fernandezsalguero et al., 1995) (FernandezSalguero et al., 1996) (Schmidt et al., 1996) (Shimizu et al., 2000), and specific alterations in the AhR control dioxin-mediated toxicity (Walisser et al., 2004) (Bunger et al., 2003).

Maternal dosing with low doses of TCDD during pregnancy is reported to cause adverse effects in the reproductive system of male offspring, notably decreased sperm count in the cauda epididymis of Holtzmann (Mably et al., 1992) or Wistar (Faqi et al., 1998) rats. These effects were used to set a Tolerable Daily Intake of 2pg kg^{-1} day^{-1} for TCDD and related compounds by the UK Committee on Toxicity (Committee on Toxicity, 2001). Subsequent studies have failed to reproduce these effects in Holtzmann rats (Ikeda et al., 2005) (Ohsako et al., 2001). We undertook studies in the Wistar(Han) rat (CRL:WI(Han)), and showed that TCDD did not cause potent developmental effects on offspring sperm levels at postnatal day 120 (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c). During the course of these studies, it became clear that there was a size polymorphism in the AhR of the CRL:WI(Han) rat (MQF, DRB, unpublished data). There was therefore a concern that the size polymorphism of the AhR protein might reflect a different AhR allele, and that the different AhR alleles might control TCDD toxicity in this system.

The Wistar(Han) (Kuopio) rat is resistant to specific toxic effects of TCDD (Pohjanvirta and Tuomisto, 1994) (Pohjanvirta et al., 1987), and one allele for resistance to the acute lethality of TCDD in a Long-Evans x Wistar(Han) cross was subsequently shown to co-segregate with an AhR allele (Pohjanvirta, 1990) (Tuomisto et al., 1999), suggesting that the AhR allele was responsible for the phenotype. The polymorphism in the Wistar(Han) (Kuopio) AhR has been characterised (Moffat et al., 2007) (Pohjanvirta et al., 1998). However, it was discovered that

the original rat strains used were an *inbred* Long-Evans line, and a small colony of outbred Wistar(Han) rats (derived from Zentralinstitut für Versuchstierzucht GmbH, Hannover, FRG). The inbred Long-Evans rats had significantly different susceptibility to the acute lethality of TCDD compared with outbred Long-Evans rats, and the small Finnish colony of Wistar(Han) rats had different susceptibility to the acute lethality of TCDD, compared to a different batch of Wistar(Han) rats (Pohjanvirta and Tuomisto, 1990), and hence the Finnish rats were named as the inbred L-E (Turku/AB) and outbred H/W (Kuopio) strains. It is therefore not clear to what extent the characteristics of the Kuopio strain of Wistar(Han) rats are representative for other Wistar(Han), or Wistar, strain rats. Given that the AhR^{hw} allele confers ~100-fold resistance to the acute lethality of TCDD in adults, there was the possibility that this AhR allele might be present, and have affected the developmental toxicity of TCDD in our study in Wistar(Han) rats.

Given that AhR polymorphisms are known to be determinative of TCDD-induced toxicity in the mouse, we therefore sought to investigate the AhR of the CRL:WI(Han) rat, whether there were any genetic polymorphisms in AhR, and characterising what those polymorphisms were. Any polymorphisms might have affected the toxicity of TCDD in our studies with this rat strain (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c), and so we investigated whether polymorphic AhR variants have altered affinity for TCDD, and whether these polymorphisms in the AhR of the CRL:WI(Han) rat are associated with susceptibility to the developmental toxicity of TCDD in two separate studies.

MATERIALS AND METHODS

All chemicals were of the highest quality available. Rats of three strains: Wistar (CRL:WI), Wistar (Han) (CRL:WI(Han)) and Sprague-Dawley (CRL:CD(SD)) were all from Charles River Laboratories, UK. Samples of CRL:WI(Han) liver were obtained from previously described studies on the developmental toxicity of TCDD (Bell et al., 2007b) (Bell et al., 2007c); samples from these studies which were analysed for RNA levels (Bell et al., 2007a) were selected for genotyping.

Western blotting. Rats were killed by barbiturate overdose and the liver perfused with ice-cold MDEG buffer (25mM MOPS, pH 7.5, 1mM DTT, 1mM EDTA, 10% glycerol). The liver was flash frozen in liquid nitrogen and stored at -80ºC for later use. An aliquot of frozen liver was homogenized with 6 volumes of MDEG buffer containing 20mM sodium molybdate. Cytosol was prepared by an initial centrifugation at 10,000g for 10 minutes, and the resulting supernatant was centrifuged again at 240,000g for 30 minutes. The cytosol was collected and protein concentration was determined by a Bradford Assay using BSA as a reference. Protein (60μg/lane) was separated on a polyacrylamide gel electrophoresis (PAGE) (either SDS-PAGE, or pre-cast gels (Invitrogen) using lithium duodecyl sulphate) and transferred to a PVDF membrane (Immobilon, USA). Signal was detected using Enhanced Chemiluminescence (ECL; GE Healthcare) and exposed to a film. The antibody against AhR has been previously described (Fan et al., 2008).

Cloning of CRL:WI(Han) AhR. Livers from CRL:WI(Han) rats expressing either the lower or higher Mr AhR were selected for gene cloning. Total RNA was extracted from frozen rat liver tissue using Trizol reagent, and PolyA+ mRNA was isolated from total RNA using Oligotex Direct mRNA Kits (Qiagen) according to manufacturers' protocol. Reverse transcription was performed with Enhanced Avian RT First Strand Synthesis Kit using oligo $(dT)_{23}$ as primer. The full length coding region of the AhR was obtained in three fragments (Fragments1-3) using PCR primers based on the sequence for CRL:WI rat AhR; the primers are shown in Table 1. JumpStart AccuTaq LA DNA Polymerase Mix (Sigma) was used for PCR and the products were subcloned into pGEM-T plasmids (Promega). The inserts were verified by double stranded DNA sequencing. Three fragments of each variant previously separately cloned in pGEM-T plasmids were used to produce full length AhR by restriction digest with appropriate enzyme and ligation. In order to facilitate *in vitro* expression of the AhR variants, a Kozak consensus sequence was introduced into 5'-end of the full length AhR by PCR with primers NOTKFW and APARV

(Table 1). The full length AhR(+Kozak) variants in pGEM-T plasmids were then used for *in vitro* expression of the AhR proteins in TNT® Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine. The expressed proteins were separated on 8% SDS PAGE. The gel was dried and exposed to a film or phosphor imaged.

Plasmid construction. Methods were essentially as described (Fan et al., 2008). Full length rat AhR variants (Wild-type, Deletion and Insertion) were cloned into pGEM-T Easy. A His_6 tag was introduced at the C-terminus by PCR with forward primer rBam-5 (AhR bases 1226-1252) and backward primers rHis-3-Del or rHis-3-Ins (Table 2). Fragments were gel-purified, and cloned into plasmid pGEM-T Easy (Promega), and checked by double strand sequencing. There was a single mutation (A2336G) in rAhR deletion variant sequence, and site-directed mutagenesis was used to change this mutation. QuikChange Site-Directed mutagenesis kit was purchased from Stratagene (USA), and mutagenesis was performed according to the manual. The plasmids pGEM-T Easy containing rAhR variants fragments with His_6 tag were double-digested with BamHI and Hind III, and then the target fragments (about 1200bps) were used to replace the wild type rAhR sequence between BamHI and Hind III sites in the plasmid pFastBac1.rAhR.His, which was constructed previously. The rAhR variants and His6 sequences were confirmed by DNA sequencing. Bac-to-Bac (Invitrogen) system was used to express recombinat AhRs in insect cells. Plasmids pFastBac1.rAhR-deletion.His and pFastBac1.rAhR-insertion.His were transformed into the bacterial strain DH10Bac (Invitrogen), and bacmids prepared by Qiagen miniprep column. The presence of AhR inserts was confirmed by PCR.

