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Gene Therapy Correction of Aldehyde Dehydrogenase 2 Deficiency

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Aldehyde dehydrogenase 2 (ALDH2) deficiency causes "Asian flush syndrome," presenting as alcohol-induced facial flushing, tachycardia, nausea, and headaches. One of the most common hereditary enzyme deficiencies, it affects 35%–40% of East Asians and 8% of the world population. ALDH2 is the key enzyme in ethanol metabolism; with ethanol challenge, the common ALDH2*2 (E487K) mutation results in accumulation of toxic acetaldehyde. *ALDH2*2* heterozygotes have increased risk for upper digestive tract cancers, compounded by smoking and drinking alcohol. We hypothesized that a one-time administration of an adeno-associated virus (AAV) gene transfer vector expressing the human *ALDH2* coding sequence (AAVrh.10hALDH2) would correct the deficiency state. AAVrh.10hALDH2 was administered intravenously to *Aldh2* knockout (*Aldh2*$^{-/-}$) and *Aldh2* E487K knockin homozygous (*Aldh2*$^{E487K+/+}$) mice. Following acute ethanol ingestion, untreated ALDH2-deficient mice had elevated acetaldehyde levels and performed poorly in behavioral tests. In contrast, treated *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice had lower serum acetaldehyde levels and improved behavior. Thus, *in vivo* AAV-mediated ALDH2 therapy may reverse the deficiency state in *ALDH2*2* individuals, eliminating the Asian flush syndrome and reducing the risk for associated disorders.

INTRODUCTION

Aldehyde dehydrogenase 2 (ALDH2) deficiency is one of the most common hereditary disorders, affecting 560 million people, 8% of the world population.[1] The highest prevalence (35%–45%) is in people of East Asian descent.[2,3] ALDH2 belongs to a superfamily of enzymes that play key roles in the metabolism of endogenous and exogenous aldehydes.[4] The enzyme is targeted to mitochondria; while ubiquitously expressed in all tissues at low levels, ALDH2 expression is most abundant in the liver, the primary organ of ethanol metabolism.[5,6] ALDH2 is the second enzyme in the ethanol metabolism pathway and functions to convert the toxic intermediate acetaldehyde to nontoxic acetate (Figure S1).[7] Mutations in ALDH2 that reduce the oxidizing ability of the enzyme result in accumulation of serum acetaldehyde.[8,9] The ALDH2 enzyme is a tetramer, and the mutant protein functions as a dominant-negative.[10,11] Heterozygotes have < 50% ALDH2 enzymatic activity and homozygotes < 4%.[12,13]

Mutations in ALDH2 are responsible for the Asian flush syndrome, characterized by facial flushing, headache, nausea, dizziness, and cardiac palpitations after consumption of alcoholic beverages.[3,14] The syndrome is caused by elevated blood acetaldehyde levels resulting from reduced ALDH2 enzymatic activity of the mutant protein.[9] The most common mutation is a glutamic acid-to-lysine substitution at position 487 (E487K), designated the *ALDH2*2* allele.[3,15] In addition to the acute Asian flush syndrome, the *ALDH2*2* variant allele is associated with a variety of neurologic, endocrine, cardiovascular, and dermatologic disorders, aberrant drug metabolism, and importantly, a marked increase in the risk of upper aerodigestive tract cancer of the oral cavity, pharynx, larynx, and esophagus.[2,3] Cigarette smoke also contains acetaldehyde, and the combination of cigarette smoking and alcohol consumption by individuals carrying the *ALDH2*2* allele represents a very high cancer risk (odds ratio 50:1) with a 25-year earlier onset of esophageal carcinoma.[1,16,17]

ALDH2 deficiency has been modeled in mice, and the molecular and clinical phenotypes closely mimic the human disorder.[12,18] The *Aldh2* knockout mouse (*Aldh2*$^{-/-}$) expresses no detectable ALDH2 protein or enzymatic activity.[12] After ethanol administration, *Aldh2*$^{-/-}$ mice have significantly higher levels of blood acetaldehyde than wild-type mice, and exposure to 2 g/kg ethanol for 8 days results in weight loss and increased mortality.[19,20] The *Aldh2* E487K knockin mouse (*Aldh2*$^{E487K+/+}$) has the lysine 487 mutation from the human *ALDH2*2* allele.[18] These mice accumulate high acetaldehyde levels in the blood when challenged with ethanol, have reduced enzymatic ALDH2 activity, and exhibit increased ethanol-related behavioral abnormalities.[9,18]

We hypothesized that genetic modification of the ALDH2-deficient liver to express the normal human *ALDH2* coding sequence would increase the oxidizing ability of the ALDH2 tetrameric enzyme toward wild-type levels, resulting in decreased acetaldehyde accumulation

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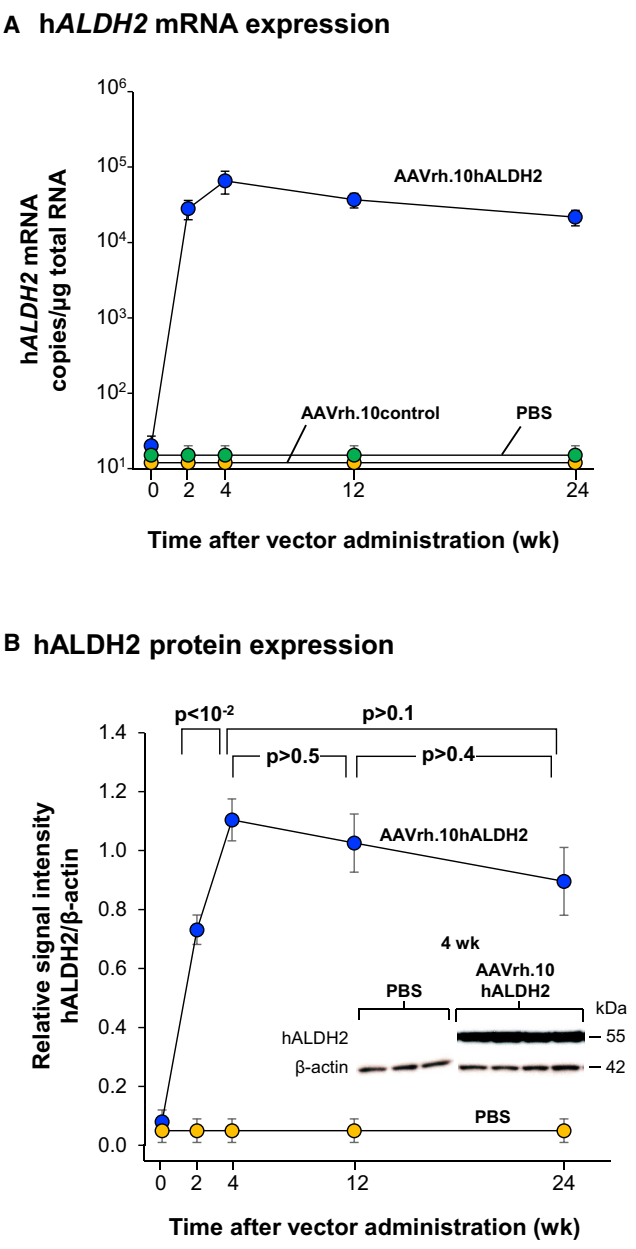

