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Aryl hydrocarbon receptor is indispensable for β-naphthoflavone-induced novel food avoidance and may be involved in LiCl-triggered conditioned taste aversion in rats

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ABSTRACT

Previous studies have shown that several aryl hydrocarbon receptor (AHR) agonists, including β-naphthoflavone (BNF), elicit avoidance of novel food items in rodents, with this behavioral response displaying a similar dose-response to hepatic induction of CYP1A1. The avoidance has been found to bear substantial similarity to conditioned taste avoidance/aversion (CTA). The present study set out to confirm the indispensability of AHR in the avoidance response, to verify whether vagal afferent fibers are involved in it, and to see if AHR signaling might interfere with the effect of the classic trigger of CTA, LiCl. To this end, globally AHR deficient (AHRKO) or vagotomized wildtype rats were treated by gavage with 60 mg/kg BNF or ip with 0.15 M LiCl (4 ml/kg), and presented with chocolate which was either novel or familiar to them. Both the avoidance response and *Cyp1a1* induction were missing in AHRKO rats. In contrast, $Ahr^{+/-}$ rats exhibited them in full, save for a single outlier. Total subdiaphragmatic vagotomy failed to interfere with the avoidance of novel or familiar chocolate or induction of *Cyp1a1*. After LiCl administration, male AHRKO rats showed a significantly mitigated suppression of chocolate consumption compared with wildtype animals (~60% *vs.* ~10% of control chocolate intake, respectively). A similar tendency was seen in females, but they were less responsive to LiCl. These findings corroborate AHR as a prerequisite of the BNF-induced novel food avoidance, prove vagal afferents unlikely mediators of this response, and imply an unforeseen involvement of AHR signaling in the thoroughly-characterized CTA instigated by LiCl.

1. Introduction

Aryl hydrocarbon receptor (AH receptor; AHR) was originally discovered some 40 years ago as the mediator of the toxicity of, and induction of xenobiotic-metabolizing enzymes by, the environmental toxicants dioxins and polycyclic aromatic hydrocarbons [1]. Structurally, it turned out to belong to the bHLH/PAS protein superfamily, which consists of some 30 members that act as environmental and physiological sensors as well as transcriptional co-activators [2,3]. Phylogenetic studies disclosed AHR to be an ancient, over 600 million-year-old protein [4], which suggested that it may also have some important physiological roles in animals. Bioengineering of AHR knockout (AHRKO) mice proved instrumental in demonstrating this. With AHRKO mice, evidence has been provided of AHR's involvement in, for example, such varied processes and entities as innate immunity of barrier organs, liver angiogenesis, male and female fertility, hematopoietic stem cell maintenance, retinal pigment epithelial cell function, tumor suppression/promotion and energy balance regulation [5–12].

Functionally, the AHR is a ligand-activated transcription factor. It resides in the cytosol in a protein complex along with several chaperones (a dimer of hsp90, AHR-interacting protein, p23). Binding of ligand causes a transformation in AHR structure, which results in translocation of the AHR into the nucleus. There it is denuded of its chaperones, and heterodimerizes with another bHLH/PAS protein, ARNT (*AHR Nuclear Translocator*; expressed ubiquitously) or ARNT2 (expressed mainly in the central nervous system [CNS]) to bind to the DNA at specific sequences (AHR response elements) located at the promoter regions of genes regulated by the AHR. The binding then triggers induction or repression of the expression of these genes [13]. One of the most sensitive indicators of activation of this canonical AHR pathway signaling is induction of the gene for cytochrome P-450 1A1, *Cyp1a1*. Enhanced capacity of CYP1A1 and other drug-metabolizing enzymes of the AHR battery mainly serves to detoxify potentially harmful xenobiotics as well as metabolizing physiological ligands of the

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AHR [14,15].

Previous studies have shown that the protective role of the AHR against chemical insults may include a behavioral response. When treated with a variety of AHR agonists, rats exhibit a conspicuous avoidance of novel food items whose presentation is temporally associated with the exposure. While the intensity of this avoidance was similar (almost total refusal to eat the novel food item) across all AHR agonists tested, its duration varied from approximately 1 day [for the short-lived physiological AHR ligand 6-formylindolo(3,2-b)carbazole (FICZ)] to over 40 days [for the long-lingering 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)] [16,17]. It was specific to AHR agonists, as activation of the constitutive androstane receptor (CAR) to the same degree (as assessed by expression of the gene for the CAR-specific drug-metabolizing enzyme, *Cyp2b1*) had no such effect. Chocolate was most often applied as the novel food item in these studies, but the avoidance phenomenon also emerged with cheese or sweet liquids, or even with a different texture of the rats' familiar feed [16,18]. Novel food avoidance was also discernible in mice though not quite as prominent as in rats [19]. It did not manifest in AHR knockout (AHRKO) mice, but the interpretation of the study outcome was impeded by the fact that control AHRKO mice ate significantly more chocolate than control wildtype (WT) mice [19]. Therefore, mediation of the avoidance response by AHR signaling awaits formal confirmation.