The bacmids were then transfected into Sf9 cells, baculovirus was harvested after 5-7 days incubation and then further amplified in infect Sf9 cells to obtain high titre virus. Virus was directly titred in a plate assay, and high titre virus stocks were used to infect Sf9 cells and express recombinant AhRs. Virus was added to Sf9 cell culture, and infected Sf9 cells were harvested by centrifugation at 500g for 10 minutes at 48 hours after infection, or as indicated, and all subsequent steps were on ice or at 4°C. Cell pellets were re-suspended in MDEG buffer (25mM MOPS, 1mM DTT, 1mM EDTA, 10% glycerol, pH7.5) containing 20mM molybdate. Cells were broken by sonication, and cell debris was removed by centrifugation at 12,000g for 10 minutes. The supernatant was further centrifuged at 200,000g for 30 minutes. The final cytosolic supernatants were divided into small aliquots and stored at -80°C.

Ligand binding assay. The method for [^{3}H]-TCDD binding to AhR was previously established by (Poland et al., 1976a), as amended by (Fan et al., 2008) for expression of AhR using baculovirus. Typically, rat liver cytosol was diluted to 5mg/ml in MDEG buffer (25mM MOPS, 1mM DTT, 1mM EDTA, 10% glycerol, pH7.5) containing 20mM molybdate. Then the sample was incubated with [^{3}H]-TCDD or [^{3}H]-TCDD plus a 200-fold excess of competitor TCAOB (Poland et al., 1976b) at 4°C overnight. After incubation, 30µl of dextran-coated charcoal suspension (67mg/ml, prepared in MDEG buffer) was added into a 200µl sample of the mixture. The suspension was incubated on ice for 10min, and then was centrifuged at 25,000g for 10min. 150µl of the supernatant was removed and radioactivity was measured in a scintillation counter. Specific binding was defined as the difference of radioactivity between without (total binding) and with competitor TCAOB (non-specific binding). For recombinant rat AhR protein, either 0.25mg or 0.5mg/ml cytosol protein was used for binding assay, and supplemented with BSA to a final protein concentration of 5mg/ml. Specific saturable binding (K_d) was characterised by using non-linear regression, using Graphpad 4/5, fitting a saturation binding isotherm.

SNP detection in genomic DNA. Genomic DNA was extracted from 20-25mg of frozen tissue with 0.6ml of lysis buffer (10mM Tris-HCl, pH 7.5; 400mM NaCl; 100mM EDTA; 0.6% SDS) containing 0.35mg of proteinase K. The sample was incubated at 60°C for 16 hours, to which 0.2ml of 5M NaCl was added at the end of the incubation, and centrifuged for 10 minutes at 10000g. The DNA containing supernatant was collected and precipitated by adding equal volume of ice cold 90% ethanol followed by centrifugation. The resulting DNA pellet was washed with cold 75% ethanol and air dried. The DNA pellet was then resuspended in 100µl water. DNA concentration was determined by measurement of a fluorescent dsDNA binding probe, PicoGreen (Molecular Probes) according to the manufacturer's instructions, using a λ DNA for the standard curve. The fluorescence in each well was measured using a plate reader (Victor, Fenland) setting at excitation 485 nm and emission 510 nm. Duplex real-time PCR was performed using Brilliant Multiplex QPCR Master Mix (Stratagene) according to the manufacturer's protocol with slight modification. PCRs were set up in a total volume of 20µl of buffer solution containing the following: 10 µl of 2x master mix; 300nM of each of both molecular beacons; 300nM of each primer (SNP66 and SNP150) and 40ng of genomic DNA. A Stratagene MX3000p cycler was used for PCR: 10 min at 95°C followed by 40 cycles of 95°C for 20 s, 52°C for 1 min, and 72°C for 30 s. The fluorescence of the molecular beacons was measured during the annealing step of the PCR cycle. The cycle number at which the signal was above the threshold fluorescence (C_t) was determined using Stratagene software. Negative controls (no

template) and positive controls of known genotype were analysed contemporaneously with each analysis.

Statistical methods. K_d values for the affinity of AhR for TCDD were compared using a t-test. Observed genotype frequencies were compared with expected allele frequencies, including those expected under Hardy-Weinberg equilibrium, using a Pearson χ^2 statistic. Previous analyses of body and organ weights and measures of reproductive toxicity in acute and chronic dosing experiments (Bell et al., 2007b) (Bell et al., 2007c) examined dose-response relationships by ANOVA methods, including mixed models with random effects terms for litter effects. These responses were reanalysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Interest was specifically in whether the different types had different susceptibility to the effects of TCDD, and this was examined by fitting interaction terms between TCDD dose and genotype. The data set from the animal studies, together with the genotyping and RNA data, is provided in Supplementary data as an Access database.

RESULTS

Whilst preparing cytosol for analysis of ligand-binding to [^{3}H]-TCDD from CRL:WI(Han) rats, it was noted that there were multiple size variants of the AhR immunodetected in Western blots, with protein sizes of ~110 and ~97 kDa (Fig. 1A). These size variants were reproducible in cytosol preparations from an individual rat; moreover, the banding pattern was unchanged by the inclusion of protease inhibitors during cytosol preparation, or to optimisation of the denaturing conditions for SDS-PAGE (Data not shown). Cytosol from Sprague-Dawley rat liver (Fig. 1A) (and C57Bl/6 and DBA/2J mouse liver, (data not shown)) consistently produced an AhR protein of the expected size, suggesting that the different protein bands were not a product of proteolysis, but were intrinsically different AhR forms. The phenomenon is not restricted to the CRL:WI(Han) rat, since the CRL:WI rat also shows two AhR size variants in hepatic cytosol (Fig. 1B).

Cloning of variant AhR cDNAs from CRL:WI(Han) rat

To determine if the differently-sized AhR protein variants were the result of different mRNA sequences, AhR cDNAs were cloned from CRL:WI(Han) rats that were known to have either a 110 kDa or 97 kDa protein product. Double-stranded sequencing of AhR clones from rats with the 110 kDa AhR protein revealed that the sequence was identical to that of the CRL:WI (AJ821851) and Sprague-Dawley rat (AAA56897), yielding a protein of 853 amino acids (Accession number AM902286). This AhR sequence is hereinafter referred to as the wild-type sequence. However, there were five variants in the AhR clones from the rat with the 97 kDa AhR protein, as shown in Fig. 2; at least two independent cDNAs of each variant were sequenced. These have a common Val497Ala point mutation, and the multiple cDNA variants are consistent with a point mutation at the end of exon 10 of the AhR, causing the use of alternative splicing sites (Pohjanvirta et al., 1998); accordingly, the nomenclature of Pohjanvirta *et al*. is used herein, and the AhR sequence encoding the 97 kDa protein is designated the mutant allele. The "Deletion" variant loses 129 bases from the end of exon 10, yielding a protein of 810 amino acids (AM902281). The "Insertion" variants use alternative splice sites with an extra 29, 126, 129 or 134 bases from the intron between exons 10 and 11, resulting in a protein of 815 amino acids (AM902282-AM902285). This evidence shows that the differently sized AhR proteins in CRL:WI(Han) rats arise from different cDNAs.

Recombinant expression and properties of variant AhR cDNAs

The recombinant cDNAs were cloned and expressed in reticulocyte lysates to determine their size, and whether the Insertion and Deletion variants were viably translated, and could be distinguished on SDS-PAGE. Fig. 3A shows that both the Insertion and Deletion cDNAs produced a protein of the expected size, but that these could not be distinguished from one another on SDS-PAGE. By contrast, the wild-type protein migrated more slowly than the variant alleles. To confirm this, the proteins were then expressed using the baculovirus expression system, and cytosolic extracts prepared and analysed by western blotting with an AhR specific antibody. Fig. 3B shows that uninfected Sf9 cells show no immunoreactive bands, confirming the specificity of the antibody. The Wild-type AhR shows a band of ~110 kDa, and the Insertion and Deletion variants show bands at ~100 kDa, which are of a similar size to each other. These findings show that both the Insertion and Deletion AhR variants are capable of giving rise to a protein product that can migrate faster than the wild-type AhR, and provides strong evidence that the faster-migrating AhR protein variant in Fig. 1 arise from both the Insertion and Deletion forms of the variant AhR in CRL:WI(Han) and CRL:WI rats.

