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Ethanol Effects On Physiological Retinoic Acid Levels

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Summary

All-*trans*-retinoic acid (atRA) serves essential functions during embryogenesis and throughout post-natal vertebrate life. Insufficient or excess atRA causes teratogenic and/or toxic effects in the developing embryo: interference with atRA biosynthesis or signaling likely underlies some forms of cancer. Many symptoms of vitamin A (atRA precursor) deficiency and/or toxicity overlap with those of another pleiotropic agent—ethanol. These overlapping symptoms have prompted research to understand whether interference with atRA biosynthesis and/or action may explain (in part) pathology associated with excess ethanol consumption. Ethanol affects many aspects of retinoid metabolism and mechanisms of action site-specifically, but no robust data support inhibition of vitamin A metabolism, resulting in decreased atRA *in vivo* during normal vitamin A nutriture. Actually, ethanol either has no effect on or increases atRA at select sites. Despite this realization, insight into whether interactions between ethanol and retinoids represent cause *vs*. effect requires additional research.

Keywords

retinol dehydrogenase; ethanol; fetal alcohol spectrum disorder; retinoic acid; vitamin A

INTRODUCTION

Two low molecular weight organic chemicals have enormous impact on human health: ethanol and retinol (vitamin A). All vertebrates require retinol, acting via its metabolite atRA, to reproduce, grow and remain healthy. Epithelial differentiation, nervous system development and function, immune system function, embryogenesis, and fertility require atRA within a fairly narrow concentration range at specific sites during restricted temporal windows (1–5). Failure to meet or regulate these demands for atRA results in birth defects, other forms of vitamin A toxicity, and increased cancer risk (6, 7). Similarly, excess ethanol consumption causes numerous pathologies, including cancer, fetal alcohol spectrum disorder (FASD), and abnormalities in many of the same processes regulated by atRA (8–10). Given the enveloping functions of atRA, and the pervasive pathologies and mechanisms of ethanol action, overlap of affected sites and processes seems inevitable (11).

Despite the unavoidable overlap of ethanol and retinoid effects, and the well-known ability of ethanol to interfere with the metabolism and function of multiple nutrients, a commonly held notion postulates that competitive inhibition of retinol's conversion into atRA contributes to the pathology of ethanol (12–15). This hypothesis requires participation of alcohol dehydrogenase(s) (Adh) in retinol metabolism under physiological conditions, and decreased tissue atRA during normal vitamin A nutriture upon ethanol exposure. Neither

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seems to be the case. Rather, ethanol either has no effect on or increases tissue atRA, depending on the site (16, 17). Ethanol affects multiple enzymes, binding proteins, and receptors important to retinoid homeostasis and atRA biosynthesis and function (11, 18, 19). Thus, ethanol effects on atRA and retinoid function are difficult to predict by examining a single component of retinoid metabolism or signaling. The complexity of maintaining retinoid homeostasis, the interactions of various participants, and compensatory reactions, all impart tissue-specificity to ethanol's effects on atRA concentrations. No determination has been made whether ethanol-induced changes in atRA contribute causally to ethanol pathology and/or FASD. A recent study of mouse limb development illustrates the complexity of the situation (20). An RAR pan-antagonist duplicated limb defects morphologically similar to those caused by ethanol, and atRA dosing prevented ethanol's effects, but gene expression assessments revealed unexplained differences between ethanol and the RAR pan-antagonist.

RETINOID HOMEOSTASIS AND MECHANISMS OF ACTION

A brief review of retinoid biosynthesis and function will frame the context of ethanol actions (21). Liver stores the majority of retinol as retinyl esters (RE) and releases retinol bound with the serum retinol binding-protein (RBP) into circulation (Figure 1). RBP interacts with the plasma membrane receptor Stra6, which mediates transfer of retinol into cells (22). The cellular retinol binding-protein (Crbp1) and lecithin:retinol acyltransferase (LRAT) couple retinol uptake to RE formation. Accretion of RE in liver and other tissues promotes storage in lipid droplets and re-use of Crbp1. In contrast to the function of holo-Crpb1 (retinol bound to Crbp1) as chaperone for RE formation, apo-Crbp1 stimulates RE hydrolysis and inhibits LRAT. The ratio holo-Crbp1/apo-Crbp1 apparently signals retinol status and directs retinol flux into and out of storage as RE, while maintaining steady-state levels of holo-Crbp1 to support atRA biosynthesis.