Although the avoidance response exhibits some features of augmented taste neophobia, it mainly appears to represent conditioned taste avoidance/aversion [CTA; discussed in length in [17]]. Conditioned taste avoidance and conditioned taste aversion are often interchangeably used terms, but conditioned taste avoidance encompasses both appetitive and consummatory phases of responding (assessed by intake measurements) whereas conditioned taste aversion only includes the consummatory phase (assessed by conditioned disgust reactions) [20]. Taste neophobia and CTA need not be mutually exclusive; on the contrary, taste neophobia may prime CTA to become engaged when suspicions of food toxicity are aroused, thereby enhancing CTA [21]. CTA is believed to usually result from the animal associating tasting a novel food to gastrointestinal (GI) discomfort. This also applies to the gold standard compound for triggering CTA in rodents, LiCl [22]. Interestingly, though, no sign of nausea was found in rats treated with TCDD, despite that one of the doses would have ultimately resulted in a fatal wasting syndrome [23]. On the other hand, in rats exposed to much lower doses of TCDD, the dose-response relationship of the novel food avoidance closely resembled that for hepatic *Cyp1a1* induction [19]. However, subsequent studies with other AHR agonists suggested that it might rather be connected with *Cyp1a1* induction in the proximal intestine [17]. In either case, if information on the induction were neurally mediated to the central nervous system (CNS), visceral afferent fibers of the vagus nerve might well be involved [24].

Hence, the present study had three main objectives. Firstly, to probe the participation of vagal afferents from the GI tract to the CNS in the avoidance response elicited by AHR agonists. Secondly, to formally verify the indispensability of the AHR in this response. Thirdly, to examine whether AHR signaling is involved in, or interacts with, the CTA effect of LiCl. These aims were pursued by means of vagotomized and AHRKO rats.

2. Materials and methods

2.1. Chemicals

LiCl and β-naphthoflavone (BNF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BNF was mixed with DMSO and sunflower oil and then heated in a + 95 °C water bath for 1 h, resulting in a suspension [final DMSO concentration 2.5% (*v*/v)]. LiCl was dissolved in distilled water to the concentration of 0.15 M.

2.2. Animals and their husbandry

For Exp. 1, Sprague Dawley (SD) rats, which had been subjected to total subdiaphragmatic vagotomy by the animal provider, were purchased from Envigo (Venray, the Netherlands). In the other experiments, AHR knockout (AHRKO) rats were used. A single pair of AHRKO rats on SD background was purchased from SAGE Labs (St. Louis, MO, USA). These rats were intended to be used as founders for a colony, but as parasitological examination had revealed that they might harbor *Entamoeba muris* protozoa in their GI tract, the founders were obtained by embryo transfer, after mating the male AHRKO rat with outbred female SD rats. The colony was maintained by heterozygote breeding. This enabled utilization of littermate WT rats as controls for the AHRKO rats. At weaning, pups were marked by auricular punching and the ear pieces were used for genotyping the rats by PCR (the primers are shown in Supplementary Table 1 and a representative PCR identification result in Supplementary Fig. 1). Based on the information sheet obtained from the supplier, the AHRKO rats were stated to have a deletion of 84 bp in exon 2 of the *Ahr* gene (although the figure enclosed indicated the size of the deletion to be 65 bp). However, our analyses revealed that the entire exon 2 (188 bp) was missing from the individual AHRKO male rat used as the ultimate founder of our colony.

The rats were housed in pairs and acclimatized to study conditions and handling for a minimum of one week. During the actual experimental periods, the rats were housed singly to enable individual intake measurements. All the cages were individually ventilated (Green Line IVC Sealsafe PLUS Rat, Techniplast, West Chester, PA, USA), and there was a 12-h light/dark cycle in the animal room with a red light on during the night. The daytime lights came on at 6 a.m. The bottom of the cages was covered with aspen wood bedding (Tapvei, Harjumaa, Estonia), and each cage enriched with a transparent red plastic hiding tube, nesting material and chew blocks (both aspen wood, Tapvei, Harjumaa, Estonia). Commercial pelleted rat chow (Teklad Global 16% Protein Rodent Diet, Teklad Diets, Madison WI, USA) and filtered, UV-irradiated tap water were available *ad libitum*. The novel food tested was solid milk chocolate [Panda (Vaajakoski, Finland) in Exp. 1 but due to its altered composition, Marabou (Mondelêz International, East Hanover, NJ, USA) in Exps. 2–4]. The animal room was air-conditioned, the temperature kept at 22 ± 1 °C and relative humidity at 38–75% (typically 50%). At the end of the experiments, the rats were euthanized with CO_2.

2.3. Experimental design

A total of 4 experiments were conducted to study the novel food avoidance response. In all experiments, the animals were fasted for ~3 h before treatment (feed but not water was withheld).