To test if there was any difference in ligand-binding functionality of the AhR alleles, a previously validated baculovirus expression system for AhR was used (Fan et al., 2008). The AhR proteins expressed in baculovirus were assayed for the ability to bind TCDD as a ligand (Fig. 4). The ligand-binding assay showed saturable and specific binding, and gave values for rat liver cytosol which are consistent with the literature (data not shown). Although the maximal amount of specific binding (B_{max}) varied between batches by two-three fold, the affinity of the expressed AhR proteins for [^{3}H]-TCDD was not significantly different. For multiple independent saturation binding assays, the dissociation constants (K_d) were: wild-type, 1.34 ± 0.32 nM (n=6 independent determinations, mean ± Standard Deviation); Insertion, 1.07±0.47 nM (n=3); Deletion 1.22±0.72 nM (n=3). Thus there were no significant differences in the ligand-binding affinity for TCDD between the variant AhRs of CRL:WI(Han) rat.

An SNP assay for AhR alleles

In order to be able to determine if the AhR alleles were able to affect susceptibility to toxic endpoints in our studies (Bell, et al., 2007a; Bell, et al., 2007b; Bell, et al., 2007c), it was necessary to develop an SNP assay to discriminate between alleles. Molecular beacon technology (Marras et al., 1999) was used to probe the allelic variant at the exon 10 junction, in a 108bp amplicon.

The Molecular Beacons were designed to discriminate between the the wild-type (G) or mutant (A) allele at the 1st nucleotide of intron 10 in the rat AhR (Table 1); note that this assay does not differentiate between the Insertion and Deletion transcripts, which are produced as a result of post-transcriptional processing. Initial hybridisation studies with the probes showed discrimination between wild-type and mutant allele oligonucleotides, with strong signal to the matching probe, minimum signal from the mismatched oligonucleotide, and hence maximum discrimination at 52°C (data not shown). The optimised PCR reaction (Fig. 5A-C) shows that both probes can be used in the same PCR reaction to yield unambiguous discrimination between wild-type and mutant alleles in genomic DNA. There was clear discrimination between wild-type, mutant and heterozygote DNA samples when using large numbers of samples (Fig. 5D), and hence this assay was validated to analyse the tissue samples available from our previous *in vivo* studies.

Analysis of the populations from the acute and chronic studies was divided up into the maternal and offspring, since the offspring represent a dependent population (Table 2). Neither of the acute study populations, nor the chronic parents, show a significant variation from a 1:2:1 ratio of genotypes (χ^2=4, 0.44 and 3.7, respectively). By contrast, the offspring in the chronic study had a ratio that was significantly different (χ^2=19, P<0.001) from the expected 1:2:1 ratio, with over-representation of the homozygous wild-type allele; this presumably reflects the genotype of the relatively small number of parents. A sample of thirty non-sibling CRL:WI(Han) rats in 2007 failed to show any significant deviation from the expected 1:2:1 ratio (χ^2=1.1). Thus it appears that the wild-type and mutant AhR alleles are equally represented in CRL:WI(Han) rats. A sampling of 51 CRL:WI rats, of unknown inter-relationships, found that there was a significant deviation from the expected 1:2:1 genotype ratio (χ^2=25)(Table 2). However, the genotype frequencies in this population did not deviate significantly from a Hardy-Weinberg equilibrium (χ^2=2.9), and thus there is no evidence for systematic skewed representation of the homozygous AhR alleles in the CRL:WI rat.

Association between AhR genotype and toxicity

In the analysis of phenotypic endpoints in the acute (Bell et al., 2007b) and chronic (Bell et al., 2007c) studies on TCDD developmental toxicity, with a view to determining if the toxicity in these studies was affected by genotype at the AhR locus, there was a prior expectation that any biologically relevant interaction between treatment and genotype would show a similar size and direction of effect between the acute and chronic studies, and would show an appropriate dose-

dependency of effect. The full dataset is provided as an access database, and the summary statistical analyses are provided as a Table for each endpoint (Supplementary Material). The principal toxic effects seen in those studies were reduction in body weight gain in the F1 animals, delay in balano-preputial separation, and total litter loss in the offspring. It was not possible to assay the animals which had died as a result of total litter loss, since no samples of the dead animals were taken, but the other measures of toxicity were examined. Maternal cytochrome P450 1A1 (CYP1A1) RNA levels (Bell et al., 2007a) showed no significant correlation with genotype in either the acute or chronic study. In the offspring, CYP1A1 RNA levels at PND70 showed no correlation with genotype in the chronic study, but there was a significant association (P~0.019) between genotypes in the acute study, with heterozygous animals having 1.7-fold, and homozygous mutant animals having 2.5-fold the level of CYP1A1 RNA as homozygous wild-type animals. Given the relatively low levels of CYP1A1 RNA in rats at PND70, the fact that the mutant appears more responsive than wild-type, the small size of the effect, and the lack of effect in the chronic study, or the mothers, it is likely that this association is due to chance. Balano-preputial separation was found to be significantly delayed by TCDD treatment in both studies (Bell, et al., 2007b; Bell, et al., 2007c), but there was no significant association between genotype and day of balano-preputial separation. Liver: body weight ratio is also a classical marker of TCDD toxicity and AhR function (Bunger, et al., 2003; Schmidt, et al., 1996), but rat genotype showed no significant association with liver: body weight ratio. There was a statistically significant association between genotype and spleen: body weight ratio in the chronic study at PND 70, but not PND120, but there was no interaction with dose, nor an effect on spleen: body weight ratio in the acute study at any age (Supplementary Material).

F_1 offspring were identified individually from PND21 onwards, and body weights after this time were affected by TCDD treatment of the dams. There was a statistically significant association between genotype and body weight in the chronic (P~0.015), but not the acute, study. However, a plot of the difference in weight between homozygous wild-type and mutant alleles by dose group, revealed no biologically plausible, or consistent, response (see Supplementary Tables, and Supplementary Figures 1 and 2). With the body weight data set, there was a significant association between post-natal day and genotype for the chronic study (but not the acute), and for group and genotype for the acute study (but not the chronic study), and a significant association between post-natal day, group and genotype for both studies. However, a plot of the data did not show any consistent or plausible effect of genotype on body weight. Thus there was

no phenotypic measurement that was associated with the genotype of the animals in a manner which could plausibly reveal a biological effect of the genotype.

DISCUSSION

We discovered that the Wistar strain rats used in our previous studies (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c) had two size variants of the AhR protein present in liver cytosol, and have shown that the size variants are the result of a genetic polymorphism of the AhR present in CRL:WI and CRL:WI(Han) rats (Fig. 1, Fig. 2). The discovery of this genetic polymorphism in the AhR raised the possibility that it could be determinative of the function of the AhR, as is known for the $AhR^{b/d}$ polymorphisms in the mouse (Poland et al., 1994). The murine AhR polymorphisms are known to affect the affinity of the AhR protein for the ligand, TCDD (Harper et al., 1988) (Poland et al., 1994), and since the binding of ligand to the receptor is a rate-limiting step for the function of the receptor, a change in the affinity of the AhR for ligand would inevitably impact upon the function of the receptor. In order to test this possibility, we undertook recombinant expression of the variant AhR proteins produced. Both deduced protein sequences gave rise to a protein product (Fig. 3), and they were both shown to encode a functional ligand-binding AhR, with affinity for TCDD that was statistically indistinguishable from the affinity of the wild-type AhR (Fig. 4). This extends the finding that the affinity for TCDD (K_d) of cytosol from Long-Evans (Turku AB) and Wistar(Han) (H/W(Kuopio)) rats was similar (Pohjanvirta et al., 1999). The ligand-binding domain of the AhR resides in a minimal domain of amino acids 228-416 (Bell and Poland, 2000) (Coumailleau et al., 1995) (Poland et al., 1994); since the polymorphisms in the rat AhR are at amino acid 497, and at the C-terminus (the transactivation domain), our data confirms that the sites of allelic variation are outside the domain responsible for ligand-binding.