The first of two dehydrogenations, conversion of retinol into retinal, limits the rate of atRA biosynthesis. Microsomes account for 80 to 94% of cellular retinal-generating capacity from holo-Crbp1, and microsomal rates exceed cytosolic rates by 5 to 20-fold (23). The microsomal retinol dehydrogenases (Rdh) that recognize retinol associated with Crbp1 have been identified as members of the short-chain dehydrogenase/reductase gene family. At least three Rdh seem physiologically important to atRA biosynthesis, based on biochemical, physiological and genetic evidence: Rdh1, Rdh10 and Dhrs9. Notably, ethanol does not function as a competitive inhibitor of Rdh, but rather stimulates activity *in vitro*. The next step involves a rapid (relative to retinol dehydrogenation) and irreversible dehydrogenation of retinal into atRA. At least three Raldh catalyze this step: Raldh1, 2, and 3 (Aldh1A1, 1A2 and 1A3).

Binding proteins of the fatty acid binding-protein gene family sequester atRA: Crabp1, Crabp2 and FABP5 (24). Crabp2 transfers atRA to RAR, whereas FABP5 transfers atRA to PPARδ/β, and Crabp1 (so far) only seems to mediate atRA catabolism (25, 26). The receptors regulate transcription and RARα, also regulates translation (27–29). Thus, selective atRA delivery, mediated by binding proteins, provides a mechanism to induce dissimilar atRA actions.

atRA regulates retinol and its own concentrations. Simultaneous induction of *Stra6*, *Crbp1*, and *LRAT* by atRA insures coupled retinol uptake and esterification with "excess" retinol directed into RE. atRA also induces *Cyp* to stimulate its catabolism (30). Finally, interactions between Rdh and Raldh provide another level of maintaining atRA concentrations. Despite Dhrs9 function as an Rdh, knocking down *Dhrs9* in primary

astrocytes increases atRA production by increasing *Raldh1* mRNA expression and activity (5).

ETHANOL DISRUPTS RETINOID HOMEOSTASIS AND SIGNALLING

The effects of acute and chronic ethanol on vitamin A homeostasis have been documented since the early 1980's and have been reviewed in detail (11, 14, 19, 31, 32). Ethanol affects multiple sites of retinoid metabolism and function outlined in Figure 1. Briefly, ethanol ingestion: 1) causes massive depletion of liver RE and alters the tissue distribution of retinol; 2) enhances liver-mediated retinol and atRA catabolism; 3) induces *Crpb1* mRNA in the embryo and postnatal brain; 4) alters *RAR* mRNA expression in testis and brain (33, 34); 5) increases *Crabp1* expression in the mouse embryo (35); 6) increases hepatic concentrations of the carotenoid cleavage enzyme, CMO1, which generates retinal (36). Generally, mechanisms of these effects have not been established, but likely reflect pleiotropic effects of ethanol on cellular processes, including gene regulation.

For the most part, distinct enzymes catalyze retinoid *vs*. ethanol metabolism, suggesting an *in*direct impact of ethanol on retinoid metabolism. Raldh1 (Aldh1a1) may represent an exception. Raldh1 (Aldh1A1) has a $K_{0.5}$ value ~150 μM for acetaldehyde and 0.8 μM for the Crbp1-retinal complex, providing potential for interaction between the two substrates (37). Blood acetaldehyde levels in rat reach <40 μM after a 5 g/kg dose of ethanol, however, whereas tissue retinal concentrations vary between 0.05 and 0.2 μM (38). These values, along with the Raldh1 kinetic constants, suggest limited possibility of competitive interaction. Furthermore, Raldh1 does not catabolize the major portion of acetaldehyde. Nevertheless, Raldh1 polymorphisms have been implicated in alcoholism and alcohol sensitivity (39). Cyp2E1 may provide another exception. This enzyme has not been implicated in atRA metabolism under physiological conditions, but higher concentrations of chlormethiazole, an inhibitor of *Cyp2E1* mRNA expression, modestly preserves RE levels in livers of rats fed ethanol for 1 month, likely through sparing retinol and atRA (40).