Figure 1. Long-Term *In Vivo* Expression of Human ALDH2 following a Single Intravenous Administration of AAVrh.10hALDH2

AAVrh.10hALDH2 (10^{11} gc), AAVrh.10control (10^{11} gc), or PBS was administered intravenously to C57BL/6 mice (n = 4/group). (A) Liver mRNA expression. (B) Liver protein expression as assessed by western analysis using an anti-HA tag antibody. β-actin was used as a loading control. Quantification was assessed on the protein bands from the western analysis. Example of western analysis of 4 week samples; each lane represents a different animal. Data are presented as means ± SEM.

and a reduction in behavioral symptoms associated with ethanol-induced acetaldehyde toxicity. The data demonstrate that both knockout and knockin ALDH2-deficient mice that were administered a single intravenous dose of a serotype rh.10 adeno-associated virus (AAV) coding for the wild-type human *ALDH2* gene showed enhanced liver ALDH2 enzymatic activity and, when challenged with ethanol, demonstrated reduced serum acetaldehyde accumulation and a marked reduction of behavioral abnormalities.

RESULTS

AAVrh.10hALDH2 Function

Because mouse and human ALDH2 protein sequences are 96% homologous (NCBI HomoloGene: https://www.ncbi.nlm.nih.gov/homologene/55480), the AAVrh.10 vector encoding the cDNA of human *ALDH2* included a hemagglutinin (HA) tag to facilitate detection (Figure S2). For the initial assessment of the *in vivo* expression of hALDH2 from AAVrh.10hALDH2, wild-type C57BL/6 mice (n = 4) were administered a single dose (10^{11} genome copies [gc]) intravenously of AAVrh.10hALDH2, AAVrh.10control (an identical construct to AAVrh.10hALDH2 but with an irrelevant transgene), or PBS. Livers were harvested at 2, 4, 12, and 24 weeks post-administration and analyzed for hALDH2 mRNA and protein expression. Sustained high levels of h*ALDH2* mRNA of at least $2.2 \times 10^4 \pm 5.0 \times 10^3$ gc/µg total RNA were detected in the liver of AAVrh.10hALDH2-treated mice (Figures 1A and S3). No h*ALDH2* mRNA was detected in PBS or AAVrh.10control-treated mice. Levels of hALDH2 protein detected in the livers mirrored the mRNA levels, with vector-derived hALDH2 detected in the liver of mice treated with AAVrh.10hALDH2 at 2, 4, 12, and 24 weeks post-administration but not in PBS-treated mice (Figure 1B). Long-term expression of hALDH2 has also been observed in $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice out to 16 weeks post-administration.[21]

AAVrh.10hALDH2-Mediated Expression in ALDH2-Deficient Mice

To assess the ability of AAVrh.10hALDH2 to correct ALDH2 deficiency, $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice were treated with AAVrh.10hALDH2 or AAVrh.10control (10^{11} gc) by intravenous administration. Four weeks later, hALDH2 mRNA, protein, and enzymatic activity levels were analyzed in the liver. Liver h*ALDH2* mRNA expression of AAVrh.10hALDH2-treated $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice was significantly higher than in mice administered AAVrh.10control (Figure 2A, $Aldh2^{-/-}$, $p < 10^{-4}$; $Aldh2^{E487K+/+}$, $p < 10^{-4}$, Figure S4A). Protein expression assessed by western analysis demonstrated hALDH2 protein only in AAVrh.10hALDH2-treated $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice and not in AAVrh.10control-treated mice (Figure 2B, $Aldh2^{-/-}$, $p < 0.03$; $Aldh2^{E487K+/+}$, $p < 0.05$, Figure S4B). Immunohistochemical staining in AAVrh.10hALDH2-treated $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice mirrored the western analysis. hALDH2-positive cells were found mainly around hepatic and portal veins in AAVrh.10hALDH2-treated mice, while no positive cells were observed in AAVrh.10control-treated mice (Figure 2C). Additionally, the $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice treated with AAVrh.10-hALDH2 demonstrated increased liver ALDH2 enzymatic activity compared to untreated mice ($Aldh2^{-/-}$, $p < 10^{-12}$; $Aldh2^{E487K+/+}$, $p < 0.0004$), with levels similar to wild-type C57BL/6 mice ($p > 0.9$; Figures 2D and S4C). Western analysis using an anti-ALDH2 antibody that recognizes both mouse and human ALDH2 showed that levels of

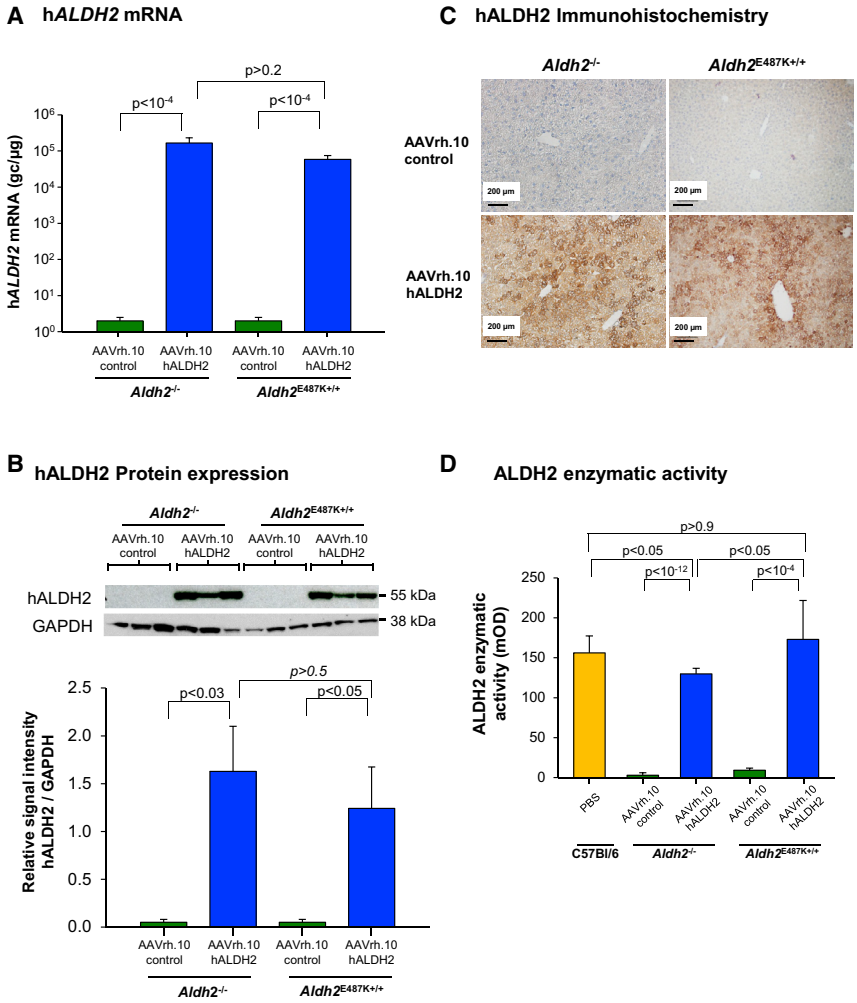

Figure 2. Treatment of *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ Mice with AAVrh.10hALDH2

