Research Paper

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Aberrant reactive aldehyde detoxification by aldehyde dehydrogenase-2 influences endometriosis development and pain-associated behaviors

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Abstract

Endometriosis affects ~176 million women worldwide, yet on average, women experience pain ~10 years from symptom onset before being properly diagnosed. Standard treatments (drugs or surgery) often fail to provide long-term pain relief. Elevated levels of reactive aldehydes such as 4-hydroxynonenal (4-HNE) have been implicated in the peritoneal fluid of women with endometriosis and upon accumulation, reactive aldehydes can form protein-adducts and/or generate pain. A key enzyme in detoxifying reactive aldehydes to less reactive forms is the mitochondrial enzyme aldehyde dehydrogenase-2 (ALDH2). Here, we tested the hypothesis that aberrant reactive aldehyde detoxification by ALDH2 underlies endometriosis and its associated pain. We determined, in the eutopic and ectopic endometrium of women with severe (stage IV) peritoneal endometriosis, that ALDH2 enzyme activity was decreased, which was associated with decreased ALDH2 expression and increased 4-HNE adduct formation compared to the eutopic endometrium of controls in the proliferative phase. Using a rodent model of endometriosis and an ALDH2*2 knock-in mouse with decreased ALDH2 activity, we determined that increasing ALDH2 activity with the enzyme activator Alda-1 could prevent endometriosis lesion development as well as alleviate pain-associated behaviors in proestrus. Overall, our findings suggest that targeting the ALDH2 enzyme in endometriosis may lead to better treatment strategies and in the proliferative phase, that increased 4-HNE adduct formation within the endometrium may serve as a less invasive diagnostic biomarker to reduce years of suffering in women.

Keywords: Gynecological pain, Chronic pelvic pain, Endometriosis, Endometrium, Lesion, Cyst, Vagina, Menstrual cycle, Estrous stage, Development, Hyperalgesia, Nociception, Rodent model, Behavior, Development, Aldehyde dehydrogenase, ALDH2, Oxidative stress, 4-HNE, Biomarker, Reactive aldehyde

1. Introduction

Endometriosis is an estrogen-dependent inflammatory condition defined by the growth of endometrial tissue in extrauterine locations (variously called lesions, cysts, ectopic growths, and implants). The condition affects ~176 million women worldwide, yet little progress has been made over the past 20 years relative to screening, detection, prognosis, and treatment.[36] The most common symptom of endometriosis is pain and 70% to 90% of women of reproductive age with chronic pelvic pain have endometriosis.[25] Painful symptoms include debilitating pelvic/abdominal pain, dyspareunia (vaginal hyperalgesia, pain during intercourse), severe dysmenorrhea (pain on menstruation), dyschezia (pain on defecation), and dysuria (pain with urination). Women with the condition also suffer from co-occurring painful conditions including interstitial cystitis/painful bladder syndrome, irritable bowel syndrome, vulvodynia, fibromyalgia, and up to 50% of these women also suffer from infertility.[11,22] A major clinical problem is that painful symptoms associated with endometriosis poorly correlate with disease extent and on average, women experience pain ~10 years before being properly diagnosed.[24,33,63] The gold standard for endometriosis diagnosis is laparoscopic visualization of the lesions preferably with histological confirmation, which is invasive and expensive. Available treatments for endometriosis include drugs and/or surgery, which tend to be ineffective over the long term and can produce unwanted side effects such as premature bone loss, vaginal dryness, and contraception. Thus, there is a need for more effective pain therapeutics and less invasive diagnostic strategies to reduce years of suffering in women with endometriosis.

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How endometriosis occurs is not fully understood and is considered an enigma. The leading hypothesis, Sampson's hypothesis, suggests retrograde menstruation underlies the disease, in which the endometrial lining (endometrium) of the uterus travels retrogradely through the fallopian tubes and implants, primarily in the peritoneal cavity.[53] However, ~90% of women experience retrograde menstruation but only ~10% have endometriosis, suggesting differences within the endometrium, possibly genetic, underlie the ability of the ectopic lesion to successfully implant, progress, and avoid immune system clearance.[28] One factor known to be involved in the pathogenesis, progression, and establishment of endometriosis is oxidative stress.[32,47] Under oxidative stress, excess reactive oxygen species are produced and as a secondary biproduct of lipid peroxidation, reactive aldehydes including 4-hydroxynonenal (4-HNE) are generated.[23] In the peritoneal fluid of women with endometriosis, elevated reactive aldehyde levels have been implicated and through accumulation, reactive aldehydes can form protein-adducts and/or generate pain.[38,43–45,52,60,68] A critical enzyme in detoxifying reactive aldehydes such as 4-HNE to unreactive forms is the mitochondrial enzyme ALDH2. Here, we tested the hypothesis that aberrant reactive aldehyde detoxification by ALDH2 underlies the painful condition of endometriosis.

2. Methods

2.1. Study design

The primary objective of this study was to evaluate the role of ALDH2 reactive aldehyde detoxification in endometriosis. First, ALDH2 activity, ALDH2 expression, and 4-HNE adduct formation were assessed in the endometrium of women with endometriosis (lesion and eutopic) compared to women without endometriosis (eutopic) to determine how ALDH2 activity was regulated. We then biochemically characterized an ALDH2*2 knock-in mouse and used a preclinical endometriosis model to determine whether ALDH2 activity influences disease development. Subsequently, we incorporated the ALDH2 activator Alda-1 and behavioral assessments to determine whether increasing ALDH2 activity could prevent endometriosis development and/or alleviate pain-associated behaviors. For the animal studies, the number per group was based on previous experience with the disease model and a power analysis. Mice were randomly placed in control and treatment groups, and experimenters were blinded to conditions. All sample processing in this study was performed concurrently on experimental and control groups using identical methods. Experimenters quantifying results were blinded. Statistical tests and number of animals or replicates for each experiment are included in the figure legends.

2.2. Human tissue samples

In total, 15 proliferative-phase endometrial tissue samples were obtained from women with histologically confirmed, severe (stage IV) peritoneal endometriosis at laparoscopy (n = 5 eutopic, n = 5 patient matched ectopic peritoneal lesions) and from women found to be free of endometriosis at surgery (n = 5 eutopic). All subjects were Caucasian, had regular menstrual cycles, and had not received steroid hormone medications within 3 months of endometrial sampling (**Table 1**). The mean age of participants was 37.8 ± 3.44 years for the endometriosis samples and 41.4 ± 2.25 years for the nonendometriosis samples. Women without endometriosis at surgery were undergoing hysterectomy or gynecological surgery for a benign condition. All endometrial samples were obtained from the University of California-San Francisco (UCSF) National Institute of Health (NIH) Human Endometrial Tissue and DNA Bank. All samples procured by the Tissue Bank are obtained after written informed consent under an approved protocol by the UCSF Committee on Human Research. University of California-San Francisco sample acquisition and storage are by established standard operating procedures.[54] Menstrual cycle phase was assigned as proliferative phase endometrium by endometrial histology according to the Noyes criteria.[49] Severe endometriosis (stage IV disease) was defined in accordance with the Revised American Fertility Society classification system in which disease stage is graded on a scale of I (minimal), II (mild), III (moderate), to IV (severe) based on endometrial tissue location, amount, depth, and associated adhesions.[59] Endometriosis biopsies were restricted to advanced-stage disease to control for gene expression differences in women with more advanced vs lesser stage disease.[3,58] Peritoneal endometriosis was defined as biopsy-proven serosal implant.

