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Pain-related behaviors and abnormal cutaneous innervation in a murine model of classical Ehlers-Danlos syndrome

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Abstract

Classical Ehlers-Danlos syndrome (cEDS) is a connective tissue disorder caused by heterozygous mutations in one of the type V collagen-encoding genes, *COL5A1* or *COL5A2*. cEDS is characterized by generalized joint hypermobility and instability, hyperextensible, fragile skin and delayed wound healing. Chronic pain is a major problem in cEDS patients, but the underlying mechanisms are largely unknown, and studies in animal models are lacking. Therefore, we assessed pain-related behaviors in haploinsufficient *Col5a1*^{+/-} mice, which clinically mimic human cEDS. Compared to wild-type (WT) littermates, 15–20 week-old *Col5a1*^{+/-} mice of both sexes showed significant hypersensitivity to mechanical stimuli in the hind paws and the abdominal area, but responses to thermal stimuli were unaltered. Spontaneous behaviors, including distance travelled and rearing, were grossly normal in male *Col5a1*^{+/-} mice, while female *Col5a1*^{+/-} mice showed altered climbing behavior. Finally, male and female *Col5a1*^{+/-} mice vocalized more than WT littermates when scruffed. Decreased grip strength was also noted. In view of the observed pain phenotype *Col5a1*^{+/-} mice were crossed with Na_v1.8-tdTomato reporter mice, enabling visualization of nociceptors in the glabrous skin of the footpad. We observed a significant decrease in intra-epidermal nerve fiber density, with fewer nerves crossing the epidermis, and a decreased total nerve length of *Col5a1*^{+/-} mice compared to WT.

In summary, male and female *Col5a1*^{+/-} mice show hypersensitivity to mechanical stimuli, indicative of generalized sensitization of the nervous system, in conjunction with an aberrant organization of cutaneous nociceptors. Therefore, *Col5a1*^{+/-} mice will provide a useful tool to study mechanisms of pain associated with cEDS.

SUMMARY

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Col5a1^{+/-} mice provide a model of classical Ehlers-Danlos syndrome, a painful heritable disease. *Col5a1*^{+/-} mice show hypersensitivity to mechanical stimuli and abnormal cutaneous nociceptive innervation.

Keywords

Classical Ehlers-Danlos syndrome; *Col5a1*^{+/-} mice; Type V collagen; Pain; Mechanical allodynia; Innervation

INTRODUCTION

The Ehlers-Danlos Syndromes (EDS) are a group of rare heritable disorders resulting from genetic defects in connective tissue biosynthesis. The current EDS classification recognizes 13 types, for most of which molecular defects have been identified in genes encoding structural components of the extracellular matrix (ECM), in particular fibril-forming collagens types I, III, V, or molecules involved in maturation, folding, secretion or supramolecular organization of these collagens (Supplementary Table S1) [20]. The syndromic presentation of joint hypermobility, skin hyperextensibility, abnormal scar formation and easy bruising is key to diagnosis, but a wide range of other systemic manifestations can be present.

One of the most common, yet intractable, complaints of EDS patients is chronic pain, which is often the primary reason for seeking medical help [37,51]. Two independent studies, in 51 [37] and 273 EDS patients [51] respectively, reported a prevalence of pain of 90%. Despite the high prevalence, the etiology of pain in EDS has barely been studied, and current understanding of underlying molecular and cellular pathways is minimal. Hence, treatment of EDS-related pain is largely inadequate [37]. EDS patients consume large amounts of medication, including non-steroidal anti-inflammatory drugs, corticosteroids, opioids, antidepressants, anti-seizure medication and muscle relaxants, or a combination thereof [6,34,37,40,51]. Yet, pain is largely refractory to these drugs, and abuse of analgesics is associated with considerable side effects and risks [11,56]. Besides pharmacological interventions, EDS patients frequently receive physiotherapy and surgical treatments, such as joint stabilization or nerve decompression [34,40], often with disappointing outcomes [34,37]. Therefore, there is an urgent need for safer and more efficacious approaches.