Exp. 1 set out to ascertain the role of afferent vagal signals from the GI tract in the novel food avoidance behavior induced by the AHR agonist BNF, and to find out the persistence of this behavioral response. A total of 24 male SD rats at the age of 8–15 weeks were used. The rats had undergone total bilateral subdiaphragmatic vagotomy one week before being delivered to our animal facilities. The experiment consisted of two parts, A and B, with A aiming at elucidating whether there is immediate avoidance of a novel food item (chocolate) upon AHR agonist exposure, and B probing its persistence. On day 0, half the rats were administered 60 mg/kg BNF ig. (4 ml/kg) and the rest the same volume of the vehicle. Immediately after the treatment, a pre-weighed ~30-g piece of chocolate was placed on the cage floor. In part A, the weight of the chocolate was measured frequently (at 6, 10, 24, 30, 48, and 54 h) until 68 h, after which the rats were euthanized. In part B, chocolate intake was measured at 6, 10, 24 and 30 h. The chocolate pieces were then removed and the rats returned to their original groups (without chocolate) for 2 weeks. After that period, the rats were moved back to their individual cages (1 h after lights off), and their chocolate pieces were reintroduced. Chocolate consumption was then monitored

for the next 24 h. Upon necropsy, the liver and kidneys were weighed, and the following tissues were sampled for subsequent RT-qPCR analyses: liver, stomach, duodenum, cecum and colon. The samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

In Exp.2, we wanted to formally verify the indispensability of AHR in the novel food avoidance behavior evoked by AHR agonists. To this end, we employed male AHRKO rats and their WT littermates at the age of 7–9 weeks. Based on findings from Exp. 1, the protocol was slightly modified to minimize the role of taste neophobia and focus on CTA in the avoidance response. On day 0, the rats were presented with chocolate. The next day, the chocolate pieces were removed and weighed to make sure that all the rats had at least tasted it (actually, all rats ate > 4 g). Half the rats of both genotypes were then exposed to BNF (60 mg/kg; 4 ml/kg) and the other half to the vehicle ($n = 7$, save for WT control, where $n = 6$), and new pieces of chocolate were placed in the cages. Chocolate intake was monitored at 8 h and 22 h. Upon necropsy, the liver was weighed, and the following tissues were sampled for subsequent RT-qPCR analyses: liver, stomach and duodenum. The samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Exp. 3 sought to find out whether AHR heterozygous ($Ahr^{+/-}$) rats resemble their WT or AHRKO counterparts with regard to the AHR-agonist-induced novel food avoidance behavior. Fourteen 8-week-old male $Ahr^{+/-}$ rats were first habituated to the presence of chocolate in their cages for 24 h. They were then administered ig. either BNF (60 mg/kg; 4 ml/kg) or the vehicle, and the pieces of chocolate were weighed and replaced with new ones of the same brand. Chocolate intake was then monitored for 24 h. Upon necropsy, the liver was weighed, and the same tissues were sampled for subsequent RT-qPCR analyses as in Exp. 2.

As Exps. 2 and 3 had confirmed AHR to be a prerequisite for the AHR-agonist-induced avoidance of novel food items and CTA to play an essential role in it, we decided to examine whether AHR also participates in the CTA elicited by the gold standard compound in these studies, LiCl. Thus, in Exp. 4, the protocol was identical to that in Exp. 3, except that WT and AHRKO rats were treated ip. (4 ml/kg) with either 0.15 M LiCl (isotonic) or 0.9% saline. Moreover, the experiment was carried out in three discrete parts to achieve a sufficient total number of both male and female animals in each group. In total, there were 14 male and 35 female AHRKO rats along with 12 male and 35 female WT rats, with an age range of 8–12 weeks (a single rat was 20 weeks old but responded in a similar fashion to the other rats of its group). After euthanasia, liver was sampled from one of the three sub-experiments for the measurement of *Cyp1a1* gene expression.

2.4. RNA isolation and RT-qPCR

For isolation of total RNA from the tissue samples, Sigma GenElute™ Mammalian Total RNA Miniprep Kit was used according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). RNA was then purified of contaminating DNA with Ambion® TURBO DNA-free™ DNase treatment and removal reagent (Life Technologies, Carlsbad, CA, USA). The concentration of total RNA was measured by a Nanodrop UV Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA purity verified by 260/280 and 260/230 nm ratios. Total RNA was reverse transcribed to cDNA at 50 °C for 1 h using M-MLV RT RNase H– Point Mutant (Promega, Fitchburg, WI, USA). For each reaction (25 µl), 100 U of the enzyme and 800 ng of RNA was used. Real-time quantitative PCR (HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX), Solis Biodyne, Tartu, Estonia) was performed on the RotorGene 3000 instrument (Qiagen, Hilden, Germany) to determine the mRNA levels of the xenobiotic-metabolizing enzyme gene *Cyp1a1* by absolute quantification using total RNA amount (20 ng/reaction) for normalization (see Supplementary Table 1 for primer sequences) [25,26]. If the RT-qPCR result was below the detection limit, a conservative approach was taken and the sample given the value of the limit.