2.3.1. ALDH2*2/*2 knock-in mouse and vaginal cytology

Animal subjects were virgin female ALDH2*2 knock-in (n = 107) and C57BL/6 wild-type (n = 107) littermate mice, aged 6 to 8 weeks. Mutant mice were homozygous ALDH2*2 (ALDH2*2/*2) knock-in mice on a C57BL/6 background generated by replacing the mouse wild-type ALDH2 allele with a mouse E487K mutant ALDH2 allele by homologous recombination. Compared to wild-type mice, the ALDH2*2 knock-in mouse has a single amino acid substitution in which adenine is substituted for guanine at the first base pair of codon 487. As a result, there is an amino acid change from glutamic acid (Glu, E) to lysine (Lys, K) that is equivalent to the E487K substitution in the ALDH2*2 human variant, in which ALDH2 enzyme activity is significantly decreased compared to that of wild-type mice.[15] Founder mice were back-crossed to the C57BL/6 background for at least 7 generations to achieve a homogeneous genetic background as previously described.[15] All mice were group housed in a temperature-controlled room (22°C), in Innovive cages lined with chip bedding and ad libitum access to rodent chow and water. Housing was in environmentally controlled conditions (room temperature ~22°C; 12-hour light/dark cycle, with lights on at 07:00). Reproductive status was determined by vaginal lavage performed ~2 hours after lights on using traditional nomenclature for the 4 estrous stages of proestrus, estrus, metestrus, and diestrus.[6] To control for the potential confound of vaginal lavage as an acute stressor, on behavioral assessments, animals were handled daily. The study and procedures were approved by the Animal Care and Use Committee as Stanford University protocol #32871 and Emory University protocol #201900201. All laboratory animal experimentation adhered to the NIH Guide for the Care and Use of Laboratory Animals.

2.3.2. Endometriosis surgery

At ~10 weeks of age, in the estrous stage of diestrus, mice were induced with endometriosis (ENDO) based on the rat model protocol originally developed by Vernon and Wilson and slightly modified by Cummings and Metcalf.[19,64] Briefly, mice were anesthetized with isoflurane (1%-3%) and placed on a heating pad to maintain body temperature (37C). An off-midline (left side) incision was made through the skin and muscle layer to expose the pelvic and abdominal organs. An ~1-cm segment of midleft

Table 1

Sample characteristics.

Sample ID	Cycle phase	Age (y)	Race	Weight (kg)	BMI (kg/m^2)	Medications/Other
Severe stage (IV) peritoneal endometriosis						
SE178	PE	47	Caucasian	84.55	46.65	Escitalopram, bupropion
SE182	PE	39	Caucasian	68.18	28.40	Cetirizine, fluoxetine
SE183	PE	29	Caucasian	61.36	22.51	None
EE181	PE	43	Caucasian	85.73	33.48	Salbutamol, mometasone, acetaminophen
EE185	PE	31	Caucasian	50.91	21.21	Methylphenidate
No endometriosis						
EN142	PE	49	Caucasian	79.38	32.01	Salbutamol, ranitidine, loratadine
EN146	PE	38	Caucasian	58.97	24.56	Fexofenadine, vitamins
EN150	PE	36	Caucasian	56.70	20.80	Levothyroxine, ibuprofen, vitamins
EN152	PE	41	Caucasian	77.56	27.60	Escitalopram
SN134	PE	43	Caucasian	123.64	46.79	Enoxaparin, warfarin, hydrocodone, docusate, iron

BMI, body mass index; PE, proliferative phase of the menstrual cycle, determined by histologic evaluation according to the Noyes criteria. Peritoneal endometriosis, defined as biopsy-proven serosal implant. Severe endometriosis (stage IV disease), defined in accordance with the Revised American Fertility Society (rAFS) classification system.

uterine horn with its attached fat was ligated proximally and distally with suture. Then, the uterine horn was excised, and the associated fat removed. Three, 2 × 2-mm pieces of the excised uterus (minus the fat) were sewn onto alternate mesenteric arteries that supply the caudal small intestine starting from the caecum using 4.0 nylon sutures. After it was confirmed that no bleeding was occurring in the abdominal cavity, the muscle layer was closed with chromic gut suture, skin incision closed with silk suture, and mice closely monitored during recovery.

2.3.3. Osmotic pump implantation and drug delivery

For Alzet osmotic pump implantation, an incision was made at the nape of the mouse neck and the pump implanted immediately below the skin layer and closed with silk suture for subcutaneous continuous delivery of Alda-1 (5 mg/kg/day), or as a control solvent only (50% polyethylene glycol [PEG] and 50% dimethyl sulfoxide [DMSO] by volume). Pumps were filled and then primed in 0.9% sterile saline at 37°C for ~24 hours before implantation.

2.3.4. Behavioral assessments

Behavior tests included measures of abdominal licking, mechanical nociception, thermal nociception, locomotor activity, and exploratory behavior, all previously shown to be altered in association with endometriosis pain-associated behaviors.[5,31,35,37] Abdominal licking has been used as an indicator of abdominal pain in various viscero-specific pain models.[18,27,31,65] Therefore, the number of times the mouse licked, groomed, and barbered the abdominal region was recorded as an indicator of local abdominal discomfort and indicator of primary/local pain. This test was conducted in a modified home cage open-field setting with bedding and the mouse first allowed a minimum of 10 minutes to acclimate. To measure changes in nociception, hind paw withdrawal threshold and thermal latency were assessed as a secondary or referred pain-associated behavior. To assess mechanical nociception, von Frey fibers were used ranging in force from 0.004 to 5.49 g using the Dixon modified up and down method.[13,21] Mice were placed individually in a plexiglass chamber on an elevated mesh screen stand and allowed to acclimate for a minimum of 10 minutes. Von Frey hairs were applied perpendicularly to the mouse hind paw plantar surface until the hair bowed and then held for approximately 3 seconds. The mechanical threshold required to elicit a paw withdrawal (50% paw withdrawal threshold) was determined. To assess for changes in thermal nociception, Hargreaves method was used to determine latency to paw withdrawal from a focused heat light source using a commercial Plantar Test Analgesia Meter (IITC Life Science).[34] Mice were placed individually in plexiglass chambers on a glass platform and allowed to acclimate for a minimum of 10 minutes. The heat stimulus was delivered to the plantar region of the mouse hind paw with an active intensity of 30%. Reaction time was measured in 0.01-seconds increments with a cutoff time of 10 seconds. A minimum of 30 seconds separated each hind paw test.

To assess for changes in locomotor activity, mice were individually placed in an automated Opto-Varimex activity monitor (Columbus Instruments) with optical beam sensors and total (ambulatory and nonambulatory), ambulatory (does not include stereotypic nonambulatory behavior, eg, grooming and digging), and vertical (rearing) counts were recorded. To assess for changes in exploratory behavior, a cardboard tunnel was included in the modified home cage open-field setting. The number of cardboard tunnel entries, amount of time spent in the tunnel (s), and number of times the mouse climbed on top of the tunnel were recorded.

Abdominal licking, locomotor activity, and exploratory behavior were assessed in separate 5-minute sessions. Behavioral tests were run in the order thought to be least stressful test to most invasive (home cage assessments [abdominal licking and exploratory behavior], locomotor activity, Von Frey, and Hargreaves) because test order may influence behavior.[42] Two experimenters, SM and MV, performed behavioral assessments (interobserver reliability, $r >$ 0.90) blinded to treatment. Because male experimenters can produce pain inhibition, both experimenters were females.[56] Because endometriosis is an estrogen-dependent condition and pain-associated behaviors are influenced by estrogen, all behavioral data are reported in the estrous stage of proestrus.[4,6,8,67] Behavioral parameters were assessed twice in proestrus twice during each 2-week period applicable (baseline, postendometriosis, and posttreatment). Testing days were ~day 5 and day 13 of each 2-week period.

2.3.5. Mouse tissue collection

At the time of sacrifice under isoflurane anesthesia, the abdominal cavity was opened and examined. When applicable, the area where the eutopic uterus autotransplants were previously sewn was investigated and sutures located to identify and measure the lesions in situ. The ectopic lesions, eutopic uterus, and liver (control) were harvested, immediately frozen in dry ice, and stored at −80C. After tissue harvesting, mice were sacrificed.

2.3.6. Mouse ectopic lesion measurement

To assess for differences in lesion size, total lesion burden was first determined. To do this, the largest diameter and the smallest diameter of each lesion were multiplied to give a value (most lesions have an ovoid shape) and then values from each lesion added to obtain a total number, the total lesion burden.[40,46] The total lesion burden was divided by the number of lesions formed to give the average lesion area for comparison between groups.

2.4. Tissue processing and analysis

Mouse and human tissue samples were homogenized in sucrose mannitol buffer, pH 7.4 (210 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, 5.0 mM MOPS) with protease and phosphatase inhibitors. Briefly, tissue homogenates were centrifuged to remove cellular debris and the supernatant was retained as the whole cell fraction for the analysis. Samples were immediately stored at −80C until further analysis.