A single intravenous administration of AAVrh.10hALDH2 or AAVrh.10control (10^{11} gc) was given to *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice (n = 10 mice/group). The liver was assessed after 4 weeks. (A) h*ALDH2* mRNA levels. (B) hALDH2 protein. Top, representative western analysis, n = 3/condition; anti-HA tag. GAPDH was used as a loading control. Bottom, quantification of protein bands from the western analysis was performed with three random samples from each group. (C) Representative immunohistochemical staining of hALDH2 in liver detected by anti-HA antibody. Scale bar is 200 μM. (D) ALDH2 enzymatic activity in liver homogenate. All values are presented as means ± SEM.

vector-derived hALDH2-HA was much higher than endogenous mALDH2 in *Aldh2*$^{E487K+/+}$ mice (Figure S5).

Acute Response to Ethanol Challenge

Aldh2$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice both developed elevated levels of blood acetaldehyde and behavioral abnormalities in response to acute ethanol challenge. To assess whether AAVrh.10hALDH2-mediated therapy could alleviate the effects of ethanol challenge in ALDH2 deficiency, both mouse models were administered a single dose of ethanol (4 g/kg) or water by intragastric gavage 4 weeks after administration of AAVrh.10hALDH2 or AAVrh.10control. Serum acetaldehyde levels of AAVrh.10hALDH2-treated *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice (n = 5) at 6 hr post-ethanol-gavage were significantly lower than that of AAVrh.10control mice (Figure 3, *Aldh2*$^{-/-}$, $p < 0.03$; *Aldh2*$^{E487K+/+}$, $p < 0.02$, Figure S6). High variability in serum acetaldehyde levels was observed in AAVrh.10control-treated ALDH2-deficient mice; however, this is consistent with studies in human *ALDH2*2* individuals after controlled ethanol ingestion.[14] All mice gavaged with water had low levels of blood acetaldehyde at the same time point.

Behavioral assessments were performed 4 weeks after AAVrh.10hALDH2 or AAVrh.10control administration to *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice (n = 10) before and 0.5, 2, 6, 10, and 24 hr post-ethanol administration by intragastric gavage (Figure S7; Tables S1–S8). At 6 hr post-ethanol-gavage, AAVrh.10hALDH2 treated *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice showed fewer behavioral abnormalities using an observational behavior score (Figures 4A and S7A) and had higher body temperature closer to normal (Figures 4B and S7B). Acute acetaldehyde toxicity results in sedation, hypoactivity, and lethargy.[2,22] AAVrh.10hALDH2-treated mice performed significantly better than mice administered the control vector in tests measuring ambulatory activity such as distance traveled on a balance beam (Figures 4C and S7C) and distance traveled in an open-field chamber (Figures 4D and S7D). Additionally, increased rearing or vertical activity was observed in AAVrh.10hALDH2 vector-treated mice, although their recovery was significantly slower compared with C57BL/6 (Figures 4E and S7E). Mice were also evaluated in tests of motor coordination and strength. AAVrh.10hALDH2-treated *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice took less time to cross a skinny beam by 46% and 72%, respectively (Figures 4F and S7F; Video S1). The time to failure for balance on a skinny rod increased by 2.8 and 5.8 s for AAVrh.10hALDH2-treated *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ treated mice, respectively, as compared to control vector-treated mice (Figures 4G and S7G; Video S2). In the screen-climb test, the time to goal was reduced by 73% for *Aldh2*$^{-/-}$ and 78% for *Aldh2*$^{E487K+/+}$ mice treated with AAVrh.10-hALDH2 vector compared with mice administered AAVrh.10control (Figures 4H and S7H; Video S3). The functional composite behavior score represents the cumulative assessment of behavior described in Figures 4C–4H; a higher score is associated with poorer performance (Table S1). Overall, AAVrh.10hALDH2-treated mice had significantly lower scores than mice treated with control vector (Figures 4I and S7I). The levels of serum acetaldehyde correlated well with

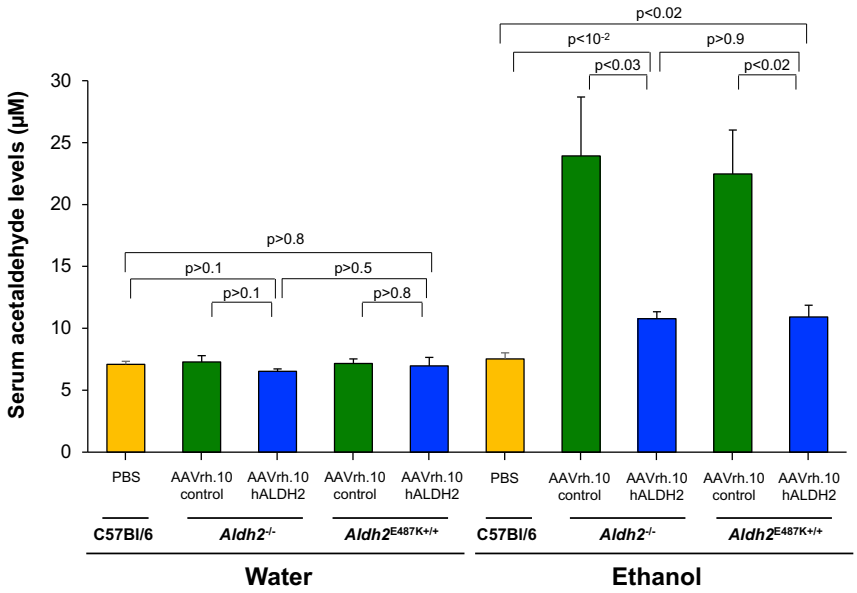

Figure 3. Serum Acetaldehyde Level of *Aldh2*$^{-/-}$, *Aldh2*$^{E487K+/+}$, and C57BL/6 Mice (n = 5/Group) 6 hr after Ethanol Exposure (4 g/kg Body Weight)

Aldh2$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice were intravenously administered AAVrh.10hALDH2 (10^{11} gc) or AAVrh.10-control (10^{11} gc). C57BL/6 mice were intravenously administered PBS. Values are presented as means ± SEM.

the observational behavior score (Figure 5A) and body temperature (Figure 5B), despite individual variability in serum acetaldehyde in the AAVrh.10control-treated groups. There was no significant difference between the treatment of *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice (Table S6). Together, the results from these assessments suggest that treatment with AAVrh.10hALDH2 can alleviate both biochemical and behavioral symptoms of acute ethanol stress in ALDH2 deficiency.