Western blotting shows that both AhR alleles are present in the CRL:WI and WI(Han) stock, but the allele frequency in Wistar strain rats was unknown. Molecular beacon technology (Marras et al., 1999) was developed for a robust and rapid SNP assay (Fig. 5). This methodology enabled the determination that both alleles were present in both CRL:WI, and CRL:WI(Han) rats, at ratios that were not divergent from the Hardy Weinberg equilibrium; thus there is no evidence for systematic under-representation of the homozygous mutant allele, for example. Given the severe physiological consequences of a null AhR allele in mouse (Bunger et al., 2003) (Fernandezsalguero et al., 1995) (Schmidt et al., 1996) (Walisser et al., 2004), it is a reasonable hypothesis that rats with a homozygous mutant of the AhR would show pathology if the AhR allele characterised herein were hypomorphic or functionally null. The absence of under-representa-

tion of the AhR null allele homozgyotes argues against the mutant alleles being functionally null or hypomorphic.

Faqi *et al*. found developmental toxicity of TCDD in Wistar rats (Bor: spf, TNO) (Faqi et al., 1998), and we undertook our studies (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c) in the CRL:WI(Han) rat since (a) it was not possible to use the Wistar strain used by Faqi (b) there was extensive background information on the CRL:WI(Han) strain in house (c) the Wistar(Han) strain is derived from the Wistar strain. Although Table 2 shows the chronic study F_1 rats had a significant difference from the equilibrium 1:2:1 AhR genotype, this was not true for the chronic P_0 dams, or in the acute study. Thus both alleles are prevalent in CRL:WI and CRL:WI(Han) populations, and these findings are likely to be informative for many Wistar colonies. However, it is important to note that inappropriate breeding strategies or chance may result in the exclusion of one of the AhR alleles from an individual Wistar colony. Studies in Wistar strain rats on the toxicity of TCDD, or similarly acting compounds, may be affected by the presence of allelic variants of the AhR (Banudevi et al., 2006) (Taketoh et al., 2007).

Toxicity endpoints from data in (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c) were correlated with rat genotype (Table 2). In view of the known pitfalls of undertaking multiple statistical tests (Ioannidis, 2005) (Ioannidis et al., 2001), the analysis was restricted to a discrete series of endpoints that were set out in advance. The AhR alleles did not have a significant association with liver: body weight ratio, and this suggests that the variant rat allele is sufficiently functional to support the normal vascularisation of the liver (Schmidt et al., 1996) (Walisser et al., 2004). There was no plausible association of AhR allele on induction of cytochrome P450 1A1 RNA, or between AhR allele and body weight or balano-preputial separation, the two most sensitive markers of TCDD-induced toxicity in the developmental studies (Bell et al., 2007c). The absence of any plausible and statistically signficant association between AhR genotype and toxicity endpoints makes a compelling case that there was no causal effect of the AhR allele on the toxicity of TCDD resulting from developmental exposure of male pups (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c). Therefore the conclusions derived from the studies of TCDD developmental toxicity do not require reinterpretation to accomodate the effect of AhR genotype, as there was no evidence to support an effect of AhR genotype on TCDD developmental toxicity (Bell, et al., 2007a; Bell, et al., 2007b; Bell, et al., 2007c).

We have described developmental toxicity of TCDD (lethality, developmental delay and depressed body weight) after a single maternal dose of 1 μg kg^{-1} (Bell et al., 2007b) or 46 ng kg^{-1} day^{-1} (Bell et al., 2007c) in CRL:WI(Han) rats; which is exquisitely sensitive in comparison to the acute lethality at >10,000 μg TCDD kg^{-1} in adult rats with the AhR^{hw} allele (Tuomisto et al., 1999). It is startling that the AhR^{hw} allele can confer such marked resistance to lethality of TCDD in adults, yet have no effect on the much more sensitive endpoint of developmental toxicity (this study). A particular strength of the approach in this study is that all of the animals shared the common genetic background of the CRL:WI(Han) rat, and this contrasts with the A, B and C lines of (Tuomisto et al., 1999), which are descended from a cross between an inbred Long-Evans and Han/Wistar(Kuopio) rats. Although the Ahr^{hw} and "B" genes have been shown to segregate with susceptibility to the acute lethality of TCDD in the Long-Evans x Han/Wistar(Kuopio) cross of (Tuomisto et al., 1999), it is clear that these genes do not determine susceptibility to all other toxicities arising from TCDD exposure (Pohjanvirta and Tuomisto, 1994). Consequently, it is entirely possible that other genetic loci could be determinative for particular TCDD-induced toxicities, and that they could differ between the parental L-E (Turku/AB) and H/W (Kuopio) strains, and in the derived lines. There is evidence for differential developmental toxicity of TCDD between the two parental lines (Huuskonen et al., 1994), and thus any differential response of the A-C lines to developmental toxicity does not necessarily demonstrate a role for the Ahr^{hw} or "B" genes (Simanainen et al., 2004). This study therefore provides clear evidence from a study in the common genetic background of the CRL:WI(Han) rat that the wild-type and mutant AhR alleles did not measurably affect multiple parameters of TCDD-induced developmental toxicity in two separate studies (Bell et al., 2007a) (Bell et al., 2007b) (Bell et al., 2007c). The disparity in the data on genetic linkage between susceptibility to TCDD-induced developmental toxicity or acute lethality makes a compelling case for using definitive transgenic approaches to determining whether the AhR^{hw} confers resistance to TCDD-induced lethality.

In summary, there is an allelic form of the AhR which is common in CRL:WI(Han) and CRL:WI rats, and the allele results in alternative splicing and C-terminal truncation, together with an amino acid mutation, in the AhR. The variant allele yields two C-terminally truncated proteins, but does not affect the affinity of AhR for the ligand, TCDD. The allele does not show a plausible association with endpoints of TCDD-induced developmental toxicity in two independent studies, and our data therefore shows that this AhR allele did not affect TCDD-induced developmental toxicity in our previous studies (Bell et al., 2007a) (Bell et al., 2007b) (Bell et

al., 2007c). Genetic linkage data show a marked difference in the association of AhR^{hw} on susceptibility with TCDD-induced acute lethality, or developmental toxicity, and this study calls into question whether the reported association with AhR^{hw} is an effect of a linked gene, or the AhR^{hw} allele.

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FIGURE LEGENDS

FIG. 1. AhR size variants in Wistar strain rats. A, 60 μg of hepatic cytosolic protein, prepared as described in materials and methods, from three CRL:WI(Han) rats (WI(Han)) and a Sprague-Dawley rat (SD) were electrophoresed on SDS-PAGE, and immunodetected with an antibody specific for the AhR LBD. Visualisation was with ECL. The size of molecular weight markers is shown in kDa (Mr, Kda). B, as for A but with CRL:WI rats (Wistar).

FIG. 2. Cartoon of cloned AhR variants from CRL:WI(Han) rat. AhR cDNAs were cloned from a CRL:WI(Han) rat with wild-type AhR protein band (Wild-type), or from an animal homozygous for the smaller protein band (Deletion, Insertion). The size of the predicted protein is shown in amino acids (aa). Exons 10 and 11 are represented by open rectangles, and the splice site between by a "V". The wild-type gene has an exon junction at 2454bp. The mutant alleles are designated as "Deletion" and "Insertion", and have a Val497Ala mutation at amino acid 497. The Deletion variant uses an alternative splice site 129bp upstream from the wild-type splice site, whereas the Insertion variants use alternative splice sites at +29, 126, 129 or 134 bp from the wild-type splice site. The intron/exon junction of the mutant WI(Han) AhRs is mutated. The site of the omitted exon sequence is shown as a light gray box for the Deletion mutation, and the extra sequence in exon 10 is shown as a black box for the Insertion mutations.