One of the most common types of EDS is the classical type (cEDS), characterized by generalized joint hypermobility, skin hyperextensibility and abnormal wound healing [20]. In cEDS, the prevalence of chronic pain has been estimated to be 76% [37,51]. cEDS is caused by heterozygous mutations in either the *COL5A1* or the *COL5A2* gene, encoding the pro- α 1- and pro- α 2-chain of type V collagen, respectively [49]. The majority of identified mutations lead to *COL5A1* haploinsufficiency due to a non-functional *COL5A1* allele, resulting in reduced availability of type V collagen in the ECM. Type V collagen is a widely expressed, quantitatively minor fibrillar collagen with distinct roles in organization and stability of the ECM [5]. At the ultrastructural level, the dermis of cEDS patients displays abnormally shaped, so-called ‘cauliflower’ collagen fibrils, reflecting the critical role of type V collagen in regulating collagen fibril diameter [12]. *Col5a1* knockout mice (*Col5a1*^{-/-}) are

embryonically lethal [52], but mice lacking one of the two *Col5a1* alleles (*Col5a1*^{+/-}) reflect the most common molecular defect. They faithfully recapitulate the human cEDS phenotype, both clinical characteristics like skin hyperextensibility and fragility, slow wound healing and reduced tendon stiffness, as well as at the ultrastructural level, with the dermis showing collagen ‘cauliflowers’ [53,55].

The lack of studies in animal models that aim to model EDS pain is a major hurdle to increasing our understanding of EDS-related pain mechanisms. Therefore, we aimed to explore pain-related behaviors in *Col5a1*^{+/-} mice and investigate the nociceptive innervation in the glabrous skin of the hind paw.

MATERIALS AND METHODS

Animals –

A total of 173 male and 174 female mice were used. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Rush University Medical Center. Mice were housed 2–5 per cage with food and water *ad libitum* and kept on 12-hour light cycles. *Col5a1* haploinsufficient mice (*Col5a1*^{+/-} on a C57BL/6 background) were kindly provided by Dr. David Birk (University of South Florida, USA) [53]. Nav1.8-Cre C57BL/6 mice (a gift from Dr. John Wood, University College London, London, UK) [42,47] were crossed with Ai9 (tdTomato) C57BL/6J mice, which robustly express tdTomato fluorescence following Cre-mediated recombination (The Jackson Laboratory, Bar Harbor, ME, USA), thereby generating Nav1.8-tdTomato mice that allow visualization of Nav1.8-positive neurons in tissues, including skin [14,29]. *Col5a1*^{+/-} mice were crossed with homozygous Nav1.8-tdTomato reporter mice to obtain Nav1.8-tdTomato^{+/-} *Col5a1*^{+/-} mice or Nav1.8-tdTomato^{+/-} *Col5a1*^{+/+} littermate control mice. All mice received a unique ear-tag for identification and were tail-clipped under 2% isoflurane anesthesia in O₂. Subsequently, DNA was extracted and genotyping was performed by PCR as described before [52].

Behavioral testing –

Male and female *Col5a1*^{+/-} mice and their wild-type (WT) littermates were tested between the age of 15 and 20 weeks. All behavioral experiments were performed during the light cycle, no earlier than 9:00 a.m. and no later than 5:00 p.m., except for monitoring of spontaneous behavior, which occurred overnight. All behavioral assessments were performed by an experimenter blinded to the genotype.

Mechanical allodynia –

Male (WT: n = 9, *Col5a1*^{+/-}: n = 12) or female (WT: n = 13, *Col5a1*^{+/-}: n = 10) mice were placed in Plexiglas cubicles (10 × 10 × 14 cm) on an elevated perforated metal grid, and were permitted to habituate for at least 20 min before testing. Mechanical allodynia was tested at the hind paws and the abdominal area with at least two days between testing. Nylon von Frey monofilaments (North Coast Medical) were applied to the plantar surface of the hind paw for 3 sec and responses were recorded. The up-down method of Dixon [10] was used to estimate 50% withdrawal thresholds of the hind paws, as described [23]. Two consecutive measures were taken on each hind paw and averaged. A decrease in withdrawal