2.5. Western blot analysis

Total protein concentration was determined using the Bradford assay according to the manufacturer's protocol. Equal amounts of protein (30 μg) for each sample were separated by SDS-PAGE on 4% to 15% polyacrylamide gels and transferred to PVDF Membranes (Bio-Rad Laboratories, Hercules, CA) and probed overnight at 4°C for specific antibodies against ALDH2 (Santa Cruz Biotechnology, Dallas, TX), 4HNE (Alpha Diagnostics, San Antonio, TX), and actin (Cell Signaling, Danvers, MA). The next day, membranes were washed and incubated with a horseradish peroxidase-linked secondary antibody (anti-goat, Santa Cruz Biotechnology or anti-rabbit, Cell Signaling) for 1 hour at room temperature. Membranes were again washed and bound antibody was detected by enhanced chemiluminescence. Images were acquired by using an Azure Biosystems c300. Image-J (NIH) software was used for the relative expression and densitometry analysis. Relative protein expressions were normalized to actin (loading control).

2.6. Enzyme activity assay

Cofactor and substrate (NAD^+ and 25 mM acetaldehyde) were added to the reaction buffer containing homogenate tissue and the conversion of NAD+ to NADH over time monitored using a spectrophotometer. For a 1 mL assay, 500 μL of 200 mM NaPPi at final concentration of 200 mM NaPPi in water (pH 9.0 (M.W. 446)), 250 μL of 10 mM NAD^+ (2.5 mM NAD^+), 100 μg protein from tissue, and homogenization buffer (0.1 M tris HCL pH 8.0% and 1% triton-X) were added and mixed to make 1 mL total volume. Absorbance (O.D.) was measured at A340 nm for 3 minutes. Then, 2.5 μL of 10 mM acetaldehyde (f.c., 25 mM) was added to the cuvette and the absorbance measured for an additional 15 minutes. ALDH2 activity was converted to μmole NADH/minute/mg of protein. As a blank control, cuvettes without tissue/sample or acetaldehyde were used. Data presented are absorbance measured during the first *2* minutes after acetaldehyde was added.

2.7. Statistical analysis

To achieve at least a 20% minimal difference between groups for a power of 95% with $\alpha < 0.05$ and $\beta < 20\%$, a minimum of 6 mice/group were used. Data are expressed as mean ± SEM. For data with only 2 groups, a two-tailed Student *t*-test was used. For data containing more than 2 groups, a one-way or two-way analysis of variance was used, followed by Tukey post hoc test as appropriate. Statistical meaningful differences were assumed for $P < 0.05$. All statistical analysis was performed using GraphPad 8.12.

3. Results

3.1. Women with endometriosis have decreased ALDH2 activity

To determine how ALDH2 activity is regulated in the endometrium of women with endometriosis, we analyzed and compared endometrial biopsies from women with severe (stage IV) peritoneal endometriosis (eutopic and patient-matched ectopic endometrium) and without endometriosis (eutopic) collected in the proliferative phase (see **Table 1** for patient and biopsy characteristics). In the eutopic and ectopic endometrium of women with endometriosis, compared to the eutopic endometrium of women without endometriosis, we determined that ALDH2 enzyme activity was decreased ($P < 0.005$ and $P < 0.0005$, respectively), which was associated with decreased ($P < 0.0005$ and $P < 0.0005$, respectively) ALDH2 protein expression (**Fig. 1A**). To determine whether decreased reactive aldehyde detoxification by ALDH2 was associated with increased protein-adduct formation, we assessed the same endometrial biopsies for 4-HNE adducts. We determined, in women with endometriosis, that 4-HNE adduct formation in the eutopic and ectopic endometrium was increased ($P < 0.05$ and $P < 0.05$, respectively), compared to the eutopic endometrium of women without endometriosis (**Figs. 1B and C**). No significant differences were found between the eutopic and ectopic endometrium of women with endometriosis, relative to ALDH2 activity, expression, or 4-HNE adduct formation (**Figs. 1A and C**). Together, these findings suggest that decreased ALDH2 activity and expression may underlie endometriosis pathophysiology and that increased 4-HNE adduct formation in the endometrium may occur as a result of decreased reactive aldehyde detoxification by ALDH2, supporting our hypothesis.

3.2. Female naive ALDH2*2 homozygote knock-in mice have decreased ALDH2 activity

To determine whether an ALDH2*2 knock-in mouse with decreased ALDH2 activity can be used as a unique tool for studying endometriosis, we biochemically characterized ALDH2 activity, ALDH2 protein expression, and 4-HNE adduct formation. Using an enzyme activity assay, we determined that relative to wild-type mice, ALDH2*2 mice had ~70% and 63% reduced liver (control) and uterus ALDH2 activity, respectively (**Fig. 2A**). Western blot revealed that, relative to wild-type mice, ALDH2*2 mice had ~60% and 62% reduced ALDH2 protein expression in the liver and uterus, respectively (**Figs. 2B and C**). Under physiological conditions, no significant differences in 4-HNE adduct formation were found between naive wild-type and naive ALDH2*2 mice liver and/or uterus, suggesting similar redox states in the absence of disease pathology (**Figs. 2B and C**). Together, these findings suggest that the ALDH2*2 mouse, with decreased ALDH2 activity and expression relative to wild-type, and similar basal levels of 4-HNE adduct formation, is a unique tool to begin to elucidate the role of reactive aldehyde detoxification by ALDH2 in endometriosis.

3.3. Decreased ALDH2 activity accelerates endometriosis development in a rodent model

To determine whether decreased ALDH2 enzyme activity contributes to lesion development, endometriosis was induced

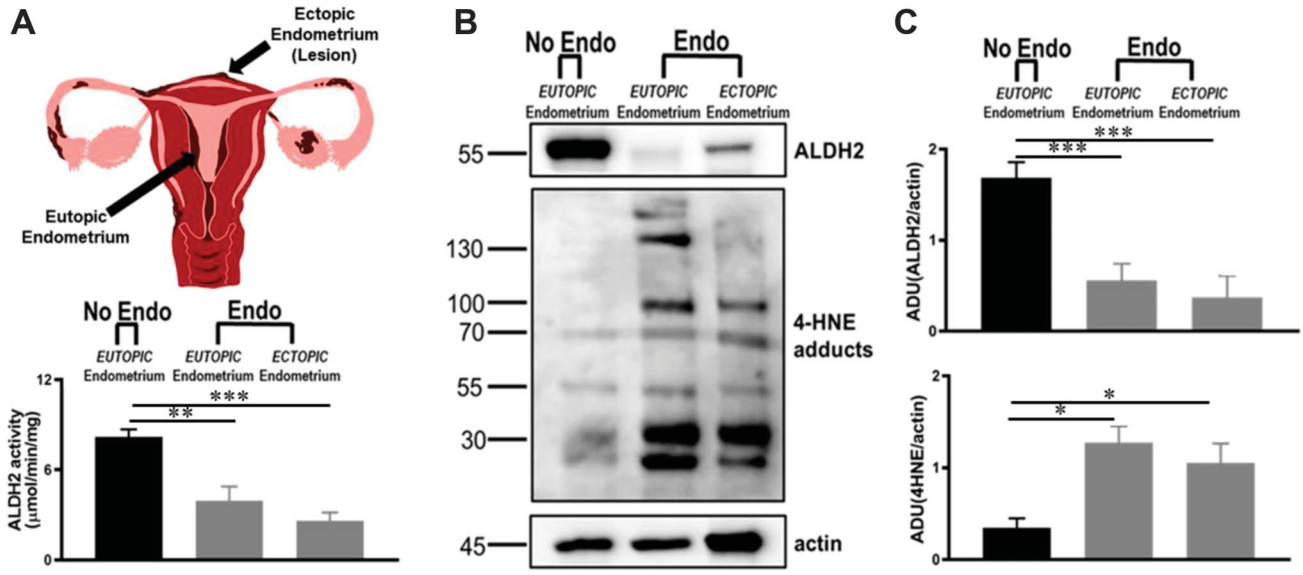

Figure 1. Molecular characterization of the endometrium of women without and with endometriosis. In the proliferative phase, ALDH2 activity of the endometrium was analyzed in women without endometriosis (No Endo: eutopic) and in women with severe (IV) peritoneal endometriosis (Endo: eutopic and ectopic lesion, patient matched) and expressed as μmol/minute/mg. The reduction of NAD+ to NADH at λ340 nm was measured using a spectrophotometer and 25 mM acetaldehyde as a substrate. Data presented are absorbance measured during the first 2 minutes after acetaldehyde substrate was added (A). (B and C) Representative Western blot of ALDH2 and 4-HNE adduct formation in the endometrium of women without endometriosis (eutopic) and with endometriosis (eutopic and ectopic lesion) (B). Western blot analysis of ALDH2 expression (top) and 4-HNE adduct formation (bottom) relative to actin as loading control (C). All data are expressed as mean ± SEM, n = 5 biological replicates/group. Assessed using one-way ANOVA followed by Tukey post hoc test. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. ANOVA, analysis of variance.