DISCUSSION

ALDH2 deficiency is one of the most common inherited enzyme deficiencies worldwide.[1,2] ALDH2 is a key enzyme for ethanol metabolism, and mutations that reduce the oxidizing ability of the enzyme result in an accumulation of toxic acetaldehyde.[2,9] The most common genetic variant, referred to as the *ALDH2*2* allele, is caused by a glutamic acid-to-lysine substitution at position 487 (E487K).[15] The E487K mutation results in greatly reduced enzymatic activity and ability to process acetaldehyde in heterozygote and homozygote individuals.[12,13] Mutations of ALDH2 and the resulting accumulation of acetaldehyde in the blood with alcohol ingestion is the cause of the Asian flush syndrome. ALDH2 deficiency has also been linked to multiple chronic diseases, including aerodigestive tract cancers, diabetes, and cardiovascular and neurodegenerative diseases.[1–3,14,23,24]

To alleviate the effects of acetaldehyde accumulation with ethanol consumption caused by ALDH2 deficiency, we have developed an AAV-mediated gene therapy strategy for a one-time administration of an AAV serotype rh.10 gene transfer vector expressing the coding sequence of the normal human *ALDH2*. The AAVrh.10 serotype was used because it predominantly targets the liver, the primary site of ethanol and acetaldehyde metabolism,[6,25,26] and has a strong safety profile.[27] We utilized the ubiquitous CAG promoter to drive high levels of transgene expression thought to be necessary for overcoming the ALDH2^{E487K} dominant-negative mutant protein. This gene therapy strategy provided near-complete correction of the ALDH2 deficiency state in two murine models of ALDH2 deficiency that mimic the Asian flush syndrome following ethanol consumption, including low ALDH2 enzymatic activity, elevated serum acetaldehyde levels, lower core body temperature, and abnormalities in behavior. The data demonstrate that delivery of AAVrh.10hALDH2 to the liver reconstitutes wild-type liver enzymatic activity levels, normalizes acetaldehyde levels, and regulates behavior after acute ethanol consumption to levels near wild-type, though further augmentation of ALDH2 expression or number of hepatocytes transduced may be required to fully correct the deficiency phenotype. The protection against the effects of alcohol coupled with the long-term gene expression study (up to 24 weeks; the last time-point evaluated) suggests that persistent protection can be achieved by a single dose.

Implications for Human Therapy

Other than the use of nutritional supplements,[28] no therapies are available to treat ALDH2 deficiency. While scaling from mice to humans has many challenges, the data in the present study supports the concept that AAV-mediated gene therapy represents a possible effective therapy for the ALDH2 deficiency state. However, the possibility of a therapy for ALDH2 deficiency raises the obvious question: given the deleterious effects of alcohol consumption, is it rational to develop a therapy that would obviate the Asian flush symptoms associated with ALDH2 deficiency, potentially promoting more alcohol consumption?

Consistent with this concern, a variety of approaches have been used to mimic the ALDH2-deficiency state as a means of mitigating alcohol use. Disulfiram (Antabuse), a FDA-approved drug, inhibits ALDH2, leading to acetaldehyde accumulation, and clinically induces facial flushing, nausea, and vertigo.[29,30] A traditional Chinese medicine, daidzin, an active isoflavane identified in the roots and flowers of kudzu (a Chinese herb), inhibits mitochondrial ALDH2 activity.[31] A pilot study involving 10 heavy drinkers suggested that use of declinol, a complex containing kudzu, gentian, tangerine peel, and bupleurum, showed statistically significant decrease in the alcohol use disorders identification test

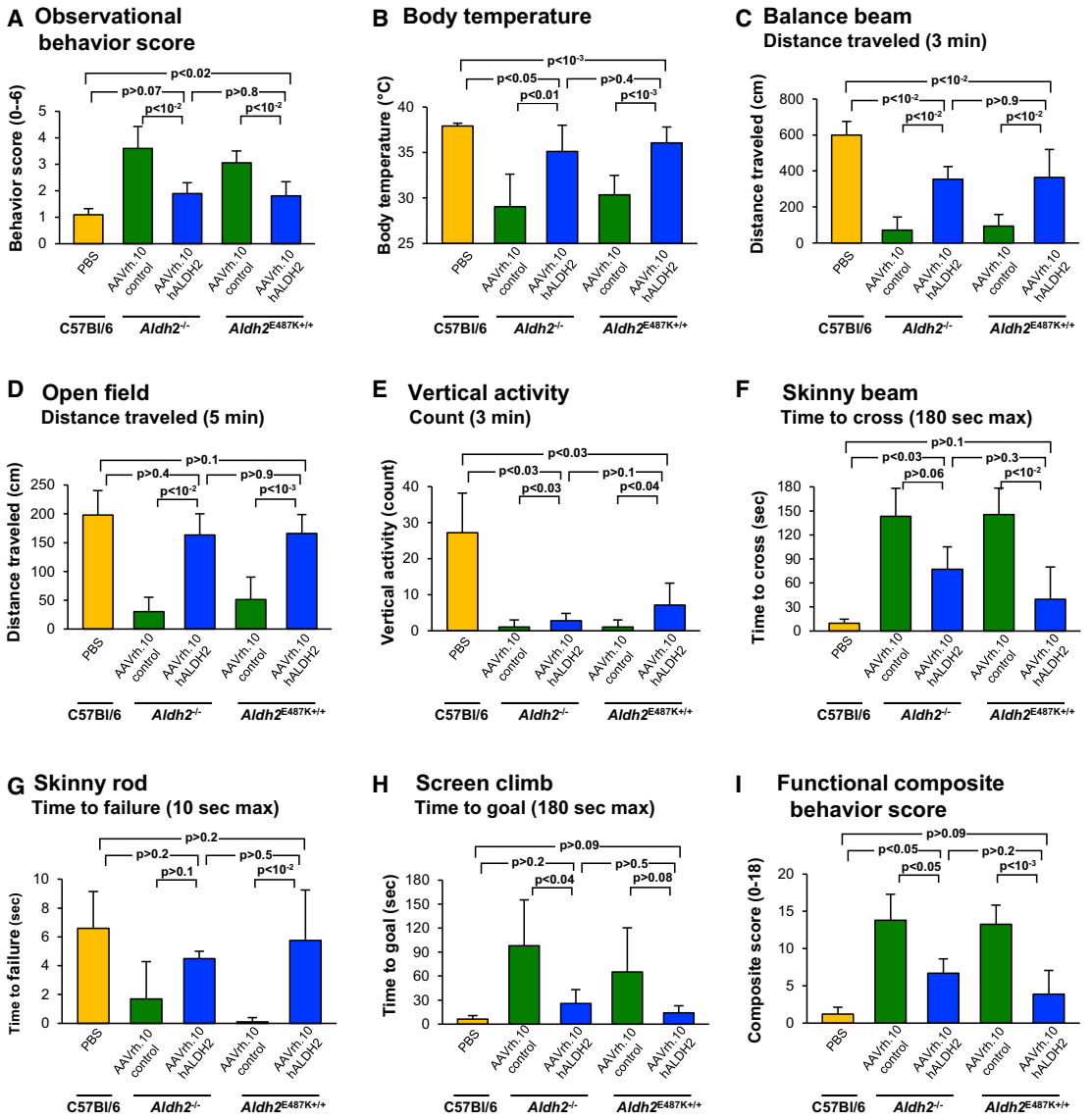

Figure 4. Behavior Tests of *Aldh2*$^{-/-}$, *Aldh2*$^{E487K+/+}$, and C57BL/6 Mice (n = 10/Group) 6 hr after Ethanol Exposure