threshold corresponds to an increase in mechanical sensitivity. Allodynia in the abdominal area was assessed as described before [33]. Monofilaments of forces 0.04, 0.4, 1 and 4g were applied to the lower abdominal area in increasing order of force for 1–2 sec for a total of 10 times with an inter-stimulus interval of at least 5 sec in male (WT: n = 10, *Col5a1*^{+/-}: n = 10) and female (WT: n = 11, *Col5a1*^{+/-}: n = 18) mice. Types of behavior that were considered a positive response included (1) sharp retraction of the abdomen; (2) immediate licking or scratching of the area of filament stimulation; or (3) jumping. Mice anticipating the stimulus by constantly looking at the fiber and responding before the actual stimulus was given were excluded from the analysis (WT male: n = 1, WT female: n = 2). Response frequency was calculated as the percentage of positive responses and data were reported as the mean percentage of response frequency $\pm$ SEM.

Thermal allodynia/hyperalgesia –

Unrestrained male (WT: n = 14, *Col5a1*^{+/-}: n = 17) and female (WT: n = 10, *Col5a1*^{+/-}: n = 16) mice were placed individually on the metal surface of the Hot Cold Plate Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA, USA), maintained at a constant temperature of 0 °C (cold) or 55°C (noxious heat). The response latency, measured as the time to observe the first nocifensive behavior, *i.e.* licking of the hind paw or jumping, was recorded. The cut-off time was set at 30 seconds at 0 and 55°C to avoid damage to the paw. Mice were first tested for cold and one week later for noxious heat (55°C) [3,25,45].

Spontaneous behaviors –

Male (WT: n = 12, *Col5a1*^{+/-}: n = 13) and female (WT: n = 14, *Col5a1*^{+/-}: n = 11) mice were individually housed overnight for 16 hours on a Laboratory Animal Behavior Observation, Registration and Analysis System (LABORAS) (Metris, Hoofddorp, The Netherlands). The LABORAS platform allows the automatic registration of different behavioral elements of mice by recording vibrations evoked by animal movements and translating these into corresponding behaviors by dedicated software [23,50]. The first hour of the test was considered an acclimatizing period during a 1-hour light cycle. Locomotive behaviors (distance travelled, climbing and rearing) were assessed during the subsequent 15-hour dark period and compared between *Col5a1*^{+/-} and WT animals, as described before [23].

Grip strength –

Grip force is used as a surrogate measure for deep tissue hyperalgesia [18] and was tested with a grip strength meter (IITC Life Science Inc., Woodland Hills, CA, USA). Male (WT: n = 19, *Col5a1*^{+/-}: n = 20) and female (WT: n = 14, *Col5a1*^{+/-}: n = 18) mice were tested in 5 rounds of 3 trials each (totaling 15 measurements), with at least 3 min in home cages between rounds. Mice were lifted by the tail and induced to grasp a round bar with a 2-mm diameter. Once the forepaws were both firmly grasping the bar, mice were gently pulled along the axis of the force sensor until they were unable to retain their grip. The 15 grip strength measurements (in grams) were averaged. Mice were weighed immediately prior to the test and grip strength analyses were corrected for the body weight of each mouse. Deep tissue hyperalgesia was defined as a decrease in the grip force.

Vocalization evoked by scruffing –

Vocalization was quantified in male (WT: n = 13, *Col5a1*^{+/-}: n = 12) and female (WT: n = 19, *Col5a1*^{+/-}: n = 10) mice by scruffing the mice 10 times with at least 1 min intervals. Audible vocalizations were recorded and a score out of 10 was obtained for each mouse tested. Mice were handled only once or twice by the experimenter before the vocalization test was performed, since pilot experiments suggested that repeated handling affected this measure.