in wild-type and ALDH2*2 mice using a validated rodent model that produces signs (fluid filled, vascularized, innervated lesions) and painful symptoms similar to that of women with endometriosis (**Fig. 3A**).[7,9,39–41] To establish and compare developmental time courses, mice were sacrificed, lesions measured, and average lesion area determined for both wild-type and ALDH2*2 mice at 1 of the 4 time points postendometriosis induction: day 1, 3, 14, or 28 (**Fig. 3B**). By day 3, ALDH2*2 mice developed a larger ($P < 0.05$) lesion area compared to the lesion area at day 1 in ALDH2*2 mice (**Fig. 3C**). However, in wild-type mice, a larger ($P < 0.0001$) lesion area was not observed until day 14, relative to wild-type lesion area day 1. At day 28, lesion area in both wild-type and ALDH2*2 mice was larger ($P < 0.0001$ and $P < 0.0001$, respectively) than their respective day 1 lesion areas, but not their respective day 14 lesions when lesion development stabilizes and endometriosis is established. Over the developmental time course, comparing wild-type and ALDH2*2 mice, at day 3, ALDH2*2 mice developed a larger ($P < 0.05$) lesion area

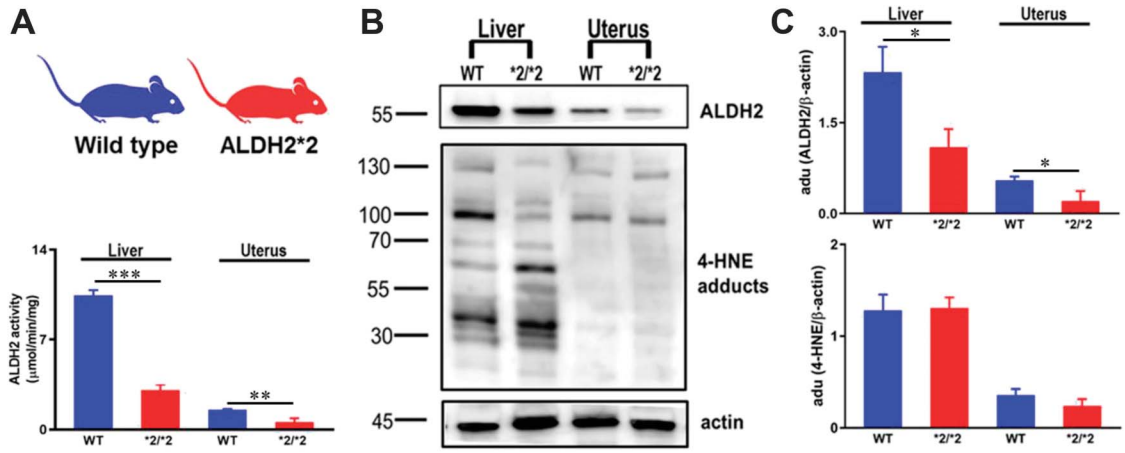

Figure 2. Molecular characterization of naive female wild-type and homozygotic ALDH2*2 knock-in mice. Wild-type C57BL/6 mice (blue) and ALDH2*2 knock-in mice (red) with a C57BL/6 background. The ALDH2*2 knock-in mouse has a single amino acid substitution in which adenine is substituted for guanine at the first base pair of codon 487. The result is an amino acid change from glutamic acid (Glu, E) to lysine (Lys, K) that is equivalent to the E487K substitution in the human ALDH2*2 variant. In proestrus, mouse liver (control) and uterus ALDH2 enzyme activity was spectrophotometrically determined by measuring the reduction of NAD+ to NADH at λ340 nm using 25 mM acetaldehyde as a substrate. Data presented are absorbance measured during the first 2 minutes after acetaldehyde substrate was added. Activity is expressed as μmol/min/mg (A). (B and C) Representative Western blot of ALDH2 protein expression and 4-HNE adduct formation in liver and uterus of wild-type and ALDH2*2 mice (B). Western blot analysis of liver and uterus ALDH2 protein expression (top) and 4-HNE adduct formation (bottom) relative to actin loading control. All data are expressed as mean ± SEM, n = 5 biological replicates/group. Assessed using two-tailed Student *t*-test test, blue and red bars indicate wild-type and ALDH2*2 mice, respectively. *$P < 0.05$, **$P < 0.0005$, ***$P < 0.0001$.

compared to wild-type mice, but at day 14 and day 28, no significant differences in lesion area were found between ALDH2*2 and wild-type mice (**Fig. 3C**). These findings suggest that the decreased ALDH2 activity of the ALDH2*2 mice accelerates early lesion development in endometriosis, relative to wild-type mice.

3.4. Decreased ALDH2 enzyme activity exacerbates endometriosis pain-associated behavior in a rodent model

To determine whether decreased ALDH2 enzyme activity contributes to endometriosis pain-associated behaviors, behavioral assessments were made in proestrus in wild-type and ALDH2*2 mice for 2 weeks before endometriosis was induced and then during the 2-week period that endometriosis becomes established (**Fig. 4A**). At baseline, no significant differences were observed between wild-type and ALDH2*2 mice relative to abdominal directed licking (events in 5 minutes period), paw withdrawal threshold, or thermal latency (**Figs. 4B–D**). Post-endometriosis, compared to baseline, both wild-type and ALDH2*2 mice had increased ($P < 0.0005$ and $P < 0.0001$, respectively) abdominal directed licking, decreased ($P < 0.0001$ and $P < 0.0001$, respectively) paw withdrawal thresholds, and decreased ($P < 0.0001$ and $P < 0.0001$, respectively) thermal latencies, relative to their respective baselines. Postendometriosis, ALDH2*2 mice compared to wild-type mice had increased ($P < 0.05$) abdominal licking suggesting that the decreased ALDH2 activity exacerbated local abdominal discomfort (**Fig. 4B**). To assess for differences in locomotor activity, an automated Opto-Varimex activity monitor recorded the total, ambulatory, and vertical counts in a 5-minute period (**Fig. 4E** and S1A and B, available at http://links.lww.com/PAIN/B66). No significant differences in locomotor activity were observed between wild-type and ALDH2*2 mice at baseline, or within groups postendometriosis relative to baseline, or between groups postendometriosis. To assess for differences in exploratory behavior, a modified home cage open-field setting with a cardboard tunnel was used. The total time spent in the tunnel (s), number of tunnel entries, and the number of times the mouse climbed on top of the tunnel were recorded in a 5-minute period (**Fig. 4F** and S1C and D, available at http://links.lww.com/PAIN/B66). At baseline, no significant differences were observed between wild-type and ALDH2*2 mice relative to total time in tunnel (s) or number of times on top of the tunnel but ALDH2*2 mice had a decreased ($P < 0.05$) number of tunnel entries relative to wild-type mice (Fig. S1C, available at http://links.lww.com/PAIN/B66). In postendometriosis ALDH2*2 mice, total time in tunnel(s) was significantly decreased compare to baseline ($P < 0.005$; **Fig. 4F**). No other significant differences were found between or within groups relative to exploratory behavior. Overall, these findings suggest that the decreased ALDH2 activity of the ALDH2*2 mouse exacerbates endometriosis abdominal pain-associated behavior.