2.5. Statistical analysis

In most cases, the normality of data distribution was assessed by Shapiro-Wilk's test and data outliers by boxplots. Normally distributed data with no extreme outliers were statistically analyzed by one-way ANOVA (if group number ≥ 3) or either Student's or Welch's *t*-test for independent samples depending on variance homogeneity and group size similarity. Severely skewed data or far outliers were attempted to resolve by data transformations (log10, square, square root). If unsuccessful, the non-parametric Kruskal-Wallis H test or Mann-Whitney *U* test was applied. In Exp. 1B, chocolate intakes over time were statistically analyzed using two-way mixed between/within subject ANOVA. In this case, data normality and outliers were assessed based on studentized residuals, homogeneity of variances by Levene's test of equality of error variances, similarity of covariances by Box's test of equality of covariance matrices, and equality of variances of differences by Mauchly's test of sphericity. The limit for statistical significance was set at $p < .05$, except for the Box's test in which it was set at $p < .001$. In Exp. 4, the experimental setting used was considered to contain too many between-subject factors (genotype, gender, exposure) to be associated with the single within-subject factor (time – *i.e.*, the two 24-h chocolate intake measurements), since this would have necessitated a four-way mixed ANOVA. Therefore, the data were separately assessed by three-way mixed ANOVAs for each gender. This approach had the advantage that the substantial difference in n (subject number) between males and females was thereby circumvented. All the analyses were carried out using the SPSS Statistics software (IBM, Version 24.0. Armonk, NY, USA).

3. Results

In Exp. 1, vagotomized male rats were administered ig. either 60 mg/kg BNF or the same volume (4 ml/kg) of the vehicle, and a piece of chocolate placed in their cages immediately upon the treatment. Their chocolate intake was then monitored for 30 h. In Exp. 1A, the rats were euthanized at that point, whereas in Exp. 1B chocolate was withdrawn from their cages for two weeks and then returned for a follow-up of 24 h. At the first encounter, the vehicle-treated rats ate less chocolate than similarly treated intact rats in our previous studies, with their cumulative intake amounting only to *ca.* 2 g by 30 h (Fig. 1, left panel). However, there was still a clear difference compared with BNF-exposed rats, which hardly nibbled the chocolate at all. This chocolate avoidance of BNF-treated rats was sustained until the end of the experiment (68 h; data not shown).

At the second encounter in Exp. 1B, all rats ate chocolate much more avidly (Fig. 1, right panel). Yet, the vehicle-treated rats consumed it significantly more by each time-point. In addition, there was a marginally significant interaction between time and treatment, $F(1.198, 11.982)$, $p = .048$, partial $\eta^2 = 0.314$, $\varepsilon = 0.399$ (Greenhouse-Geisser correction).

Cyp1a1 expression was increased in the BNF-treated groups at termination in both sub-experiments (Table 1). This induction was especially pronounced in cecum and colon where it amounted to some 200-fold on day 3 post-exposure and persisted high at 15 days in Exp. 1B. It was lowest and subsided most swiftly in the liver where only a 10-fold induction was detected on day 3 and no statistically significant difference between the BNF and vehicle groups existed any longer on day 15. In stomach and duodenum, the induction level remained stable (about 20-fold) at both time-points, but there was considerable interindividual variation in stomach values in Exp. 1A.

In Exp. 2, we tested the BNF-induced novel food avoidance response in AHRKO rats. To this end, the experimental design was slightly modified from that in Exp. 1A, with the aim of focusing on CTA-like properties of the response and minimizing the role of taste neophobia in

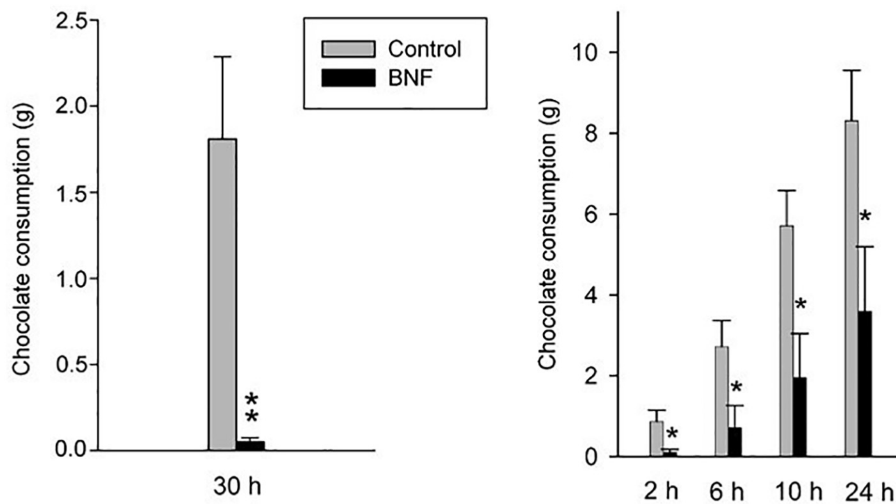

Fig. 1. *Left panel:* Cumulative chocolate consumption by vagotomized rats over 30 h following exposure to BNF or vehicle (mean + SEM; $n = 11–12$). The asterisks denote a statistically significant difference ($p = .004$). The figure compiles data from the first encounter with chocolate in Exps. 1A and 1B. *Right panel:* Cumulative chocolate consumption in Exp. 1B by vagotomized rats 2 weeks after their initial habituation to chocolate (mean + SEM; $n = 6$). The asterisks denote a statistically significant difference ($p < .05$).