Skin innervation –

Hind paws were collected from 15-week old male (WT: n = 6, *Col5a1*^{+/-}: n = 6) and female (WT: n = 5, *Col5a1*^{+/-}: n = 5) *Col5a1*^{+/-} x Nav1.8-tdTomato mice and their WT littermates. Footpads were postfixed in 4% paraformaldehyde (PFA) for 2 days and stored in 30% sucrose at 4°C. The glabrous skin of the footpad was embedded in O.C.T. (Optimum Cutting Temperature, Tissue-Tek), frozen on dry ice and cut into transverse 20 µm cryosections. Sections were postfixed in 4% PFA for 20 min and immediately counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 20 min both at room temperature, and mounted with hard-set vectashield. Sections were imaged using a LSM880 confocal microscope and dedicated software (Zeiss, Oberkochen, Germany). Images were subsequently processed and analyzed using the Fiji software [39] for the four following parameters: (1) Intra-epidermal nerve fiber density was determined as the number of Nav1.8-positive nerve fibers crossing the epidermis in function of the epidermal-dermal junction length. The epidermal-dermal junction was outlined by a blinded experimenter, and the number of nerves crossing this junction was counted by two blinded experimenters (DS and ZM), as previously described [22]. Counts were obtained for 3 individual locations in one footpad per mouse using the ImageJ Cell Counter plug-in and scores from both blinded experimenters were averaged; (2) The total density of Nav1.8-positive nerve fibers present in the skin sections was determined by obtaining the ratio of the Nav1.8-positive area in the skin and the total area representing the skin; (3) Diameters of the nerves were measured by using the DiameterJ plug-in [13]; and (4) Total length of the Nav1.8-positive nerve fibers in the skin was calculated by manually tracing the nerves using the Simple Neurite Tracer plug-in [19]. Analyses were done on the maximal intensity projection images of the Z-stacks of at least five individual locations throughout the footpad per mouse and lengths were averaged for each mouse. Individual Z-stacks were compiled into 3-dimensional reconstructions and movies were created using the Volocity software (v6.3, Perkin Elmer, Groningen, The Netherlands).

Knee histopathology –

At age 30 weeks, mice (previously used for behavioral testing), were anesthetized by ketamine and xylazine (100 and 10 mg/kg; i.p.) and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Hind legs were collected from male (WT: n = 2, *Col5a1*^{+/-}: n = 2) and female (WT: n = 4, *Col5a1*^{+/-}: n = 6) mice, postfixed in 4% PFA for 3 days at 4°C, decalcified for 2 weeks in 14% EDTA at room temperature, and stored in 30% sucrose in PBS at 4°C. Knees were embedded in O.C.T. and cryosectioned into 20-µm coronal sections. Knee sections were subsequently stained with 0.04% (w/v) toluidine blue and were evaluated for cartilage degeneration, based on modified

Osteoarthritis Research Society International recommendations, by an experimenter blinded to the genotype [24]. Briefly, two 20- μ m midjoint sections (150–200 μ m apart) as well as one section from the anterior part of the knee and one from the posterior knee were used to score the medial femoral condyles and tibial plateaux for severity of cartilage degeneration. For each cartilage surface, scores were assigned individually to each of 3 zones (inner, middle, outer) on a scale of 0–5, with 5 representing the most damage (maximal summed score of 30 for femoral plus tibial cartilage degeneration). For each knee, the scores were averaged for the two midjoint sections and for the anterior/posterior sections.

Statistical analysis –

For mechanical allodynia in the hind paw, 50% withdrawal threshold data was log-transformed prior to performing the unpaired two-tailed *t* test in order to allow the use of parametric statistics. For mechanical allodynia in the abdominal area, area under the curve (AUC) was calculated prior to unpaired two-tailed *t* test. The non-parametric Mann-Whitney test was performed to compare the vocalization data. The body weight data were analyzed by two-way ANOVA. For all other datasets, unpaired two-tailed *t* test was used to compare two groups at a single time point. All data are expressed as mean $\pm$ S.E.M., with *n* indicating the number of animals. *P* values less than 0.05 were considered significant. Statistical calculations were performed using GraphPad Prism 5 (La Jolla, CA, USA).

RESULTS

General animal health –

The body weight of *Col5a1*^{+/-} mice was monitored every 3 weeks over a 30-week period. Despite a significant effect of age (*p* < 0.0001), no overt differences could be seen in body weight between female wild-type (WT) and *Col5a1*^{+/-} mice. In male mice, the body weight significantly increased with age (*p* < 0.0001) in both WT and *Col5a1*^{+/-} mice, but *Col5a1*^{+/-} mice showed a significant lower body weight compared to WT mice (*p* < 0.0001) (Supplementary Fig. S1).