FIG. 3. Expression of recombinant AhR variants from CRL:WI(Han) rat. A, Wild-type, Insertion (Ins) and Deletion (Del) variants of the CRL:WI(Han) AhR were cloned into a vector, and transcribed in a reticulocyte lysate system in the presence of [^{35}S]-methionine. The resulting products were electrophoresed, and the gel subjected to phosphor imaging. Molecular weight markers are shown in kDa (Mr, kDa). B, Wild-type, Insertion (Ins) and Deletion (Del) variants of the CRL:WI(Han) AhR were cloned into pFASTBAC1, and then used to make baculovirus as described in materials and methods. The viruses were used to infect cells, and compared with uninfected cells (Sf9), by SDS-PAGE of 10μg of cytosolic protein, followed by western blotting with an AhR-specific antibody. Visualisation was with ECL. The size of molecular weight markers is shown in kDa (Mr, Kda).

FIG. 4. Saturation ligand-binding analysis of AhR variants. Wild-type, Insertion (Ins) and Deletion (Del) variants of the CRL:WI(Han) AhR were cloned into baculovirus as described in Fig. 3, and used to prepare cytosol under optimised conditions (see materials and methods). Saturation ligand-binding to TCDD was performed, and the specific binding to TCDD (in nmol/

mg cytosolic protein) is plotted against the concentration of TCDD in nanoMolar. Experimental determinations were performed in triplicate, and results are shown as mean +/- Standard Deviation; where error bars are not visible, the error is less than the size of the symbol. The Wild-type is shown as a closed circle, the Insertion as an open triangle, and the Deletion mutant as an open square. The values for B_{max} and K_d for the experimental data shown were determined by non-linear curve fitting, and are shown above the graph, as the mean estimate, with 95% confidence interval in square brackets.

FIG. 5. An SNP assay for genotyping AhR variants in CRL:WI(Han) rats. Wild-type (closed square) or mutant (open circle) probes, or no template control for wild-type (NTC1, vertical line) or mutant (NTC2, cross), were used for PCR analysis against 40 ng of wild-type (A), homozygous mutant (B), or heterozygous mutant (C) CRL:WI(Han) genomic DNA. Both probes were present in the same reaction. Cycle number is shown on the X-axis, and the increase in fluorescence in that cycle is shown on the Y-axis. D, a panel of ~90 DNA samples was analysed by real time PCR for the wild-type and mutant allele. The change in fluorescence for the terminal reaction cycle is shown for the wild-type allele on the X-axis, and for the mutant allele on the Y-axis. Wild-type samples (+/+) are shown as a closed circle, mutant samples (mut/mut) as an open circle, and heterozygous samples (+/mutant) as a half-filled circle. No template control is shown by a cross.

TABLES

TABLE 1: Oligonucleotide sequences

Oligonucleotide name	Oligonucleotide sequence
Fragment 1 forward	ATGGCCAGCCGCAAGCGGCGC
Fragment 1 reverse	GCAAGGGATCCATTATGGGAGAGAAAGG
Fragment 2 forward	CTGGAGAGGCTGTACTGTACGAGAT
Fragment 2 reverse	TGCTGCTGCTGAAGCTGC
Fragment 3 forward	TGAATTCAGCTTGCCAGCA
Fragment 3 reverse	TACAGGAATCCGCTGGGTGT
rBam-5	CTTTCTCTCCCATAATGGATCCCTTGC
rHis-3-Del	TAAGCTTCTAGTGATGGTGATGGTGATGCAGGAATCCGCTGGGT-GTGATATC
rHis-3-Ins	TAAGCTTCTAGTGATGGTGATGGTGATGTTCCCTGTA-GAAAGCCCTTATCTTGC
NOTKFW	CGGCCGCCACCATGGCCAGCCGCAGCAAGCGGCGC
APARV	GGGCCCTTACAGGAATCCGCTGGGTGTGATA
SNP66 (f)	CAGTACAGCCCTGAGATTCCAG
SNP150 (r)	ACACAATAGACTACACGGAGATGC
WtMB	FAM-CACCCGCTAAGCAAGGTAAGGGCTCGGGTG-BHQ1
MutMB	HEX-CACCCGCTAAGCAAGATAAGGGCTCGGGTG-BHQ2
Wt-Temp	AGCCCTTACCTTGCTTAG
Mut-Temp	AGCCCTTATCTTGCTTAG

Sequence of oligonucleotides used in this study. The designation of the oligonucleotide is shown together with the corresponding sequence. HEX (6-Hexachloro-Fluorescein-CE Phosphoramidite) and FAM (6-carboxyfluorescein) represent the 5’ fluorescent groups, and the BHQ1/2 groups at the 3’ end are “Black Hole Quencher” moieties.

TABLE 2: Genotyping of rat samples

	Wild-type	Heterozygous	Mutant	Ratio
P_0 acute (124)	27	73	24	1:2.7:0.86
F_1 acute (200)	48	98	54	1:2.04:1.13
P_0 chronic (78)	26	38	14	1:1.48:0.55
F_1 chronic (200)	76	87	37	1:1.16:0.5
CRL:WI(Han) (30)	8	17	5	1:2.11:0.63
CRL:WI (51)	26	24	1	1:0.92:0.039

Tissue samples were analysed by PCR for the presence of wild-type, homozygous mutant or heterozygous mutant alleles, as described in Fig. 5. Animal samples were parental GD16 or 21 kills (P_0) or offspring (F_1) from the acute dose (Bell et al., 2007b) (Acute) or chronic dose (Bell et al., 2007c) (Chronic) studies. The total number of animals is indicated in brackets. Thirty non-sibling CRL:WI(Han) were obtained from Charles River for a sampling of the stock, and CRL:WI animals were of unknown sibling status were obtained over a period of months, prior to analysis. The number of wild-type, heterozygous or homozygous mutant animals are shown, and this is shown as a ratio in the right-most column, with the wild-type ratio set as one.