Table 1
Fold-induction (gene expression in BNF-treated/vehicle-treated) of *Cyp1a1* in the liver and GI tract of vagotomized rats in Exps. 1A (3 days from exposure) and 1B (15 days from exposure).

	Liver	Stomach	Duodenum	Cecum	Colon
Exp. 1A					
Mean	9.57*	24.5	17.7**	210**	150**
SEM	4.97	14.7	3.12	40.0	40.9
Exp. 1B					
Mean	2.45	17.5**	21.3**	90.3**	72.4**
SEM	0.70	5.69	8.11	29.7	25.4

The asterisks denote a statistically significant difference (one: $p < .05$; two: $p < .01$). $N = 6$.

it. Thereby, the rats were allowed to gain access to the novel food item, chocolate, for 24 h prior to exposure to BNF or vehicle. This also enabled us to verify that all rats had truly tasted the chocolate before the treatment.

When rats were presented with chocolate one day before the exposure, there were no statistically significant differences in their consumption of this novel food item among the four subgroups over 24 h (Fig. 2, left panel; $p = .256$). Upon exposure, WT rats responded to BNF by reducing their chocolate intake down to about 15% of control consumption and their own previous intake level (Fig. 2, left panel). In AHRKO rats, in contrast, BNF failed to exert any discernible effect on chocolate consumption. This divergence emerged already at the earlier measurement time-point at 8 h (data not shown).

Cyp1a1 expression was induced by over 2200-fold in the livers of BNF-treated WT rats (Fig. 2, right panel), whereas no *Cyp1a1* induction existed in AHRKO rat livers. This was also true of the other two tissues analyzed, stomach and duodenum, in which WT rats exhibited *ca.* 80- and 25-fold inductions of *Cyp1a1*, respectively (data not shown).

To see whether a single allele of the *Ahr* gene would suffice to bring about the WT-like response to BNF, *Ahr* heterozygous rats ($Ahr^{+/-}$) were tested in Exp. 3. At their first encounter with chocolate, rats of both treatment groups ate similar amounts of it [9.86 ± 0.34 and 9.17 ± 0.60 g (mean ± SEM; $n = 6–7$; $p = .321$)]. After BNF exposure, all rats in this group (save for one) consumed only negligible amounts of chocolate during the 24-h observation period (0.35 ± 0.16 g *vs.* 5.1 ± 1.13 g for the controls; $p = .003$). However, one of the BNF-treated rats was removed from the experiment as an extreme outlier, as it ate chocolate voraciously: 11.1 g! It clearly was not fully AHR deficient since it displayed a 1090-fold induction of *Cyp1a1* in its liver. Yet, its AHR function may have been compromised also in this respect since the remaining rats of the BNF group had a 9260-fold (± 3040; mean ± SEM) hepatic *Cyp1a1* induction level. Subsequent determination of AHR mRNA abundance in the liver and duodenum showed that this particular rat exhibited 83 or 67%, respectively, of the mean expression levels of its group partners.

As LiCl has been the most common chemical agent to elicit CTA in rodents for decades, it was of great interest to examine whether AHR is

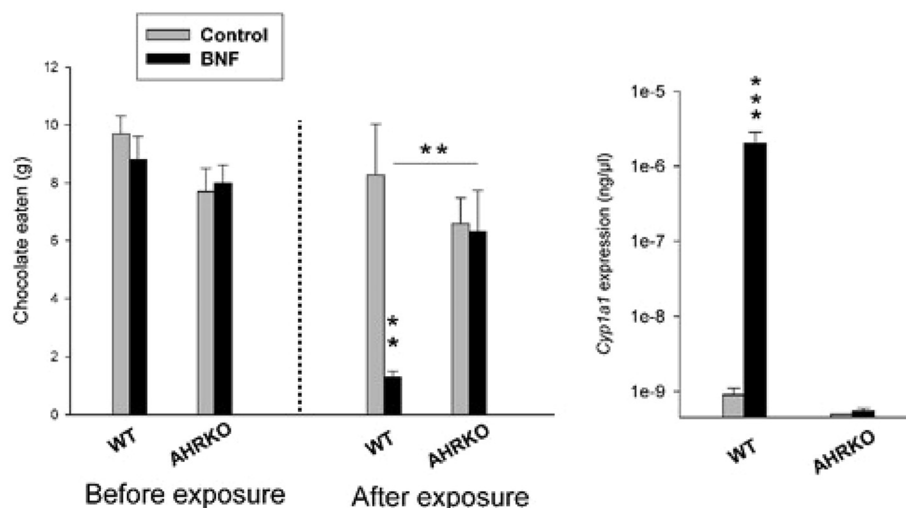

Fig. 2. *Left panel:* One-day chocolate intake of WT and AHRKO rats immediately prior to and after exposure to 60 mg/kg BNF or the same volume of vehicle (mean ± SEM; $n = 6–7$) in Exp. 2. The asterisks denote statistically significant differences ($p < .01$). *Right panel:* Expression of *Cyp1a1* in the livers of the same rats. The asterisks denote a statistically significant difference ($p < .001$).