Aldh2$^{-/-}$ and *Aldh2*$^{E487K+/+}$ were intravenously administered AAVrh.10hALDH2 (10^{11} gc) or AAVrh.10control (10^{11} gc). C57BL/6 mice were intravenously administered PBS. Mice were challenged with ethanol in water (4 g/kg body weight) by intragastric gavage 4 weeks after vector administration. (A) Observational behavior score (0–6): 0, walking around normally; 1, rearing; 2, sedation; 3, mild ataxia (dysfunction of hind limbs); 4, severe ataxia (inability to move); 5, loss of righting reflex; and 6, death.[9] (B) Body temperature. (C) Balance beam; distance traveled in 3 min. (D) Open-field chamber; total distance traveled in 5 min. (E) Vertical activity; count in 3 min. (F) Skinny beam; time to cross the beam (maximum 180 s). (G) Skinny rod; time to failure (maximum 10 s). (H) Screen climb; time to goal (maximum 180 s). (I) Functional composite behavior score. Behavior at each time point in tests shown in (C)–(H) was scored (0–3) using the indicated parameter data; see Table S1 for scoring parameters of the behavioral tests. The composite score is the sum of parameter scores for each behavior test. All values are presented as means ± SEM. See Figure S3 for all data and all time points and Tables S3–S6 for all statistical analyses.

(AUDIT) scores.[32] CVT-10216, a highly selective, reversible ALDH2 inhibitor, increased acetaldehyde and reduced alcohol preference in moderate and high alcohol-drinking rat models.[33] Finally, genetic approaches have been assessed to silence ALDH2 expression, including phosphorothioate nucleotides, adenovirus-delivered antisense mRNA, or ribozyme and short hairpin RNA (shRNA) strategies.[34–37] In contrast, Alda-1, a small molecule activator of ALDH2, enhances the catalytic activity of ALDH2, protects ALDH2 enzymatic activity from inactivation, and restores the enzymatic activity of the E487K mutant ALDH2.[2] In a clinical study, 4-methylpyrazole (fomepizole), an alcohol dehydrogenase inhibitor, decreased blood and salivary acetaldehyde levels and the flushing reaction from ethanol consumption in *ALDH2*2* individuals.[38]

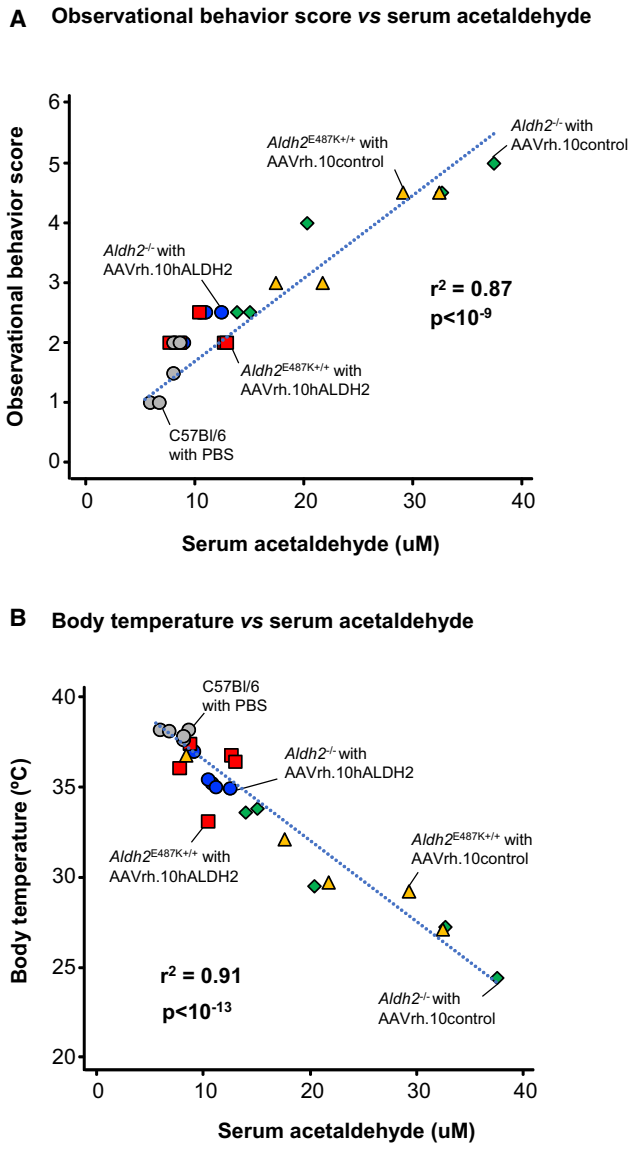

Figure 5. Correlation of Observational Behavior Score and Body Temperature with Serum Acetaldehyde Levels

Serum acetaldehyde levels, observational behavior score, and body temperature of *Aldh2*$^{-/-}$, *Aldh2*$^{E487K+/+}$, and C57BL/6 mice (n = 5/group) were measured 6 hr after intragastric ethanol exposure (4 g/kg body weight) and 4 weeks after intravenous administration of AAVrh.10hALDH2 (10^{11} gc) or AAVrh.10control (10^{11} gc). C57BL/6 mice were intravenously administered PBS. (A) Observational behavior score (from Figure 4A) versus serum acetaldehyde levels (from Figure 3) of individual mice. (B) Body temperature (from Figure 4B) versus serum acetaldehyde levels (from Figure 3) of individual mice.

While ALDH2-deficiency-mimicking alcohol aversion therapies are an interesting approach to reducing alcohol consumption, there is extensive evidence that the ALDH2-deficiency state has significant risks, sufficient to seriously consider the concept that correction of the deficiency state would have benefits that would outweigh the risks of enabling alcohol consumption in affected individuals that would no longer have to deal with the unpleasant Asian flush syndrome. Indeed, numerous studies have demonstrated that enhancing ALDH2 activity, either with overexpression or small molecule activators, can ameliorate the deleterious effects of exogenous and endogenous aldehydes and provide protection against both acute and chronic disorders stemming from acetaldehyde toxicity and oxidative stress (reviewed in Chen et al.[2]).