ETHANOL IMPACT ON RA

An impact of ethanol on the concentrations of atRA likely would have a large impact on processes governed by vitamin A. Yet, until recently no analytically robust test has reported an effect of ethanol on serum and tissue atRA concentrations during *normal* vitamin A nutriture. The recent application of LC/MS/MS assays has allowed direct quantification of ethanol's effects on serum and tissue atRA (41). LC/MS data showed that acute dosing of ethanol (2 × 5 g/kg, 2 hr apart) to postnatal day 4 rat pups caused cerebellar atRA levels to *increase* ~5-fold 2 hr after the second dose (16). A second study quantified atRA in a variety of mouse tissues and brain areas after both acute and chronic ethanol exposure (17). Only the highest single ethanol dose (3.5 g/kg) affected atRA, and the affect was an *increase* in hippocampus, testis, and liver, and no change in serum or kidney. Chronic exposure (6.5% ethanol diet, one month) increased atRA in testis, serum, hippocampus and cortex, and had no impact on atRA in the other tissues and brain areas assayed (Table 1). Additionally, exposure of pregnant dams to ethanol starting on e13 increased atRA in proportion to dam BAC% in the hippocampus and cortex of e19 embryos (Table 2). These data illustrate several points: 1) short-term effects of ethanol on atRA do not predict longer term effects; 2) in no instance did ethanol decrease serum or tissue atRA; 3) effects were tissue specific; 4) serum atRA levels did not predict tissue atRA after chronic ethanol exposure; 5) ethanol ingestion by dams increases embryo atRA at select sites in the developing brain. The last point suggests a connection between FASD and the impact of ethanol on atRA, because even modest increases in atRA cause toxicity, especially in the embryo (42).

MECHANISMS OF ETHANOL EFFECTS ON RA

The results of the two studies detailed above may seem enigmatic: increased atRA; tissue-specific impact of ethanol on atRA; differences in acute *vs*. chronic exposure. Considering these effects in context of retinoid homeostasis, as outlined in Figure 1, however, produces some insight.

The liver reacts to ethanol exposure by mobilizing RE, which increases hepatic retinol and provides additional substrate to drive atRA biosynthesis (Figure 2). With time, ethanol induces expression of two genes in liver that encode enzymes involved in atRA biosynthesis, *Dhrs9* (~3-fold) and *Raldh3* (~5-fold) (17). But chronic increases in atRA and ethanol induce atRA catabolism, such that the elimination $t_{½}$ of atRA decreases nearly 9-fold. The result is a short-term increase in liver atRA, with atRA catabolism eventually offsetting the increase in retinol concentrations and *Dhrs9* and *Raldh3* induction. In contrast, the hippocampus reacts to both acute and chronic ethanol exposure with increases in atRA, driven in the short term by increased retinol and maintained in the longer term by increased expression of *Stra6* and *Crbp1*, insuring increased retinol uptake. Also, Raldh1 activity increases, which contributes to increased atRA, even though the amount of Raldh1 protein decreases. Thus, understanding the impact of ethanol on atRA requires longer-term study, quantification of atRA, assessment of multiple contributors to atRA homeostasis, and assessments of activity (not just protein expression assays).

ADH1 CONTRIBUTES TO RETINOL TOXICITY

Ethanol effects have been attributed to reduced tissue atRA *via* inhibition of Adh. This conclusion originated from reports that the same human liver and testis enzymes catalyze oxidation of ethanol and retinol *in vitro*, and low ethanol concentrations competitively inhibit retinol oxidation (43, 44). This conclusion has persisted as a result of indirect estimation of atRA *in vivo*, rather than quantification with validated analytical assays, and by assessing ethanol's impact on the metabolism of pharmacological amounts of retinol dosed in a bolus (13, 15, 45, 46). Indirect estimation relies on a RARβ response element driving a *Lacz* reporter. This approach has multiple design and practical limitations, does not generate "real time" data, and has not been validated as an assay for atRA. Siegenthaler *et al*. compared reporter data to LC/MS generated data and concluded that the former was "not useful" to assess atRA in the developing cortex (47). Additionally, it is not clear how the reporter could distinguish interference with atRA biosynthesis from cumulative ethanol effects on the multiple processes required to produce a blue tint.

Participation of Adh in retinol metabolism under physiological conditions has not been verified and seems unlikely. Adh do not recognize the physiological form of retinol, *i.e*. holo-Crbp. Unbound retinol occurs in low nM concentrations *in vivo*, and is more likely to associate with membranes than occur solvated in the aqueous medium. Adh recognition of high concentrations of unbound retinol *in vitro* does not inform that Adh function with physiological concentrations of intracellular retinol. Consistent with this, studies of the various *Adh*-isozyme-null mice have not reported pathology, retinoid metabolism changes (including in atRA), or gene expression changes related to atRA deficiency. The mechanisms responsible for the limited phenotypes of *Adh*-isozyme-null mice remain unclear. In contrast, physiological participation of Rdh1 and Rdh10 in atRA biosynthesis has been verified by direct quantification of retinoids, pathology, gene expression changes, and/or mechanistic studies.