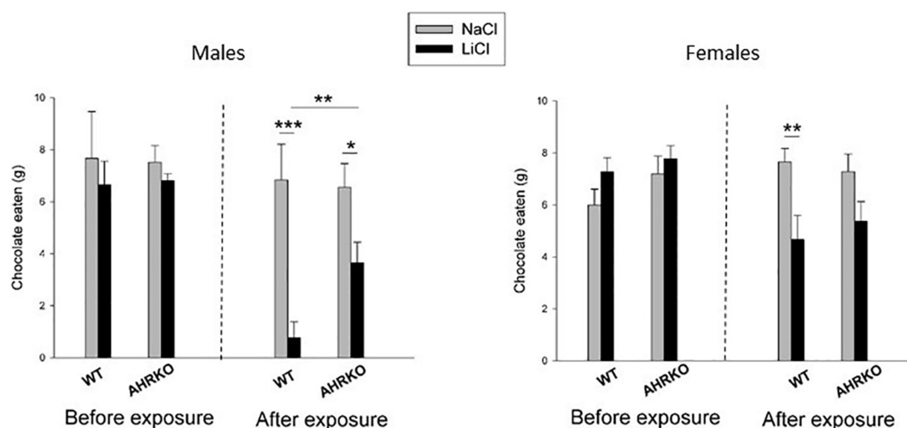

Fig. 3. One-day chocolate intake of WT and AHRKO rats immediately prior to or after exposure to 0.15 M LiCl or 0.9% saline (mean ± SEM; n = 6–7 for males and 16–19 for females) in Exp. 4. The asterisks denote a statistically significant difference (one, p = .033; two, p = .009 [in both cases]; three p = .001).

involved in, or can interfere with, its action. In Exp. 4 we therefore exposed WT and AHRKO rats to 4 ml/kg of 0.15 M LiCl or 0.9% saline. The same protocol was followed as in Exps. 2 and 3, except that also female rats were included in the study.

Prior to LiCl exposure, there was no difference in chocolate consumption between either the genotypes or the two treatment groups within genotype; also rats of both genders ate it in similar amounts (Fig. 3). Treatment with physiological saline did not affect that intake level. However, male WT rats responded to LiCl administration by diminishing their chocolate intake precipitously down to some 10% of that in the control group. A less prominent, though significant, drop occurred in male AHRKO rats to *ca.* 60% of control. The difference between genotypes in the resultant chocolate intake levels after LiCl exposure was also significant (p = .009).

In female rats, LiCl affected chocolate intake less prominently. Rats treated with LiCl ate about 60 and 75% of the amounts consumed by their controls for WT and AHRKO rats, respectively. The difference from their saline-treated counterparts was significant in WT (p = .009) but not in AHRKO rats (p = .073).

Three-way mixed ANOVA conducted gender-wise over the data showed a significant 3-way interaction [genotype x exposure x time; F (1, 20) = 6.059; p = .023; partial η^2 = 0.233] in males. Subsequent analysis revealed a statistically significant simple two-way interaction of genotype x exposure after treatment [F(1, 20) = 7.933; p = .011] but not before treatment [F(1, 20) = 0.038; p = .848]. Statistical significance of simple simple main effects post-treatment was accepted at a Bonferroni-adjusted alpha level of 0.025. There was a significant simple simple main effect of LiCl [F(1, 20) = 14.790; p = .001] but not of saline [F(1, 20) = 0.001; p = .982]. Pairwise comparisons with adjusted p values proved that chocolate intake after LiCl treatment was significantly higher in AHRKO than WT rats (p = .001). In females, the three-way interaction was not significant [F(1, 66) = 1.248; p = .268; partial η^2 = 0.019).

LiCl did not affect hepatic *Cyp1a1* gene expression (data not shown).

4. Discussion

As an animal species unable to vomit, the rat has had to resort to alternative strategies in attempting to avoid exposure to poisonous substances. One of these is pica in the form of eating clay (or kaolin in laboratory conditions), which adsorbs noxious compounds and whose intake increases in rats in response to visceral malaise [27]. A more effective strategy is based on exposure prevention. For this purpose, two complementary behavioral responses have developed in the course of evolution: taste neophobia and CTA. Of these, taste neophobia represents an innate reluctance to eat food items with a novel flavor in large amounts until they become familiar. CTA, in turn, refers to the ability of the animal to associate post-ingestive nausea or intestinal discomfort (unconditional stimulus) to a novel taste or flavor (conditional stimulus) and subsequently to remember this association. It thus constitutes a form of learning [28]. It should be noted that CTA is by no means confined to rats alone; on the contrary, it is a widespread safety strategy against deleterious foods in all animals, including humans [21].