3.5. Increasing ALDH2 activity prevents endometriosis development in a rodent model

To determine whether increasing ALDH2 enzyme activity could prevent lesion development, we incorporated with our rodent endometriosis model, the small molecule Alda-1[N-(1,3-benzodioxo-5-ylmethyl)-2,6-dichlorbenzamide] that selectively increases ALDH2 activity by correction of the ALDH2*2 mutant structural deficit.[50] Beginning the day of endometriosis surgery, in wild-type and ALDH2*2 mice, Alzet osmotic pumps were implanted for continuous delivery of Alda-1 (5 mg/kg) or DMSO PEG50/50% (control). Mice were then sacrificed, lesions measured, and average area determined for both wild-type and ALDH2*2 mice at 1 of the 2 developmental time points: day 3 or 28 (**Fig. 5A**). By day 3, ALDH2*2 mice treated with Alda-1 had smaller ($P < 0.0005$) lesion areas compared to DMSO-treated ALDH2*2 mice (**Fig. 5B**). By day 28, both wild-type and ALDH2*2 mice treated with Alda-1 had smaller ($P < 0.005$ and $P < 0.05$, respectively) lesion areas compared to respective DSMO-treated groups (**Fig. 5C**). Comparing wild-type and ALDH2*2 mice, treated with DMSO at day 3, ALDH2*2 mice lesion area was larger ($P < 0.05$) than that of wild-type mice (**Fig. 5B**) but by day 28, no significant differences were found between DMSO-treated wild-type and ALDH2*2 mice relative to lesion areas. Comparing wild-type and ALDH2*2 mice, treated with Alda-1, no significant differences in lesion area were found at day 3 or day 28 (**Figs. 5B and C**). Overall, these findings suggest that Alda-1 prevents the accelerated lesion development seen at day 3 in ALDH2*2 mice, relative to wild-type mice (**Fig. 3C**), and that by day 28, Alda-1 prevents lesion development in both wild-type and ALDH2*2 mice, supporting the involvement of ALDH2 activity in endometriosis development.

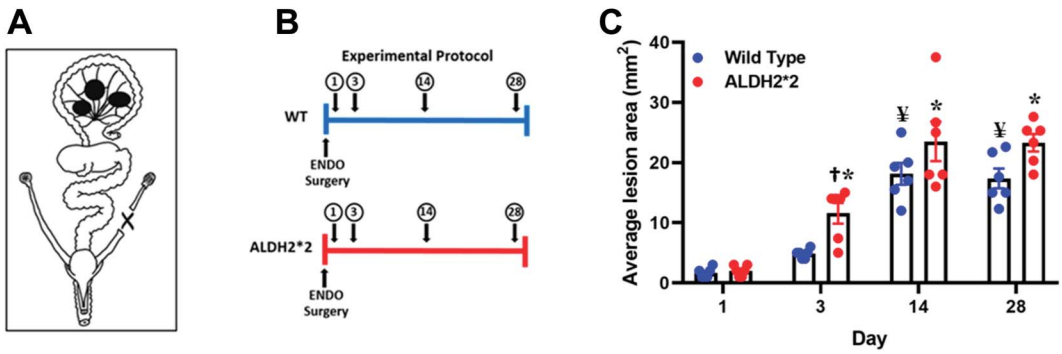

Figure 3. The influence of decreased ALDH2 activity on endometriosis development in a rodent model. Three equal pieces of eutopic uterus with endometrium are sutured onto alternate cascading mesenteric arteries. These autotransplants form ectopic lesions and symptoms similar to women with endometriosis (A). (B and C) Experimental protocol: endometriosis surgery was performed in wild-type (n = 24) and ALDH2*2 (n = 24) female mice and then at day 1, 3, 14, or 28 mice sacrificed and lesions measured (n = 6/group/genotype) (B). The average lesion area was determined for wild-type and ALDH2*2 mice at each time point in the developmental time course (C). All data are expressed as mean ± SEM. Assessed using two-way ANOVA followed by Tukey post hoc test, blue and red circles indicate wild-type and ALDH2*2 mice, respectively. †$P < 0.05$ vs wild-type day 3, *$P < 0.0001$ vs ALDH2*2 day 1, ¥$P < 0.0001$ vs wild-type day 1. ANOVA, analysis of variance.

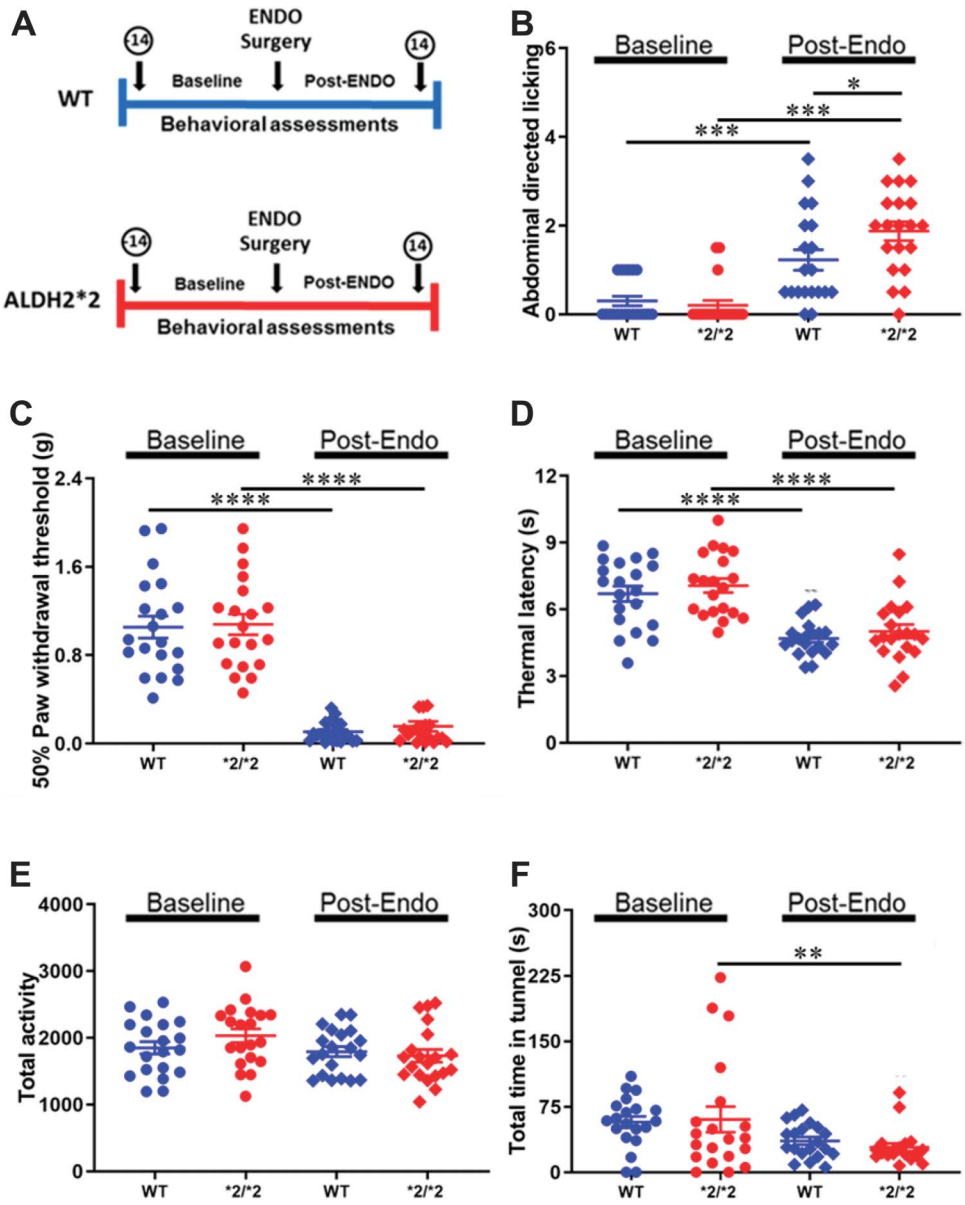

Figure 4. The influence of decreased ALDH2 activity on endometriosis pain-associated behaviors in a rodent model. (A–F) Experimental protocol: behavioral parameters were assessed for 2 weeks before and after endometriosis surgery in wild-type (n = 20) and ALDH2*2 mice (n = 20) in proestrus (~day 5 and day 13 of both periods) (A). As a measure of abdominal discomfort or primary pain, the number of times the mouse licked, groomed, or barbered the abdominal region was recorded (B). As an indicator of secondary referred pain, changes in nociception were assessed. Hind paw mechanical withdrawal threshold (C) and thermal latency (D) were assessed by von Frey hairs and Hargreaves method, respectively. Locomotor activity and exploratory behavior were measured by the total activity (E) and total time spent in tunnel (F). Abdominal licking, locomotor, and exploratory behavior were assessed in 5-minute sessions. Abdominal licking and exploratory behavior were assessed in a modified home cage open-field setting. All data are expressed as mean ± SEM. Assessed using 2-way ANOVA with Tukey post hoc test; blue data points indicate wild-type mice, red data points indicate ALDH2*2/*2 mice, circles indicate baseline assessments, and diamonds indicate postendometriosis assessments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$. ANOVA, analysis of variance.