Decreased serum atRA after ethanol dosing or in *Adh1*-null mice has been observed after administration of toxic retinol doses (50 to 100 mg/kg) (46). A dose of 50 mg/kg delivers ~300-fold more than the recommended daily intake of retinol and drives serum atRA ~1600-

fold higher than the steady-state value of ~2 pmol/ml in wild-type mice. Although this high serum atRA concentration decreases ~87% after ethanol dosing and in the *Adh1*-null mouse, the atRA concentrations in both remain ~200-fold higher than normal. These data indicate that Adh1 contributes to converting massive doses of retinol into toxic amounts of atRA, but do not address physiological function for Adh in atRA biosynthesis.

A recent study of ethanol's effects on *Xenopus* embryogenesis showed that manipulating Adh did not affect expression of atRA-responsive genes, in contrast to manipulating Raldh2 (48). Although these data illustrate lack of participation of Adh in atRA generation, it is not certain how they relate to the complex process of FASD during mammalian embryogenesis. Conversion of retinal into atRA involves at least three Raldh, and Raldh2 has not been shown to metabolize acetaldehyde *in vivo*, or to react to ethanol dosing in mammals.

CONCLUDING REMARKS

Increasing insight into the physiological aspects of retinoid metabolism, along with analytically robust atRA assays, have improved ability to examine the impact of ethanol on atRA concentrations. Generating data that reflect realistically the vitamin A/ethanol interaction entails quantification of atRA in tissues, after chronic ethanol dosing to vertebrates, maintained *via* normal vitamin A nutriture. Understanding mechanism involves evaluating multiple enzymes, binding proteins, transport proteins and receptors. The complexity of retinoid homeostasis and compensatory reactions does not allow extrapolation from ethanol effects on a single gene or protein (especially *in vitro* or in non-vertebrate models) to tissue atRA status.

Abnormal concentrations of atRA could contribute to ethanol's toxicity, including FASD. Modest excesses of atRA can produce teratogenic CNS effects and emerging insight indicates that atRA affects learning and cognitive abilities, in addition to nervous system development and neuron specification. To address this issue, future research must distinguish ethanol's pathology caused by changes in atRA from the pathology of ethanol changing vitamin A homeostasis as a secondary effect. Cause and effect have not been established: the distinction has not been made between retinoid-dependent processes responding to *vs*. contributing to the pathology of ethanol.

Abbreviations

Adh	alcohol dehydrogenase
atRA	all-*trans*-retinoic acid
Crabp	cellular RA binding protein
Crbp	cellular retinol binding-protein
FASD	fetal alcohol spectrum disorder
LRAT	lecithin:retinol acyltransferase
RAR	retinoic acid receptor(s)
RBP	serum retinol binding protein
Rdh	retinol dehydrogenase
REH	retinyl ester hydrolase