Our previous studies have demonstrated that activation of AHR signaling reliably elicits a behavioral response in rodents manifesting itself as avoidance of novel food items. This response displays properties consistent with both taste neophobia and CTA, can be triggered by a wide variety of AHR agonists inclusive of toxic xenobiotics and a physiological AHR activator, occurs more intensely in rats than mice, and is variably attenuated by preceding habituation to the "novel" food item (with chocolate avoidance being affected less than that of saccharine or cheese) [16,17,19]. Moreover, its persistence depends on the AHR agonist, and appears to show a correlation with the elimination half-life of the agonist. For example, repeated weekly testing with chocolate revealed that in rats, aversion to the slowly-metabolized TCDD does not begin to dissipate until 6 weeks after exposure to a single low dose [16]. An especially intriguing finding was that in the rat lines employed, hepatic induction of drug-metabolizing enzymes (foremost CYP1A1) exhibited a conspicuously similar dose-response to that for the novel food avoidance [19]. Furthermore, a potent inducer of the phenobarbital battery of drug-metabolizing enzymes, 2,4,6-tryphenyldioxane-1,3 (TPD), failed to cause the novel food avoidance, attesting to the specificity of the response to AHR signaling [17].

As vagal afferents are important channels for conveying feeding-related information from the GI tract to the brainstem, it was of great interest to see if total bilateral subdiaphragmatic vagotomy would modify the novel food avoidance response. At their first encounter with chocolate, vagotomized rats treated with the vehicle consumed only some 25% of the amount intact rats had done in our previous studies or were observed to do here (compare Fig. 1, left panel, with Figs. 2–3). This may not be highly surprising, because subdiaphragmatic vagotomy has been reported to reduce intake of sweet-tasting solutions in rats [29], which could be explained by a recent unexpected finding that the gut-to-brain vagal axis is an integral component of the neuronal reward pathway [30]. However, when the rats regained access to chocolate two weeks later, the ones in the vagotomized control group increased their intake to the normal level (Fig. 1, right panel). This suggests augmented taste neophobia in vagotomized rats, which has also been recorded earlier using saccharine and water in a two-bottle selection test [31]. Upon treatment with the potent AHR agonist BNF, chocolate intake dropped practically to zero at the first encounter and displayed delayed recovery at the second one (reaching merely 50% of control consumption by 24 h; Fig. 1). Since these outcomes are similar to the

responses exhibited by BNF-treated intact SD rats [17], vagotomy failed to overtly interfere with the AHR agonist-triggered avoidance of novel foods.

Previously, non-consistent outcomes have been reported from attempts to modulate CTA by vagotomy. While radiation-induced CTA was not influenced by subdiaphragmatic vagotomy [32], intragastric administration of copper sulfate was more effective in reliably producing a taste aversion in vagotomized rats than in their sham-operated controls, and this aversion could be prevented by lesions of the area postrema in the hindbrain [33]. In contrast, CTA due to motion-induced sickness caused by vertical axis rotation failed to develop in rats with complete, selective gastric vagotomy [34]. On the other hand, angular acceleration-instigated CTA was only delayed in subdiaphragmatically vagotomized rats [31]. Perhaps most interestingly, LiCl was equally effective in eliciting CTA in both intact and subdiaphragmatically vagotomized rats [35]. Thus, vagal afferents appear to have a role in some but not all cases of general malaise- or GI discomfort-associated food aversions. Plausibly, in other cases involving visceral organs, information from the periphery is mediated to the CNS through afferent autonomic fibers in the splanchnic nerves [36] or by humoral signals.

We previously observed a strikingly similar dose-response for augmented hepatic CYP1A1 catalytic activity and the avoidance of novel foods in rats exposed to the extremely powerful AHR agonist TCDD [19]. Our subsequent studies showed that this avoidance response appeared to be even more closely associated with induction of the *Cyp1a1* gene in the proximal GI tract [17]. The present findings may lend further support to this latter view, because in BNF-treated vagotomized rats retested with chocolate two weeks after its initial presentation and still displaying its diminished intake, hepatic *Cyp1a1* expression level did not deviate significantly any longer from the vehicle group whereas its GI expression levels did. On the other hand, AHR activation may only be needed at the acquisition phase of the CTA-like response and not for its maintenance or later expression.

Our second experiment provided convincing formal substantiation to the indispensability of AHR in BNF-induced avoidance of novel foods. Rats with global AHR deficiency failed to show both chocolate avoidance and *Cyp1a1* induction. In the future, this information can be utilized in probing the critical target tissue(s) of AHR agonist action by tissue-specific knockouts. For example, the nature of the relationship between the avoidance response and *Cyp1a1* induction in the liver or GI tract could be addressed with animals in which AHR signaling has been eliminated in the liver or intestine alone [37].