3.6. Increasing ALDH2 activity alleviates endometriosis pain-associated behaviors in a rodent model

To determine whether increasing ALDH2 enzyme activity could alleviate pain-associated behaviors once endometriosis is established, Alda-1 (or DMSO PEG50/50% control) treatment was delivered through Alzet osmotic pump beginning 2 weeks postendometriosis and continued for 2 weeks (**Fig. 6A**). Behavioral assessments were made before and after endometriosis was induced and then again posttreatment. Compared to postendometriosis assessments, Alda-1-treated wild-type and ALDH2*2 endometriosis mice had decreased ($P < 0.05$ and $P < 0.05$, respectively) abdominal directed licking, increased ($P < 0.05$ and $P < 0.005$, respectively) mechanical paw withdrawal threshold, and increased ($P < 0.005$ and $P < 0.05$, respectively) thermal latency (**Figs. 6B–D**). In DMSO-treated wild-type and ALDH2*2 endometriosis mice, no significant differences were found in abdominal directed licking, paw withdrawal threshold, or thermal latency compared to their respective postendometriosis assessments (**Figs. 6B–D**). Alda-1-treated wild-type and ALDH2*2 endometriosis mice, compared to their respective DSMO groups, had decreased ($P < 0.05$ and $P < 0.0001$, respectively) abdominal directed licking and an increased

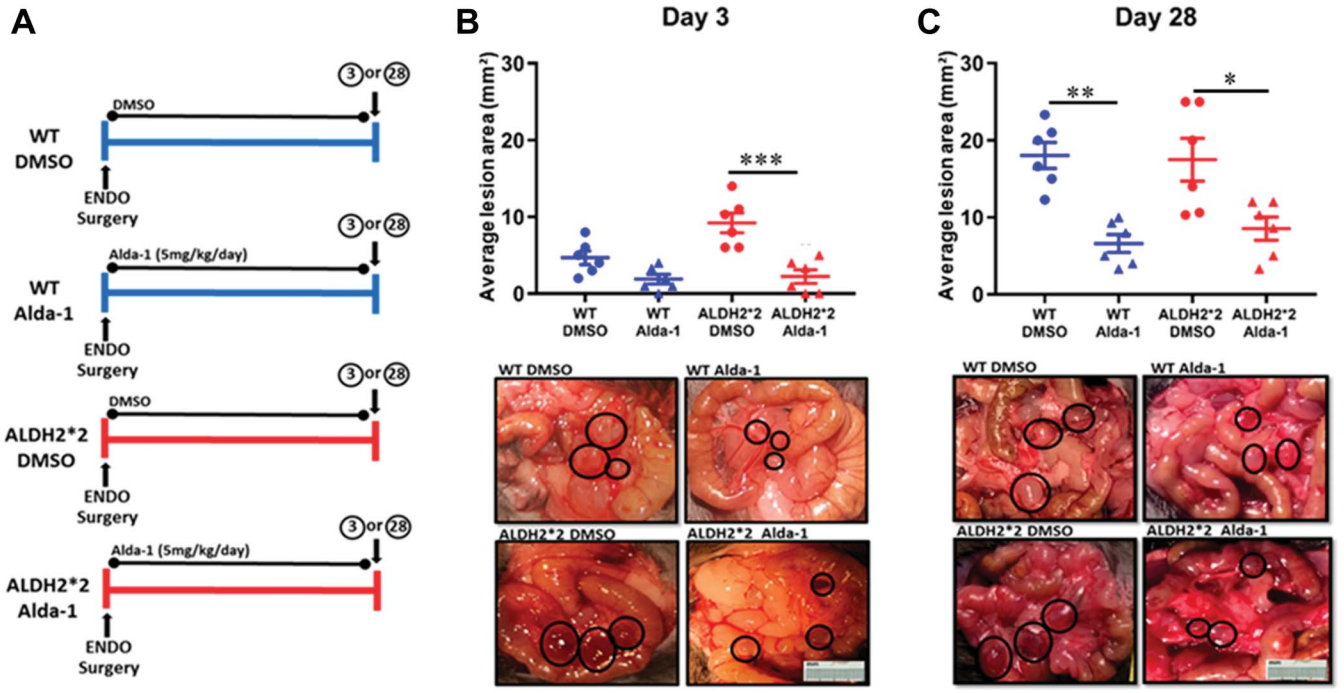

Figure 5. The influence of increased ALDH2 activity on endometriosis development in a rodent model. (A–C) Experimental protocol: endometriosis surgery was performed in female wild-type (n = 24) and ALDH2*2 mice (n = 24). Mice were treated for 3 days or 28 days with Alda-1 (5 mg/kg/day or as a control 50-50 DMSO-PEG) through Alzet pump delivery beginning the day of surgery (n = 6/group/genotype/treatment) (A). Average lesion area and representative lesions in wild-type and ALDH2*2 mice treated with Alda-1 or DMSO control for 3 days (B) or 28 days (C). All data are expressed as mean ± SEM. Assessed using one-way ANOVA followed by Tukey post hoc test; blue data points indicate wild-type mice, red data points indicate ALDH2*2 mice, circles indicate DMSO-treated, and triangles indicate Alda-1-treated. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. ANOVA, analysis of variance.

($P < 0.005$ and $P < 0.005$, respectively) mechanical paw withdrawal threshold (**Figs. 6B and C**). Relative to thermal latency, no significant differences were found between wild-type endometriosis mice treated with DMSO or Alda-1; however, Alda-1-treated ALDH2*2 endometriosis mice had an increased ($P < 0.05$) thermal latency compared to their respective DMSO group (**Fig. 6D**).

To determine whether Alda-1's alleviation of endometriosis pain-associated behaviors was related to locomotor activity, posttreatment total, ambulatory, and vertical counts were compared to postendometriosis assessments. No significant differences were found posttreatment in wild-type or ALDH2*2 mice treated with Alda-1 or DMSO compared to their respective total, ambulatory, and vertical postendometriosis counts (**Fig. 6E**, S2A and B, available at http://links.lww.com/PAIN/B66), suggesting that Alda-1's alleviation of pain was independent of locomotor activity. Furthermore, no significant differences were found between Alda-1-treated wild-type and ALDH2*2 endometriosis mice, compared to their respective DMSO groups, in locomotor activity.

To determine whether Alda-1 treatment influenced exploratory behavior, the total time spent in the tunnel (s), number of tunnel entries, and the number of times the mouse climbed on top of the tunnel, assessments posttreatment were compared to post-endometriosis (**Figs. 6F**, S2C and D, available at http://links.lww.com/PAIN/B66). Alda-1-treated ALDH2*2 mice, compared to post-endometriosis, had a decrease in total time in tunnel and number of tunnel entries ($P < 0.05$ and $P < 0.05$, respectively), suggesting Alda-1 pain alleviation in ALDH2*2 mice may be associated with decreased exploratory behavior (**Fig. 6F**, S2C, available at http://links.lww.com/PAIN/B66). No other significant differences were found in number of tunnel entries, total time in tunnel, or number of times on top of the tunnel in Alda-1- or DMSO-treated wild-type and ALDH2*2 endometriosis mice, relative to respective postendometriosis assessments (**Figs. 6F**, S2C and D, available at http://links.lww.com/PAIN/B66). Furthermore, no significant differences in locomotor activity were found between Alda-1-treated wild-type and ALDH2*2 endometriosis mice, compared to their respective DMSO groups. Overall, these findings suggest that increasing ALDH2 activity with Alda-1 can alleviate endometriosis pain-associated behaviors in both wild-type and ALDH2*2 mice without influencing locomotor activity but in ALDH2*2 mice, exploratory behavior may be influenced.