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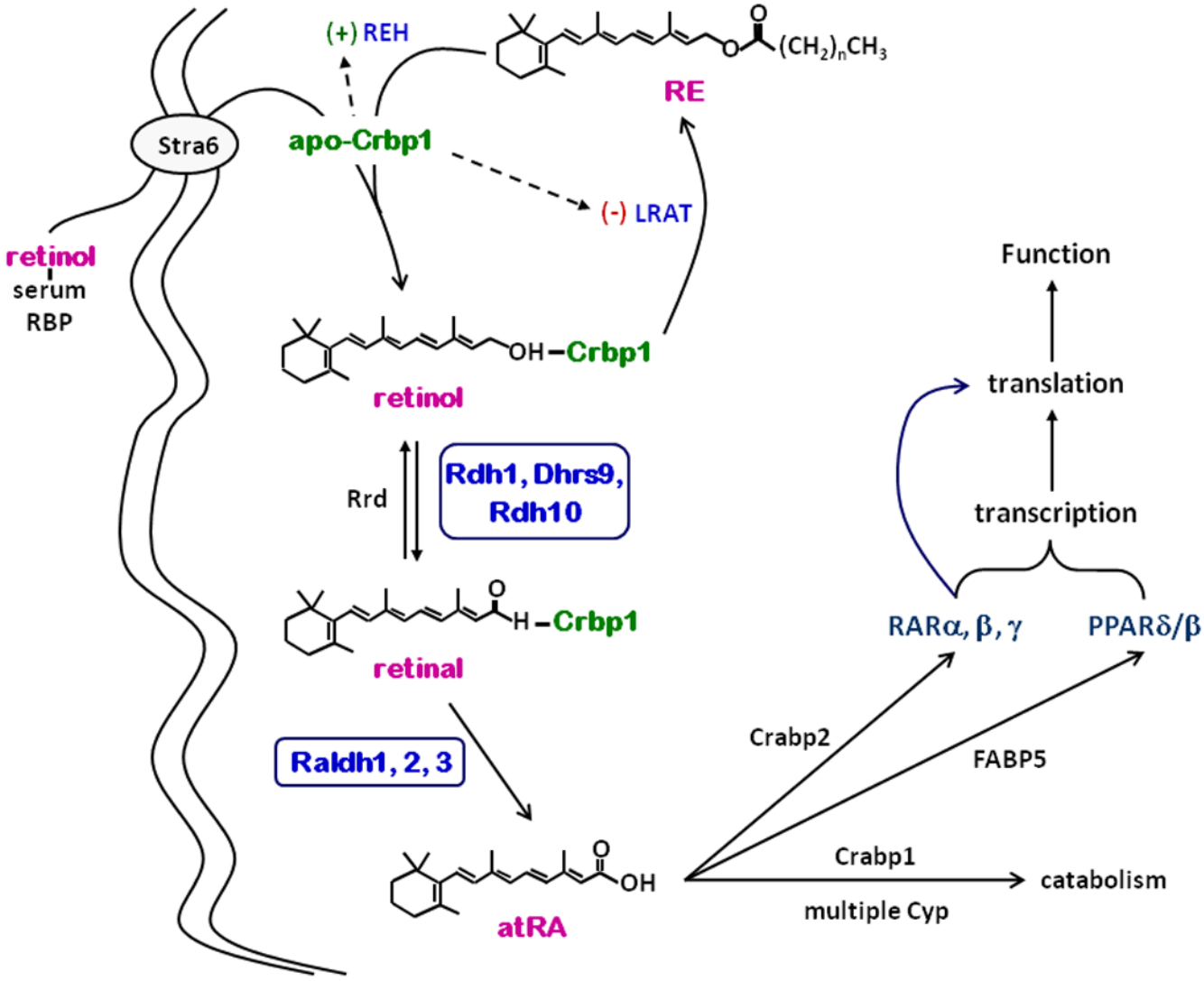

Figure 1.

Retinoid homeostasis. Serum Rbp-transported retinol enters cells *via* Stra6-mediated uptake. Net uptake requires Crbp1 and/or LRAT. Incoming retinol undergoes LRAT-catalyzed esterification into RE. REH mobilizes RE to provide retinol for atRA biosynthesis. Three members of the short-chain dehydrogenase/reductase gene family catalyze the first and rate-limiting step of atRA biosynthesis: Rdh1, Dhrs9 and Rdh10. Retinal reductases, Rrd, reduce retinal into retinol. Three members of the Aldh gene family catalyze the second and irreversible step: Raldh1, 2, and 3 (aka Adlh1A1, 1A2, and 1A3). Three members of the FABP gene family determine intracellular disposition of atRA: Crabp1, Crabp2, and FABP5. atRA regulates translation and transcription *via* four nuclear receptors: RARα, β, and γ and PPARβ/δ. Genes regulated by atRA include *Stra6*, *Crbp1*, *LRAT*, *Raldh*, and *Cyp*.