First, while early studies demonstrated a reduction of alcohol consumption and abuse with ALDH2 deficiency compared to those with normal *ALDH2* alleles,[39] social, cultural, and economic factors in the past few decades have led to an increase in alcohol dependence and abuse among the populations of *ALDH2*2* carriers.[40] There has been a significant rise in the proportion of heavy drinkers who are carriers of the *ALDH2*1/*2* genotype in East Asian countries.[2,40] For example, in Japan, 26% of heavy drinkers are *ALDH2*2* heterozygotes.[41] Importantly, in simulation tests, *ALDH2*2* heterozygotes have significantly impaired driving ability after moderate alcohol consumption.[42] It is anticipated that, without intervention, the health risk and healthcare burden caused by *ALDH2*2* heavy alcohol drinkers will become much more severe in the next few decades.[43]

Second, in addition to obviating the relationship of ALDH2 deficiency to acute alcohol effects, there is extensive data demonstrating a central role of ALDH2 deficiency in many pathologies. The toxic acetaldehyde that accumulates in ALDH2 deficiency with alcohol consumption is categorized as a group I human carcinogen.[44] There is epidemiologic evidence that ALDH2 deficiency is linked to psychiatric and personality disorders, drug addiction, cognitive disorders, Parkinson's and Alzheimer's diseases, peripheral neuropathy, a higher sensitivity to pain, risk for diabetes, osteoporosis, cardiac ischemia, stroke, nonalcoholic fatty liver, reactive airway disease, severity of Fanconi anemia, dermatitis, radiation-induced dermatitis, hepatocellular carcinoma, and impaired metabolism of some drugs, including nitroglycerin, acyclovir, 5-nitrofuran, and acetaminophen.[2,3] In addition, *ALDH2*2* heterozygotes have a well-documented 7- to 12-fold increased risk of cancer of the oral cavity, pharynx, larynx, and esophagus.[23] ALDH2 not only metabolizes acetaldehyde as part of the ethanol metabolism pathway, but also metabolizes numerous other aldehydes, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), products of lipid peroxidation from oxidative stress, and acrolein, an environmental aldehyde found in car exhaust, tobacco smoke, and other pollutants.[2,45] The toxicity of these aldehydes stems from their ability to diffuse through cell membranes and form adducts with DNA, proteins, and lipids that disrupt their function,[46–48] and a rapid clearance mechanism is necessary to prevent cell and tissue damage.

Our data shows AAVrh.10hALDH2 gene therapy provides near wild-type levels of functional ALDH2 enzyme and alleviates the symptoms caused by acute alcohol administration. Long-term expression of the *ALDH2* gene after vector administration for at least 6 months suggests that gene therapy treatment with

AAVrh.10hALDH2 for individuals with the *ALDH2*2* genotype may provide long-term protection against some of the alcohol-related chronic effects of elevated acetaldehyde levels such as esophageal cancer from ALDH2 deficiency. While the persistent correction of ALDH2 deficiency does have the theoretical risk that alleviation of the Asian flush syndrome could encourage alcohol consumption, the overall burden of ALDH2 deficiency on human health, particularly the marked increase risk for cancer, supports the concept of developing gene therapy for ALDH2 deficiency.

MATERIALS AND METHODS

Study Design

Research Objective

The objective of this study was to determine if a single administration of AAVrh.10hALDH2 corrects the biochemical and behavioral abnormalities associated with ALDH2 deficiency upon acute administration of ethanol in the $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mouse models.

Research Subjects or Units of Investigation

$Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice were used as models of ALDH2 deficiency. C57BL/6 mice were used as wild-type controls; C57BL/6 is the background for both $Aldh2^{-/-}$ and $Aldh2^{E487K+/+}$ mice.

Experimental Design

The study used $Aldh2^{-/-}$, $Aldh2^{E487K+/+}$, and C57BL/6 mice treated with AAVrh.10hALDH2 or AAVrh.10control gene therapy vectors, or PBS. Experimental vector- or control-treated animals were then challenged with ethanol (4 g/kg by intragastric gavage) or water. Mice were evaluated by a panel of behavioral tests including observational score, body temperature, distance traveled on balance beam, distance traveled in open-field chamber, vertical activity, time to cross skinny beam, time to failure on skinny rod, and time to goal on screen climb. After sacrifice, liver h*ALDH2* mRNA was evaluated by TaqMan qRT-PCR and hALDH2 protein by western analysis and immunohistochemistry, ALDH2 enzymatic activity was quantified using a commercially available assay kit, and serum acetaldehyde levels were measured by liquid chromatography/mass spectrometry after derivatization.

Randomization

Mice were randomly assigned to treatment versus control groups.

Blinding

Scoring and assessments were performed in a blinded manner by investigators.

Sample Size

Sample sizes were chosen to provide statistical power for the data with allowing for an outlier result while giving a reasonable chance that substantial effects can be seen. For long-term expression studies, n = 4 mice were selected because this number allows the possibility of seeing a 3-fold difference in parameters with an alpha of 0.05 and a power of 0.9 if the variance in the parameter is 25%. For behavioral analysis in the acute ethanol challenge, n = 10 mice were selected because this number allows the possibility of seeing a 2-fold difference in parameters with an alpha of 0.05 and a power of 0.9 if the variance in the parameter is 30%. Each of these mice was also used for quantitation of mRNA and protein in liver and ALDH2 enzymatic activity. For the serum acetaldehyde analysis, n = 5 mice were used because this number allows for the possibility of seeing a 2-fold difference in parameters with an alpha of 0.05 and a power of 0.9 if the variance in the parameter is 20%.

Rules for Stopping Data Collection and Selection of Endpoints

Acute ethanol challenge studies were performed 4 weeks after vector administration as determined by the peak expression for the AAVrh.10hALDH2 vector by 2 weeks.

Data Inclusion/Exclusion Criteria and Outliers

Data from all mice enrolled in the ethanol challenge studies are reported.

Replicates

The long-term expression and acute ethanol challenge studies were each performed one time with n = 4 and n = 10 mice per group, respectively. TaqMan assay for mRNA and ALDH2 enzymatic assays were run in duplicate for each mouse in the study. Western analyses were run once for each animal in the study, and serum acetaldehyde measurements were run on n = 5 randomly selected mice from the study.

Mouse Models of ALDH2 Deficiency

The *Aldh2* knockout homozygous ($Aldh2^{-/-}$) mice, backcrossed with C57BL/6 mice, were obtained from the Department of Environmental Health, University of Occupational and Environmental Health (Kitakyushu, Japan).[12] The $Aldh2^{E487K}$ knockin homozygous mice ($Aldh2^{E487K+/+}$), a humanized model of ALDH2 deficiency, were obtained from the Department of Chemical and Systems Biology, Stanford University School of Medicine (Stanford, CA, USA).[18] All mice were housed in microisolator cages, and all food and water were autoclaved. Mice were bred as pairs (one female with one male) or trios (two females with one male). C57BL/6 mice, $Aldh2^{-/-}$, and $Aldh2^{E487K+/+}$ were not littermates, but all mice used in the studies were age matched. In order to generate homozygous mice, mice were bred as heterozygous pairs, with genotyping of pups at 3 weeks of age by PCR reaction (Transnetyx, Cordova, TN, USA). The primers for $Aldh2^{-/-}$ genotyping were as follows: forward, 5′-GGACGTAGACAAGGCAGTGAAG-3′; reverse, 5′-CCCTACCC GGTAGAATTCGATATCA-3′. The primers for $Aldh2^{E487K+/+}$ genotyping were as follows: forward, 5′-GGAGCTGGGCGAGTATGG-3′; reverse, 5′-GAGTCTGAAGGCTGTGTACGTA-3′.[12,18] All experiments conformed to the relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Because alcohol consumption in the East Asian population is dominated by males,[49] we utilized male mice for all studies.