Mechanical sensitivity –

Sensitivity to mechanical stimulation was assessed in *Col5a1*^{+/-} and WT mice. Mechanical stimulation of the hind paws showed a decreased paw withdrawal threshold in male (*Col5a1*^{+/-}: 0.38 $\pm$ 0.041 g vs. WT: 0.58 $\pm$ 0.064 g, *p* = 0.0291) and female (*Col5a1*^{+/-}: 0.42 $\pm$ 0.063 g vs. WT: 0.60 $\pm$ 0.035 g, *p* = 0.0167) *Col5a1*^{+/-} mice, compared to age-matched WT littermates (Fig. 1A–B). In order to evaluate the presence of generalized sensitization, mechanical allodynia was also evaluated in the abdominal area. This revealed an increased response frequency in *Col5a1*^{+/-} mice compared to age-matched WT littermates, both in male and female mice (Fig. 1C–D). Area under the curve (AUC) analysis indicated that response frequencies were significantly increased in male (*Col5a1*^{+/-}: 140.3 $\pm$ 9.779 vs. WT: 105.6 $\pm$ 9.170, *p* = 0.0186) and female (*Col5a1*^{+/-}: 148.7 $\pm$ 5.864 vs. WT: 123.7 $\pm$ 9.089, *p* = 0.0223) *Col5a1*^{+/-} mice, compared to WT littermates (Fig. 1E–F). Taken together, these results indicate the presence of widespread hypersensitivity to mechanical stimuli in *Col5a1*^{+/-} mice.

Thermal sensitivity –

Next, sensitivity to cold (0°C) and noxious heat (55°C) was evaluated. The majority of the mice outperformed the cold test with a withdrawal latency of 30s for 0°C (Fig. 2A–B). No significant differences in the latency to withdraw from a noxious heat stimulus at 55°C could be observed between male and female *Col5a1*^{+/-} and WT mice (Fig. 2C–D).

Spontaneous behaviors –

In order to evaluate signs of functional physical impairment, spontaneous behavior was assessed during a 15-hour overnight period on a LABORAS platform. *Col5a1*^{+/-} mice travelled a similar distance compared to age-matched WT littermates (Fig. 3A–B). In the same experimental setting, the average number and total time climbing and rearing (standing on their hind limbs) were recorded. The average number of rears and climbs per hour were similar between *Col5a1*^{+/-} and WT mice in both males and females (Supplementary Fig. S2). The time spent rearing also remained unaltered between *Col5a1*^{+/-} and WT mice of both sexes (Fig. 3C–D). Overall, female mice spent more time climbing compared to male mice regardless of the genotype (Fig. 3E–F). We did, however, observe a significant decrease in the time female *Col5a1*^{+/-} mice spent climbing, compared to WT mice (*Col5a1*^{+/-}: 12748 ± 1345 s vs. WT: 17920 ± 1885 s, *p* = 0.0453) (Fig. 3F). This difference was not observed for male mice (*Col5a1*^{+/-}: 3684 ± 506.6 s vs. WT: 3847 ± 676.5 s, *p* = 0.8473) (Fig. 3E).

Grip strength analysis –

Because the observed difference in climbing between female *Col5a1*^{+/-} and WT mice could be indicative of reduced grip strength, we performed grip strength analysis. Grip force was significantly decreased in male *Col5a1*^{+/-} mice compared to their WT littermates (*Col5a1*^{+/-}: 82.00 ± 2.480 vs. WT: 92.68 ± 1.849, *p* = 0.0015) (Fig. 3G), but this difference was no longer significant after correcting for body weight (*Col5a1*^{+/-}: 2.774 ± 0.1008 vs. WT: 2.979 ± 0.07179, *p* = 0.1091) (Fig. 3H). *Col5a1*^{+/-} female mice also displayed a significantly lower grip force (*Col5a1*^{+/-}: 72.57 ± 1.608 vs. WT: 89.28 ± 1.757, *p* = <0.0001), which was apparent regardless of correction for their body weight (*Col5a1*^{+/-}: 3.338 ± 0.09111 vs. WT: 3.898 ± 0.09375, *p* = 0.0002) (Fig. 3I,J).