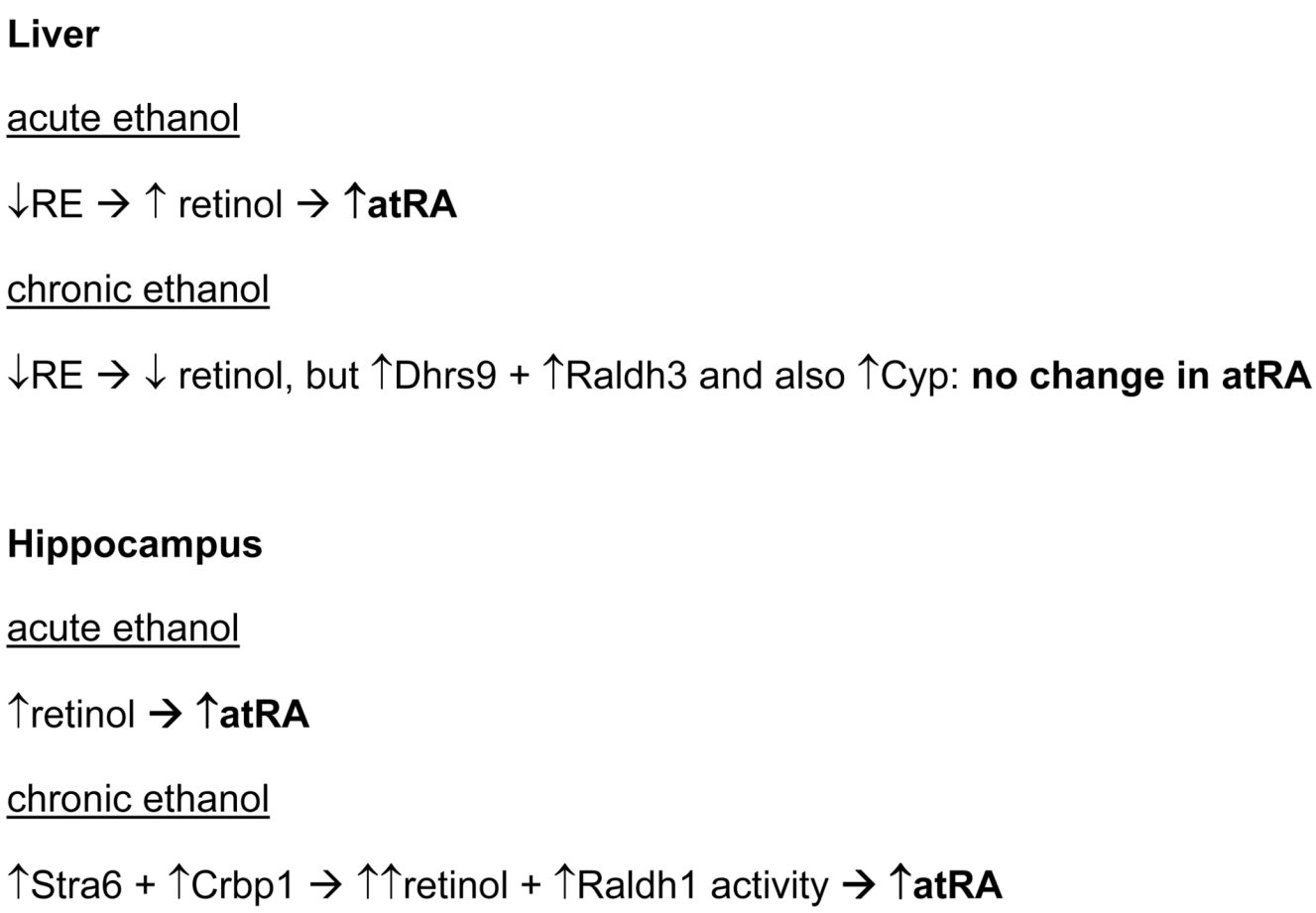

Figure 2.
Impact of acute and chronic ethanol exposure on atRA in liver and hippocampus.

Table 1

atRA concentrations (pmol/g) in mouse tissues after 1-month feeding a liquid diet ± ethanol.

Diet	liver	serum	testis	kidney	Hp	Cx	Ob	Cb	St	Th
control	5.6 ± 0.4	2.2 ± 0.4	8.3 ± 0.4	3 ± 0.3	23 ± 3.6	13. 9 ± 1.5	77 ± 21	55 ± 4	78 ± 33	81 ± 6
ethanol	6.2 ± 0.9	24 ± 2*	14 ± 0.8*	3.3 ± 0.2	511 ± 253*	37.5 ± 10*	79 ± 13	52 ± 7	90 ± 33	60 ± 10

Data are means ± SE. Mice were fed liquid diets. The “ethanol” diet contained 6.5% ethanol. atRA concentrations were quantified by LC/MS/MS.

Abbreviations: Hp, hippocampus; Cx, cortex; Ob, olfactory bulb; Cb, cerebellum; St, striatum; Th, Thalamus.

P values for serum, testis, hippocampus and cortex were <0.0001, <0.0002, ~0.02 and <0.05, respectively.

Table 2

Effects of dam blood alcohol on mouse embryo atRA (pmol/g) concentrations.

	control	L1	L2	L3	L4
BAC%	0	0.01	0.025	0.1	0.13
hippocampus	20 ± 1	30 ± 2	47 ± 3	81± 4	422 ± 8
cortex	13 ± 3	29 ± 3	112 ± 3	210 ± 4	650 ± 11

Data are means ± SE. Five individual dams were exposed to a control liquid diet or a liquid diet with 6.5 % ethanol from e13 through e19.