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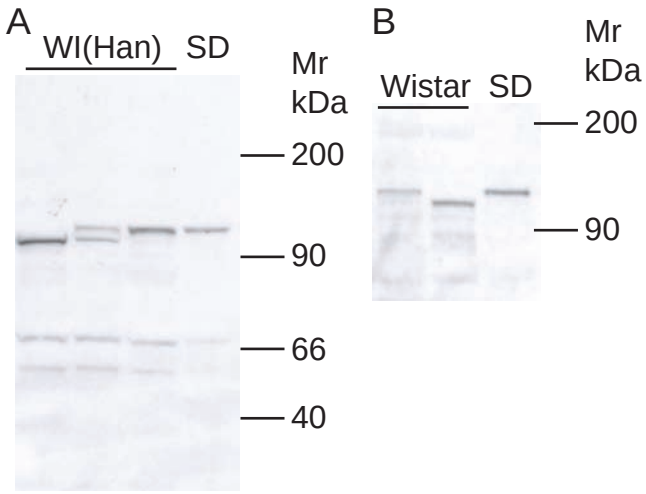
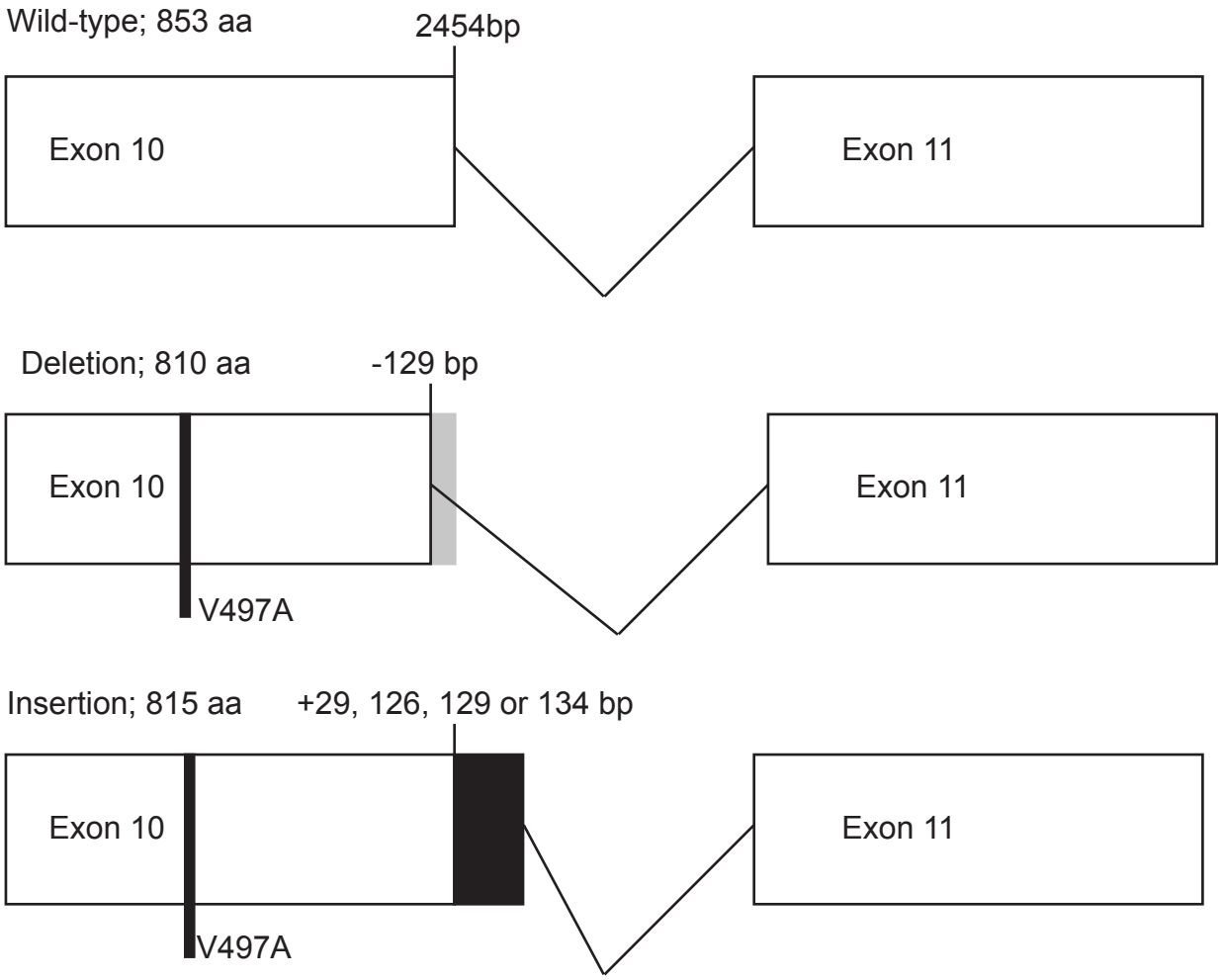


Figure 1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Exon 10
Exon 11
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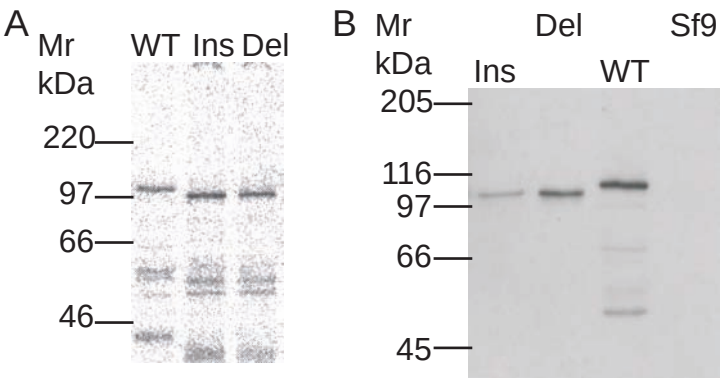
V497A
Insertion; 815 aa
+29, 126, 129 or 134 bp
Exon 10
Exon 11
V497A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Sf9
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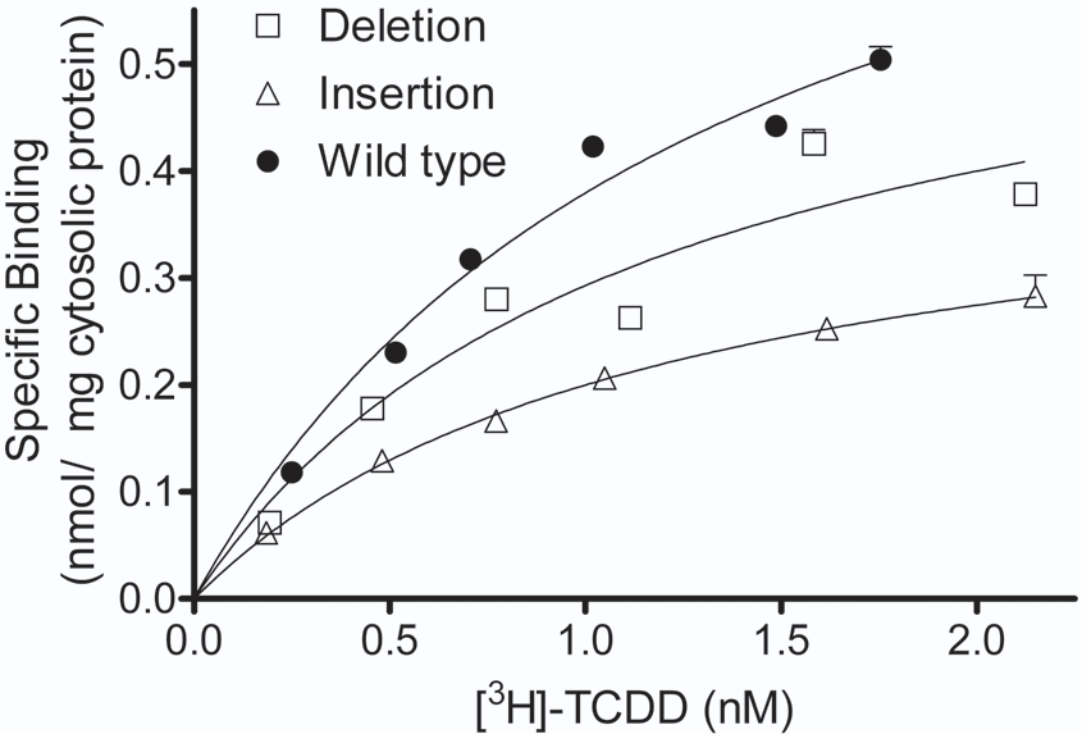
116
97
66
45

Figure 3

	Wild-type	Ins	Del
B_{max} (nmol/mg cytosolic protein)	0.89 [0.73-1]	0.44 [0.38-0.49]	0.63 [0.45-0.81]
K_d (nM)	1.35 [0.89-1.8]	1.19 [0.9-1.48]	1.15 [0.47-1.84]



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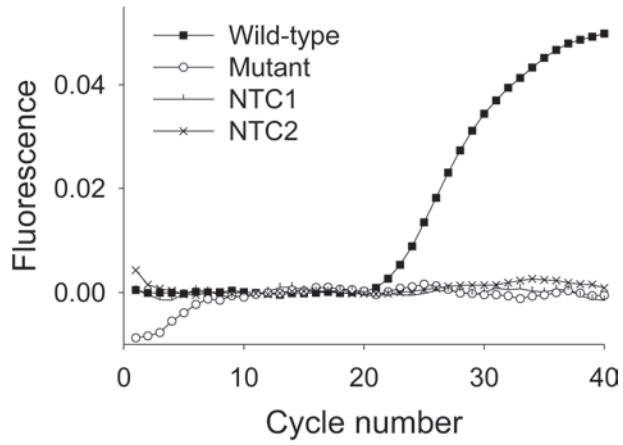
Wild-type
Mutant
NTC1
NTC2
Fluorescence
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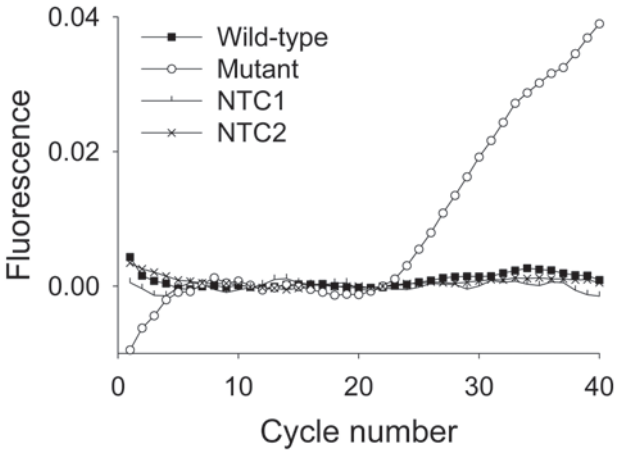
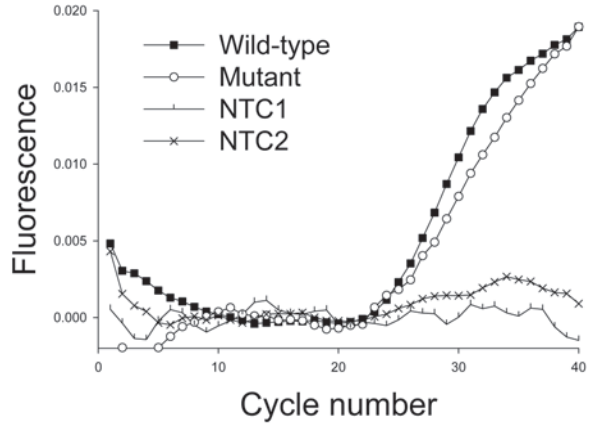
0.00
0
10
20
30
40
Cycle number