To determine whether Alda-1's alleviation of endometriosis pain-associated behaviors was associated with lesion parameters, the average area, number of lesions, and total lesion burden were analyzed and compared posttreatment. In wild-type and ALDH2*2 mice treated with Alda-1, no significant differences were found in average lesion area, relative to respective DMSO-treated mice (Fig. S2E, available at http://links.lww.com/PAIN/B66). However, in both Alda-1-treated wild-type and ALDH2*2 mice, the number of lesions was decreased ($P < 0.0001$ and $P < 0.0001$, respectively) compared to respective DMSO-treated mice (**Fig. 6G**). To account for the reduced lesion number, the total lesion burden or total amount of ectopic growth for each group was analyzed and compared. In Alda-1-treated wild-type mice, total lesion burden was significantly decreased ($P < 0.0001$) relative to that of DMSO-treated mice; however, similar lesion burdens were found in Alda-1- and DMSO-treated ALDH2*2 mice (**Fig. 6H**). Combined, these findings suggest that in established endometriosis, Alda-1 pain alleviation is associated with a decreased lesion number and burden in wild-type mice and a reduced lesion number in ALDH2*2 mice.

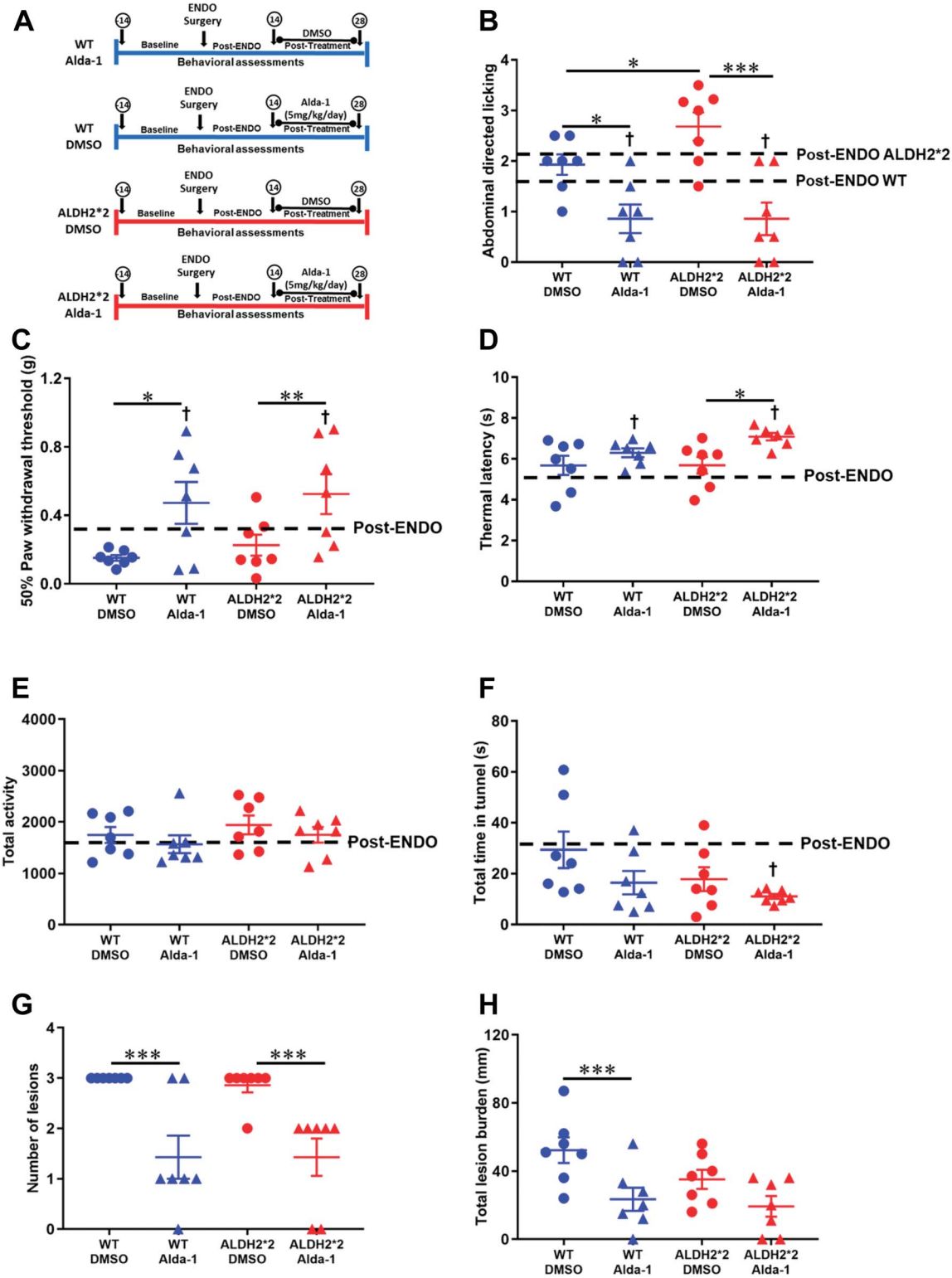

Figure 6. The influence of increased ALDH2 activity on endometriosis pain-associated behaviors in a rodent model. (A–F) Experimental protocol: Endometriosis surgery was performed in wild-type (n = 14) and ALDH2*2 mice (n = 14). At day 14 postendometriosis, Alda-1 treatment (5 mg/kg/day or 50-50 DMSO-PEG control) began and continued for 2 weeks using Alzet osmotic pump (n = 7/group/genotype) (A). Presented behavioral parameters were assessed in proestrus 2 weeks postendometriosis surgery and 2 weeks posttreatment (~day 5 and day 13 of both periods). Abdominal licking (B), paw withdrawal threshold (C), thermal latency (D), total activity (E), and total time in tunnel (F) were assessed. At the time of sacrifice, lesions were assessed to compare number of lesions (G) and total lesion burden (H) between groups. All data are expressed as mean ± SEM. Behavioral data were assessed using two-way ANOVA followed by Tukey post hoc test. Lesion data were assessed using one-way ANOVA followed by Tukey post hoc test. Blue data points indicate wild-type mice, red data points indicate ALDH2*2/*2 mice, circles indicate DMSO-treated, and triangles indicate Alda-1-treated. †$P < 0.05$ vs postendometriosis, *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$. ANOVA, analysis of variance.

4. Discussion

This study investigated the role of ALDH2 reactive aldehyde detoxification in endometriosis. In women with endometriosis, elevated peritoneal fluid reactive aldehyde levels are implicated and through accumulation, reactive aldehydes can form tissue protein-adducts and also generate pain.[38,43–45,52,60,68] Our results show that in the proliferative phase, women with severe (IV) peritoneal endometriosis have decreased ALDH2 activity and expression in the endometrium (eutopic and ectopic) compared to endometrium (eutopic) of women without endometriosis, suggesting altered ALDH2 activity may underlie endometriosis disease pathology. Further supporting this hypothesis, in women with endometriosis, 4-HNE adduct formation was increased in the endometrium (eutopic and ectopic) of women with endometriosis compared to the endometrium (eutopic) of women without endometriosis, suggesting protein-adducts may form as a result of the decreased reactive aldehyde detoxification by ALDH2.

Using a rodent model of endometriosis and an ALDH2*2 knock-in mouse, with reduced ALDH2 activity and expression and similar basal levels of 4-HNE adducts, relative to wild-type mice, we provide evidence that early in endometriosis development, decreased ALDH2 activity accelerates lesion development and exacerbates pain-associated behavior. We further determined that increasing ALDH2 activity with the enzyme activator, Alda-1, could prevent early lesion development. Once endometriosis was established, we determined that increasing ALDH2 activity with Alda-1 treatment could alleviate endometriosis pain-associated behavior that was associated with a decreased lesion number in both wild-type and ALDH2*2 mice and a decreased lesion burden in wild-type mice. Combined, our findings from women with endometriosis and a preclinical rodent model support our overall hypothesis that aberrant reactive aldehyde detoxification by ALDH2 underlies the painful condition of endometriosis.