AAV Vectors

The AAVrh.10hALDH2 vector is comprised of the nonhuman primate-derived rh.10 capsid and an expression cassette including the 5′ and 3′ AAV2 inverted terminal repeats, the cytomegalovirus (CMV) enhancer, chicken-β-actin promoter and intron, rabbit β-globin splice acceptor (CAG promoter), the human *ALDH2* coding sequence with a HA tag, and rabbit β-globin polyadenylation signal (Figure S2). The AAVrh.10 serotype predominantly transduces the liver, but transgene expression is also detected in other organs.[25,26] The vector was produced using 293T cells as described previously.[50] In brief, the pAAV-CAG-hALDH2 expression plasmid (600 μg) and the AAVrh.10 packaging-Ad helper hybrid plasmid pPAK-MArh.10 (1,200 μg) were co-transfected into 293T cells using PEI transfection reagent (Polysciences, Warrington, PA, USA). At 72 hr post-transfection, cells were harvested and lysate prepared using five cycles of freeze-thaw. The cell lysate containing the virus was clarified by centrifugation at 3,500 rpm for 15 min. The AAVrh.10hALDH2 vector was purified from the crude viral lysate by iodixanol gradient and QHP anion exchange chromatography (GE Healthcare, Piscataway, NJ, USA), concentrated using a BioMax 100K membrane concentrator (Millipore, Billerica, MA, USA) and stored in PBS (pH 7.4) at −80°C.

Vector genome titers were determined by TaqMan qPCR using a CAG specific primer-probe set (forward primer, 5′-GTCAAT GGGTGGAGTATTTACGG-3′; reverse primer, 5′-AGGTCATG TACTGGGCATAATGC-3′) (Applied Biosystems, Foster City, CA, USA). The purified AAVrh.10hALDH2 vector was digested with proteinase K in the presence of 0.5% SDS plus 25 mM EDTA at 70°C for 1 hr followed by inactivation of the protease at 95°C for 15 min. The vector was then used as a template for TaqMan analysis using a pAAV-CAG-hALDH2 plasmid DNA standard of known copy number to generate a standard curve. The AAVrh.10control vector expresses an irrelevant transgene; for the long-term expression study, the control transgene was human alpha 1-antitrypsin,[50] and for the efficacy studies was anti-anthrax protective antigen antibody.[51]

Long-Term Expression

To demonstrate that the AAVrh.10hALDH2 vector expressed hALDH2 on a persistent basis, male C57/Bl6 mice (n = 4), age 6 to 8 weeks, were injected intravenously (tail vein) in 100 μL with a one-time dose of AAVrh.10hALDH2 (10^{11} gc), AAVrh.10control (10^{11} gc), or PBS. This is equivalent to 4 to 5 × 10^{12} gc/kg for a 20- to 25-g mouse. Mice were euthanized at 0, 2, 4, 12, and 24 weeks post-vector-administration using CO_2 inhalation followed by cardiac perfusion with 40 mL cold PBS (pH 7.4). Livers were collected and immediately frozen on dry ice. To quantify h*ALDH2* mRNA levels, frozen livers were homogenized in 750 μL lysis buffer (10 mM HEPES-KOH [pH 7.4], 5 mM mannitol, and 1% Triton X-100 in water) using a pestle mixer (VWR International, Radnor, PA, USA). The homogenate was centrifuged at 10,000 × *g* for 5 min, and supernatant was collected and stored in aliquots at −80°C until use. mRNA was isolated from 200 μL homogenate aliquots of liver (RNeasy, QIAGEN, Valencia, CA, USA) and stored at −80°C until use. cDNA was prepared from 1 μg mRNA by reverse transcription (Applied Biosystems, Waltham, MA, USA). The reverse transcription reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 500 μM deoxynucleoside triphosphate (dNTP), 2.5 μM random hexamer, 10 U RNase inhibitor, and 32 U reverse transcriptase in a final volume of 25 μL. The PCR conditions were as follows: 25°C for 10 min; 42°C for 60 min, and 95°C for 5 min. The h*ALDH2* mRNA and murine 18S RNA levels were assessed by TaqMan qPCR using FAM-dye-labeled h*ALDH2* specific primer-probe sets (Assay ID: Hs01007998_ml) and murine 18S specific primer-probe sets (Life Technologies, Waltham, MA, USA). The standard curve was generated with the pAAV-CAG-hALDH2 plasmid DNA as the standard. The total amount of transgene mRNA was normalized to the total amount of RNA. To assess hALDH2 protein levels in liver, 20 μg of protein from stored liver homogenate from three randomly selected samples per group was loaded on a 4%–12% Bis-Tris mini-SDS gel (Novex) and transferred to a polyvinylidene difluoride membrane. Expression of hALDH2 was evaluated by incubation of the membrane with mouse monoclonal anti-HA (Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-β-actin (C4; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5,000), and peroxidase-conjugated goat-anti-mouse (Santa Cruz) antibody. For quantification of protein expression, four animals were analyzed from each group at each time point (week 2, 4, 12, and 24) and PBS. All images were analyzed following the same protocol. The acquired western analysis images quantified with ImageJ.

Efficacy Studies of Acute Alcohol Administration to ALDH2-Deficient Mice

Male *Aldh2*$^{-/-}$ and *Aldh2*$^{E487K+/+}$ mice (n = 10), age 6 to 10 weeks old, were injected intravenously (tail vein) in 100 μL with a one-time dose of AAVrh.10hALDH2 (10^{11} gc) or AAVrh.10control (10^{11} gc). C57BL/6 mice were administered PBS. Four weeks later, a single dose of ethanol (4 g/kg) was administered by intragastric gavage using a reusable, straight, 20G stainless-steel feeding needle with a 2.25-mm ball (South Point Surgical Supply, Coral Springs, FL, USA). Following ethanol administration, the AAVrh.10hALDH2 and AAVrh.10control mice and PBS-treated wild-type C57BL/6 mice were assessed for 0 to 24 hr for behavior, at 6 hr for serum acetaldehyde levels, and after sacrifice for liver mRNA, hALDH2 protein (by western and immunohistochemistry), and ALDH2 enzymatic activity. The hALDH2 mRNA and protein assays were as described above; the other assays were as described below.

Immunohistochemistry

Paraformaldehyde-fixed livers were embedded in paraffin and cut into 5-μm sections. Detection of vector-derived hALDH2 expression in the liver was carried out by an anti-HA antibody (Histowiz, Brooklyn, NY, USA). Following sectioning, anti-HA immunohistochemical staining, and counterstaining with hematoxylin, digital images of liver cross-sections were acquired using a 20× objective.