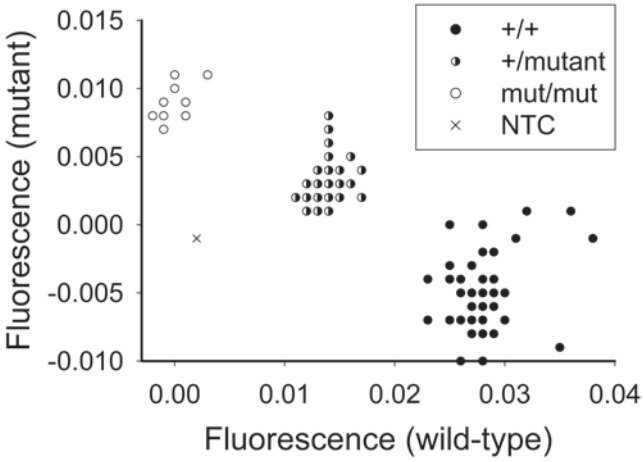
B


Wild-type
Mutant
NTC1
NTC2
Fluorescence
0.04
0.02
0.00
0
10
20
30
40
Cycle number

C


Wild-type
Mutant
NTC1
NTC2
Fluorescence
0.020
0.015
0.010
0.005
0.000
0
10
20
30
40
Cycle number

D


+/+
+/mutant
mut/mut
NTC
Fluorescence (mutant)
0.015
0.010
0.005
0.000
-0.005
-0.010
0.00
0.01
0.02
0.03
0.04
Fluorescence (wild-type)

Supplementary Material

Term	p-value	
	Acute	Chronic
Group	<0.001	<0.001
Type	0.35	0.21
Group.Type	0.97	0.82

TABLE 1: Balano-preputial separation. The dose-response relationship for effects on balano-preputial separation was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There is no significant effect of genotype on survival probabilities, and no interaction with dose group implies that genotype does not affect susceptibility to dose, in either the acute or chronic study.

Term	p-value			
	F_1 CYP1A1 RNA		Maternal CYP1A1 RNA	
	Acute	Chronic	Acute	Chronic
Group	<0.001	<0.001	<0.001	<0.001
Type	0.019	0.739	0.073	0.984
Group.Type	0.368	0.64	0.905	0.819

TABLE 2: CYP1A1 RNA levels. The dose-response relationship for effects on CYP1A1 RNA levels was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There is a significant effect of dose group, and no significant interaction of genotype with dose group in all four samples. The effect of genotype is not significant in the Chronic F1, or the maternal liver in either study; however, there is a significant effect of genotype in the Acute study F1 samples. The heterozygotes show a 1.7-fold increase in CYP1A1 RNA level over wild-type, and the mutants show a 2.5-fold increase. This is discussed further in the results text.

Term	p-value	
	Acute	Chronic
Group	0.655	0.427
Type	0.055	0.212
Group.Type	0.001	0.795

TABLE 3: Maternal liver weight. The dose-response relationship for effects on maternal liver weight was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There is no significant effect of group, or genotype, on maternal liver weight in either the Acute or Chronic study. The interaction between Group and Genotype is not significant in the chronic study, but is significant in the acute study. Consequently, the predictions from the regression model are shown.

	Genotype		
Group	Wild-type	Heterozygous	Mutant
1	11.63	10.64	11.72
2	10.42	11.66	9.58
3	9.49	11.17	12.86
4	12.77	10.63	12.55

TABLE 4: Predictions of maternal liver weight from regression model. The regression model predictions for liver weight with genotype are shown for the acute study data.

Hence, although the interaction between group and genotype for maternal liver weight is statistically significant, including genotype as a predictor in the model shows that there is no consistent or biologically plausible effect of genotype upon response to dioxin.

	Bodyweight (log scale)		
Kill	Term	Acute	Chronic
10 weeks	Group	<0.001	0.231
	Type	0.027	0.301
	Group.Type	0.005	0.581
17 weeks	Group	0.287	0.369
	Type	0.747	0.376
	Group.Type	0.864	0.451

TABLE 5: F_1 bodyweights. The dose-response relationship for effects on F_1 terminal body weight was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There is a significant effect of group, genotype and group-genotype interaction in the acute study at 10 weeks, but not at 17 weeks, nor in the chronic study.

	F_1 liver: bodyweight ratio		
Kill	Term	Acute	Chronic
10 weeks	Group	0.737	0.323
	Type	0.218	0.988
	Group.Type	0.362	0.669
17 weeks	Group	0.185	0.252
	Type	0.778	0.286
	Group.Type	0.638	0.524

TABLE 6: F_1 liver: bodyweight ratio. The dose-response relationship for effects on F_1 liver: body weight ratio was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There is no significant effect of group, genotype or group-genotype interaction on liver: bodyweight ratios in the F_1 animals in either study.

	Spleen: bodyweight ratio		
Kill	Term	Acute	Chronic
10 weeks	Group	0.212	0.025
	Type	0.813	0.015
	Group.Type	0.311	0.603
17 weeks	Group	0.593	0.107
	Type	0.571	0.132
	Group.Type	0.360	0.663

TABLE 7: F_1 spleen: bodyweight ratio. The dose-response relationship for effects on F_1 spleen: body weight ratio was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