Overall, our preclinical findings suggest that targeting the ALDH2 enzyme may be effective for the alleviation of endometriosis-associated pain, particularly primary/local abdominal pain. Early on, ALDH2*2 endometriosis mice, with reduced ALDH2 activity, develop larger lesions and exacerbated abdominal pain-associated behaviors, relative to wild-type mice, suggesting the lesion's reduced ability to detoxify reactive aldehydes within the peritoneal cavity, and contributes to endometriosis pain-associated behaviors. With reduced ALDH2 activity and therefore, reduced reactive aldehyde detoxification, elevated levels of reactive levels such as 4-HNE can influence lesion innervation, to induce pain.

In rodent models and women with endometriosis, lesions develop a sensory and sympathetic nerve supply, which opens a 2-way line of communication between the peripheral lesions and spinal cord allowing central nervous system engagement and the generation of pain.[7,39-41,57] Often coexpressed on sensory nerves are the nociceptive ion channels transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1), where they integrate noxious stimuli and generate pain in inflammatory conditions.[29,55] In women with endometriosis, lesion TRPV1 and TRPA1 mRNA and immunoreactivity are upregulated and positively correlated with painful symptom severity, compared to the eutopic endometrium of controls with no pain.[10] Moreover, mRNAs encoding TRPA1 and TRPV1 are increased in the peritoneum of women with endometriosis and chronic pelvic pain, compared to the peritoneum of healthy women with no pain.[30] Therefore, elevated reactive aldehydes, such as the endogenous TRPA1 agonist 4-HNE, implicated in the peritoneal fluid of women with endometriosis, could activate TRPA1 channels on sensory nerve fibers in peripheral lesions and/or the peritoneal cavity, in concert with TRPV1 channels, to drive increased pain signaling.[20] If true, increasing reactive aldehyde detoxification by ALDH2 with Alda-1 may reduce endogenous reactive aldehyde sensitization of TRPA1, to reduce pain. Furthermore, topical or local administration (i.p.) of Alda-1 may alleviate endometriosis-associated primary abdominal/pelvic pain, reducing the risk of side effects common in oral/systemic routes of drug administration.

Our findings further suggest that ALDH2 activity influences lesion development and maintenance, which has implications for surgery-based treatment strategies for endometriosis. In mice, decreased ALDH2 activity exacerbated lesion development, whereas increasing ALDH2 activity prevented lesion development. In women with endometriosis, laparoscopic lesion excision alleviates pain in ~50% of cases.[16] However, in the ~50% of women in whom surgery is successful, ~25% have a return of the lesions that is frequently accompanied by a return of the pain.[1,16,62] If our preclinical findings are translational, then in women in whom surgery is successful, Alda-1 treatment during and/or after laparoscopic lesion excision may be effective in preventing the return of the lesions and/or pain. Our approach may also influence pain in patients with chronic overlapping pain conditions such as fibromyalgia syndrome (FMS).[2,10,61] Supporting this idea, Giamberardino et al. (2017) determined that visceral pain, including pelvic pain from endometriosis, is a triggering factor for FMS pain in comorbid patients and that effective treatment, here lesion ablation, decreases FMS pain.[17]. This study highlights in comorbid patients the important role of peripheral nociceptive input from lesions in triggering/maintaining FMS pain, a central sensitization syndrome.[26] Then, in line with our hypothesis, increasing ALDH2 activity to reduce algogenic input to the central nervous system by lesion sensory afferents should decrease FMS pain.[66] However, in comorbid patients with endometriosis pelvic pain that is centralized and independent of peripheral input, for example, women in whom lesion excision does not relieve pain, our approach may not influence FMS pain.

Findings from this study also provide potential insight into mechanisms underlying endometriosis etiology. The decreased ALDH2 activity and expression in the eutopic and ectopic endometrium of women with endometriosis suggests that ALDH2 activity and expression differences may help explain why 90% of women have retrograde menstruation but only 10% of women develop endometriosis. The decreased reactive aldehyde detoxification by ALDH2 in women with endometriosis is likely key in reactive aldehyde accumulation, promoting an environment of oxidative stress and therefore, extrauteral endometrium (lesion) implantation and survival.[32,47] The concomitant increase in 4-HNE adducts in the endometrium of women with endometriosis provides preliminary support for 4-HNE adduct formation as a diagnostic biomarker, in the proliferative phase. The gold standard for endometriosis diagnosis is laparoscopic lesion visualization preferably with histological confirmation, which is invasive and expensive.[28] Therefore, biopsy of the eutopic endometrium or endometrial curettage with subsequent 4-HNE analysis may serve as a less invasive diagnostic biomarker. Overall, a better understanding of the differences between women with and without endometriosis is critical in the development of more effective treatment and diagnostic strategies.

Limiting the applicability of our findings, all endometriosis biopsies in this study were from women with peritoneal

endometriosis, excluding other endometriosis subtypes (ovarian endometriomas and deep infiltrating endometriosis) involving different etiologies that may require different diagnostic and treatment strategies.[48] All biopsies were from the proliferative phase of the menstrual cycle to control for differences within the menstrual cycle; however, we did not control for potential gene expression differences within the days of the proliferative phase.[12,51] Limitations to our study also include biopsy sample size (n = 15) and lack of patient pain scores. In a larger future study, we will assess patient pain using a visual analogue scale (VAS) and analyze additional endometriosis subtypes and days within the proliferative phase. Our analysis will also include biopsies from additional races, particularly East Asians of which ~45% carry the ALDH2*2 point mutation that results in reduced ALDH2 activity. Our findings in ALDH2*2 mice may be translatable to women with the ALDH2*2 variant; however, a direct link between the E487K mutation and endometriosis must be established in future studies. Furthermore, cell-specific ALDH2 characterization in endometrial tissue will be performed because this is yet to be explored.

Animal study limitations include the inability to distinguish between the contribution of reduced uterus and liver ALDH2 activity to endometriosis pathology, which would require a conditional uterine knockout model. Although desirable, it was beyond the scope of the original study to determine the relationship between ALDH2 and 4-HNE adduct formation in the endometriosis mouse model. Our primary objective was to establish aberrant ALDH2 activity in women with endometriosis and then, in a preclinical endometriosis model, manipulate ALDH2 activity to determine its role in lesion development and pain-associated behaviors. However, other studies have established the relationship between ALDH2 activity, 4-HNE, and Alda-1 in other tissues.[14] Future studies will further test our hypothesis and assess the relationship between ALDH2, 4-HNE, and Alda-1 in women and mice with endometriosis.

Overall, our findings suggest the ALDH2 enzyme as a novel target for the painful condition of endometriosis, and Alda-1 as a potentially therapeutic. Furthermore, we provide preliminary support for the development of increased 4-HNE protein-adduct formation in the endometrium as a biomarker to reduce diagnostic delay. Further research is needed to improve our understanding of the role of ALDH2 in endometriosis to potentially reduce years of suffering in women.

Conflict of interest statement

S.L. McAllister and E.R. Gross are listed as co-inventors on the patent WO 2018/204673 "Methods and Compositions for Treating Endometriosis and Endometriosis-Associated Symptoms" filed by Stanford University. P. Sinharoy is currently an employee of AstraZeneca Pharmaceuticals. No other potential conflicts of interest are declared.

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Author Contributions: S.L. McAllister conceived the idea, designed the study, performed molecular and behavioral experiments and rodent surgeries, analyzed and interpreted the data, made the figures, and wrote the manuscript. P. Sinharoy optimized molecular protocols, performed molecular experiments, and contributed to the experimental design, interpretation of the data, and writing of the manuscript. M. Vasu performed molecular and behavioral experiments and made illustrations. E. R. Gross conceived the idea and contributed to the experimental design, interpretation of the data, and writing of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B66.

Supplemental video content

Video content associated with this article can be found online at http://links.lww.com/PAIN/B67.

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