We found here that *Ahr* heterozygous rats may retain their full responsiveness to BNF in terms of the novel food avoidance and *Cyp1a1* induction. However, there was one outlier rat which did not show the behavioral response at all and whose hepatic *Cyp1a1* expression level was not elevated as substantially as in its group partners. Although *Ahr* gene expression also proved to be slightly lowered in this rat, more likely explanations for the anomalous responses may be more remarkably decreased AHR protein abundance, a functional defect in the AHR signaling pathway, or a deviation in BNF pharmacokinetics. A previous paper reported that compared with WT animals, $Ahr^{+/-}$ mice expressed approximately 30% of the AHR mRNA in the liver [38]. The mouse model appears to differ in this regard from its rat counterpart as we did not see lowered *Ahr* expression in our heterozygous rats (data not shown).

It is important to note that the experiments in AHRKO and $Ahr^{+/-}$ rats further ascertained the role of CTA in the novel food avoidance response. To this end, the rats had access to the novel food item, chocolate, for 24 h before the onset, and were thus accustomed to it at the exposure. The fact that both WT and $Ahr^{+/-}$ rats still displayed avoidance, and at least to the same degree as in the case of LiCl in the next experiment, attests to a crucial involvement of CTA in the behavioral response. However, this does not preclude contribution by enhanced taste neophobia because it may be intimately interwoven with CTA [21], and we have earlier provided evidence of its participation (see discussion in [17]).

Given that LiCl is the gold standard chemical agent used experimentally for decades as the unconditional stimulus in bringing about CTA, we were intrigued to see whether global AHR deficiency might modify its effect. Our experiments yielded two major findings. Firstly, evidence was obtained that functional AHR signaling is required for LiCl to exert its full impact on CTA. This was clearer in males, but a similar propensity was discernible (based on pairwise comparisons) in females, too. Secondly, especially in WT, but also in AHRKO rats, there was a distinct gender difference in responsiveness to LiCl, with male rats exhibiting greater sensitivity than females. In keeping with this, a previous study showed that 1) gonadally intact male rats display a more robust CTA response to LiCl than intact female rats, 2) gonadectomy essentially eliminates this sex divergence, and 3) the male or female pattern of CTA responsiveness can be elicited in gonadectomized rats (irrespective of their original gender) with implanted sex hormone pellets [39]. The fact that this well-established gender difference was reproduced in our study in both WT and AHRKO rats provides further strong evidence in support of the view that our experimental setting did indeed measure the classic CTA response to LiCl.

The modulation of the CTA-triggering effect of LiCl by AHR signaling is a novel and unexpected finding. The anatomic level at which this cross-talk takes place is unknown at present. As described above, the aversion responses to both AHR agonists and LiCl seem to be refractory to total subdiaphragmatic vagotomy. However, while the CTA triggered by LiCl is generally believed to require an element of GI distress [40], no clear evidence of this was found in studies addressing the role of nausea in TCDD-induced hypophagia [23]. It is thus conceivable that the site of interaction is not peripheral. At the CNS, two critical targets for LiCl are the area postrema and the parabrachial nuclei [41–43], which are interconnected in the brainstem [44]. In the parabrachial nuclei, calcitonin-gene-related peptide (CGRP) neurons have been identified as sufficient and necessary for establishing CTA [45,46] and they are activated by LiCl [45]. Perpetual CGRP neuron activation can result in a life-threatening body weight loss due to anorexia [47], and a recent study has established these neurons as key mediators of cancer-induced appetite suppression [48]. Although next to nothing is known about the effects of AHR signaling in the brainstem, it is noteworthy that the hallmark of acute TCDD toxicity is a wasting syndrome, which predominantly results from severe hypophagia [49]. Screening of AHR, ARNT and ARNT2 mRNA expression levels and sites in the rat CNS revealed that AHR and ARNT2 are expressed in area postrema; while parabrachial nuclei were not analyzed, high abundance of AHR and ARNT2 mRNA was detected in the nucleus of the solitary tract [50]. This nucleus and the parabrachial nuclei constitute the first and second central relays in the rodent taste pathway, respectively [51,52]. Hence, the modulation by AHR signaling of the LiCl-induced CTA may occur in the hindbrain, and this aspect warrants further studies.

Taken together, the present study demonstrates that the BNF-induced novel food avoidance requires AHR signaling but not vagal afferent fibers. In the rat AHRKO model, heterozygous animals retain their full AHR agonist responsiveness. Male and female rats differ markedly in their sensitivities to CTA evoked by LiCl, and AHR signaling can interfere with the CTA effect of LiCl. Future studies should be directed at elucidating the location and mechanism of this interference as well as the relationship between induction of xenobiotic-metabolizing enzymes and the avoidance of novel foods by AHR agonists.

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