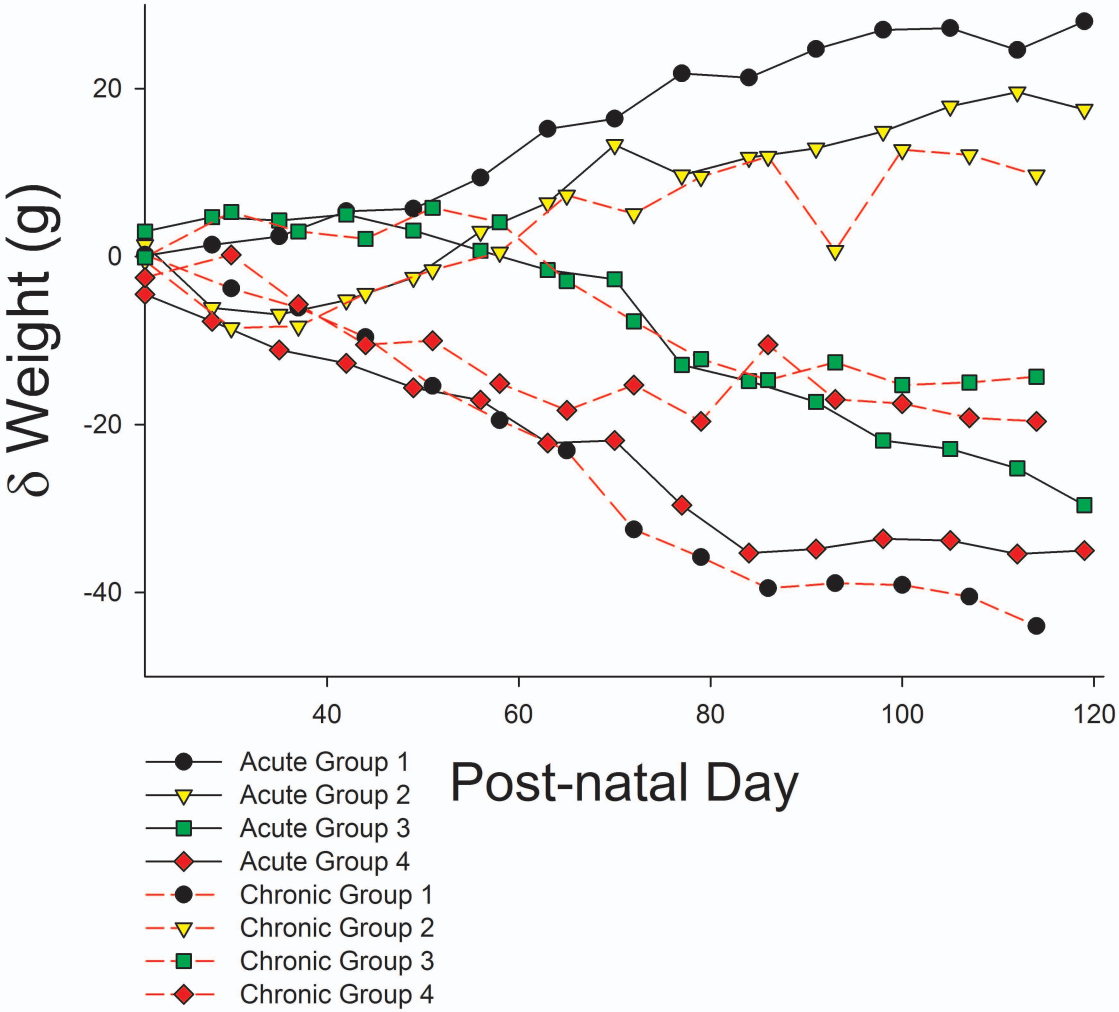
There was a significant effect of dose group, and genotype, in the chronic study at the 10 week kill, but not at 17 weeks, and there were no significant effects in the acute study on these parameters.

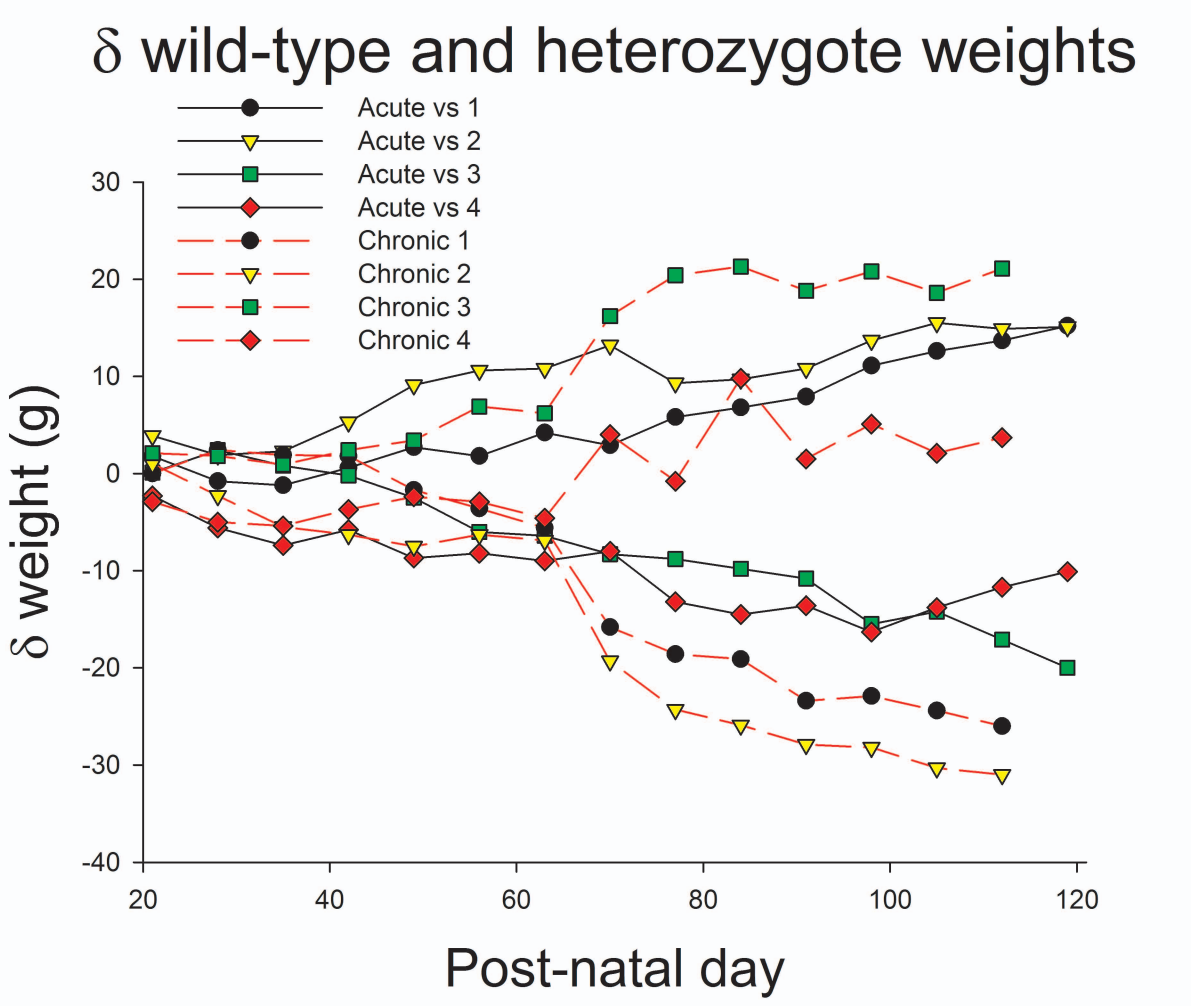
	F_1 Serial bodyweights (log scale)	
Term	Acute	Chronic
PND	<0.001	<0.001
Group	<0.001	0.092
Genotype	0.144	0.015
PND.Group	<0.001	<0.001
PND.Genotype	0.79	<0.001
Group.Genotype	0.002	0.418
PND.Group.Genotype	<0.001	<0.001

TABLE 8: F_1 serial body weights. he dose-response relationship for effects on F_1 serial body weights was examined using ANOVA methods, using mixed models with a random term for litter effects; these responses were analysed, for those animals and tissues for which genotyping had been carried out, under the same models but with an additional factor representing the genotype as wild type, mutant or heterozygous. Group indicates TCDD treatment group, Type indicates genotype, and Group.Type indicates an interaction between Group and genotype.

There are multiple significant effects of PND, Group and genotype in both studies, and interactions between these variables. To determine if these effects were likely to be plausible, the difference between the weight of the wild-type group, and the homozygous (supplementary Figure 1) or heterozygous (Supplementary Figure 2) mutants was plotted for each dose group as a function of post-natal day for each study. Supplementary Figure 2 shows absolutely no biologically plausible relationship between dose group, genotype, and post-natal day; moreover, the magnitude of changes is from +20g to -30g. Supplementary Figure 1 also shows changes within this range of values, and it is noticeable that the acute and chronic control groups are the most widely separated values in this analysis. There is therefore no plausible relationship between the genotype, and with serial body weight.

δ wild-type and mutant weights




δ wild-type and heterozygote weights
Acute vs 1
Acute vs 2
Acute vs 3
Acute vs 4
Chronic 1
Chronic 2
Chronic 3
Chronic 4
30
20
10
0
-10
-20
-30
-40
δ weight (g)
20
40
60
80
100
120
Post-natal day