ALDH2 Activity Assay

The activity of ALDH2 was analyzed using the colorimetric mitochondrial aldehyde dehydrogenase (ALDH2) activity assay

kit (ab115348; Abcam, Cambridge, MA, USA) according to the manufacturer's protocol using 200 μg of total protein from liver homogenate. C57/Bl6 mice injected with PBS were used as controls.

Acetaldehyde Assay

Acetaldehyde measurements were performed at the Proteomics Resource Center, Rockefeller University[9,52] for n = 5 mice per group. Acetaldehyde levels were determined through derivatization with dinitrophenylhydrazine (DNPH) using butyraldehyde-DNPH (Supelco, Bellefonte, PA, USA) as an internal standard. Blood (120 μL) was deproteinized by addition of an equal volume of cold acetonitrile (ACN) and centrifuged for 30 min at 3,500 × *g* at 4°C. Forty-eight microliters of supernatant was mixed with 2 μL of 10 mM ^{13}C-acetaldehyde, and then 15 μL of 16 mM DNPH in ACN and 5 μL of 1 M citric acid (pH 4.0) were added. After incubation at 25°C for 30 min, the reaction was quenched with 60 μL of 0.1% formic acid in water, and samples were kept in −80°C until analysis. Prior to liquid chromatography/mass spectrometry analysis (LC/MS), 49 μL of sample was spiked with 1 μL of butyaldehyde-DNPH standard for a final 20 pm/μL. Acetaldehyde-DNPH (Sigma-Aldrich) was used as an external calibrant to verify retention time. Samples were analyzed by direct injection onto a Dionex U-3000 HPLC system and Thermo Scientific TSQ Vantage triple-quad mass spectometer. Chromatographic separation was performed using a Thermo Scientific Acclaim 120 C18 (2.1 × 150 mm) column at a flow rate of 200 μL/min, using formic acid in water as buffer A and 0.1% formic acid in ACN as buffer B. The gradient was 0 to 6 min 5% buffer B, to 6.5 min 40% buffer B, to 7 min at 80% buffer B, to 11 min at 80% buffer B, return to 5% buffer B at 11.5 min, and re-equilibration of the column to 15 min at 5% buffer B. Analysis was performed in the negative mode with acetaldehyde-DNPH monitored using a parent mass of 223 and transition ions of 46, 122.1, and 181.1; ^{13}C-acetaldehyde-DNPH monitored using a parent mass 225 and transitions of 46, 122.1, and 181.1; and butyaldehyde-DNPH monitored using a parent mass of 251 and transitions of 46, 122.1, and 181.1. Quantitation was performed using a known amount of ^{13}C-acetaldehyde standard in the sample and an external calibration curve of acetaldehyde-DNPH prepared in parallel. Rate constants of acetaldehyde consumption were determined by fitting time and concentration values to a first-order decay model. Butyaldehyde-DNPH was used to evaluate instrument performance.

Behavioral Assessments

Baseline behavioral assessments were performed 3 weeks after administration of AAVrh.10hALDH2, AAVrh.10control, or PBS, prior to ethanol exposure. Four weeks after vector administration, mice were administered 4 g/kg ethanol (Sigma-Aldrich) in water by intragastric gavage, and behavior was assessed using the eight tests described below at 0.5 (observational behavior score and body temperature only), 2, 6, 10, and 24 hr after ethanol administration.

Observational Behavior Score

Mouse behavior was observed and scored as described by Jin et al.:[9] 0, walking around normally; 1, rearing; 2, sedation; 3, mild ataxia (dysfunction of hind limbs); 4, severe ataxia (inability to move, body shaking); 5, loss of righting reflex; and 6, death.

Body Temperature

Rectal temperature was measured using homeothermic blanket systems with flexible probe (Harvard Apparatus, Holliston, MA, USA).

Balance Beam

A polyurethane-coated wooden beam 3 cm in width and 1 m in length was fixed to a wooden stand 80 cm above the ground. The mouse was placed at the edge of the wooden beam, and distance walked in 3 min was measured. If the mouse fell from the beam, it was placed back at the same point and testing continued. If the mouse fell five times, testing was aborted for safety.

Open-Field Chamber: Total Distance and Vertical Activity

Mice were placed in the center of a large plexi-glass cage (40 cm × 40 cm) with two sets of 16 light beam arrays in the horizontal X, Y, and vertical Z axes. Mice were habituated to the room for 10 min prior to each test. The distance traveled and vertical activity count were registered at any given moment by breaks in the light beam array caused by the mouse movement. Each test lasted for 5 min. Total distance traveled was measured by the number of horizontal beam breaks, and vertical activity was collected by the height and number of vertical beam breaks.

Skinny Beam

A polyurethane-coated wooden beam 1.5 cm in width and 30 cm in length was fixed to a wooden stand 15 cm above the ground. The mouse was placed at the edge of the wooden beam and the time to walk to the opposite edge was recorded. If the mouse fell from the beam, it was placed back at the same point and testing continued. If the mouse did not arrive to the opposite side within 3 min, the time was recorded as 180 s.

Skinny Rod

A hardwood round dowel 9.6 mm in diameter was placed horizontally above a large open plastic storage box. Each mouse was placed on the rod, and the time to fall from the rod was recorded. If the mice were able to stand on the rod for 10 s or more, time was recorded as 10 s.

Screen Climb

The screen-climb test apparatus comprises a 0.2-cm^2 wire grid (screen) mounted in a wooden frame (1 m × 1 m), which was treated with multiple applications of polyurethane to seal the wood. The screen stands against a wall at 45 degrees and has a drawn circle of 40 cm in diameter in the middle of the screen to allow for easy measurement of mouse upward or downward movements. Mice were placed in the center of the circle on the screen, and the time to climb out of the 30-cm circle in any direction was recorded. The maximum time allowed was 180 s.

Functional Composite Behavior Score

Behavior at each time point in every test was scored from 0–3 according to the parameters shown in Table S1. Composite score is the sum of the parameter scores for each behavioral test.

Statistical Analysis

All data are presented as means ± SEM unless otherwise stated; the "n" value for each group is stated in the figure or figure legend. Differences between groups were analyzed using an unpaired two-tailed Student's t test to compare all combinations of two groups independently. The behavior score and body temperature after ethanol exposure were also correlated to acetaldehyde levels. These correlations were evaluated by regression analysis and ANOVA. R^2 values > 0.6 indicate a strong relationship between the test groups. p values < 0.05 were considered significant for all comparisons.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.08.004.

AUTHOR CONTRIBUTIONS

Y.M. conducted experiments, acquired and analyzed data, and wrote the manuscript; K.M.S. designed research studies, conducted experiments, analyzed data, and wrote the manuscript; J.R., E.Z.F., and S.C. conducted experiments and acquired data; O.E.P. designed research studies, analyzed data, and wrote the manuscript; R.G.C. designed research studies, analyzed data, and wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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