Vocalization –

During routine handling and identification of the mice, we observed that *Col5a1*^{+/-} mice often produced audible vocalizations. Therefore, we evaluated and quantified this vocalizing behavior in response to scruffing in a blinded manner, and found a significant increase in the number of vocalizations in the *Col5a1*^{+/-} mice versus WT littermates, both in male (*Col5a1*^{+/-}: 5.6 ± 0.82 vs. WT: 1.5 ± 0.51, *p* = 0.0014 and female (*Col5a1*^{+/-}: 7.0 ± 0.93 vs. WT: 4.5 ± 0.62, *p* = 0.0267) mice (Fig. 4A–B).

Skin innervation –

To assess if changes in peripheral innervation in *Col5a1*^{+/-} mice might contribute to the observed increased mechanical sensitivity, we crossed these mice with Na_v1.8-tdTomato reporter mice, which express tdTomato in all neurons that express the voltage-gated sodium

channel, Nav1.8 [42,47]. This neuronal subset comprises approximately 75% of dorsal root ganglion sensory neurons, including >90% of C-nociceptors, as well as a small fraction of A δ -nociceptors and A β afferents. [42] We then analyzed the nociceptive innervation of the glabrous skin of the footpad. This revealed disorganization of the Nav1.8-positive fibers in male and female *Col5a1*^{+/-} mice, with apparently less protrusion of the nerve fibers into the epidermis (Fig. 5A–H, higher magnifications are shown in Fig. 5 I–L).

This effect was quantified by calculating the intra-epidermal nerve fiber density, which was significantly decreased in both male (*Col5a1*^{+/-}: 0.01390 $\pm$ 0.001827 vs. WT: 0.02004 $\pm$ 0.001884, *p* = 0.0414) and female (*Col5a1*^{+/-}: 0.01491 $\pm$ 0.001281 vs. WT: 0.02064 $\pm$ 0.001582, *p* = 0.0226) *Col5a1*^{+/-} mice compared to WT littermates (Fig. 6A,B). To determine if the decreased intra-epidermal nerve fiber density is a consequence of a general reduction of nerve fibers, the total volume of nerve fibers in the dermis was quantified. This analysis did not show a significant difference in the total dermal nerve density in male and female *Col5a1*^{+/-} mice (Fig. 6C,D). In addition, it was noted that in *Col5a1*^{+/-} mice, fewer nociceptive nerve fibers tend to cross the epidermal-dermal junction, they also appear to run more often parallel to this junction compared to the nerve fibers of WT littermates, as can be clearly seen in a 3-dimensional reconstruction (Supplementary Video S1–S4). Quantification of the nerve fiber diameters revealed a similar size of the nerve fiber diameters between *Col5a1*^{+/-} and WT mice but showed a lower overall nerve fiber frequency, indicative of a lower number of nerves in *Col5a1*^{+/-} mice (Fig. 6E,F). Finally, the total length of the nerve fibers in the skin was quantified and revealed a significant decrease in nerve fiber length in male (*Col5a1*^{+/-}: 1503 $\pm$ 87.33 μ m vs. WT: 2144 $\pm$ 174.8 μ m, *p* = 0.0083) and female (*Col5a1*^{+/-}: 1588 $\pm$ 122 μ m vs. WT: 2274 $\pm$ 55.62 μ m, *p* = 0.0009) *Col5a1*^{+/-} mice (Fig. 6G,H).

Knee histopathology –

A recently developed conditional tendon- and ligament-specific *Col5a1* knockout (*Col5a1*^{ten/ten}) mouse model revealed that complete absence of type V collagen in these tissues results in early-onset knee osteoarthritis at postnatal day 30 [48]. Therefore, we assessed whether *Col5a1* haploinsufficiency also renders *Col5a1*^{+/-} mice prone to developing premature knee osteoarthritis, which is associated with pain-related behaviors in mice [23]. Histological analysis of the medial compartment of the knees of 30-week old WT and *Col5a1*^{+/-} mice revealed no signs of cartilage damage in the midjoint (WT: 2.75 $\pm$ 0.85 and *Col5a1*^{+/-}: 2.0 $\pm$ 0.26, *p* = 0.3455) or the anterior/posterior sections (males: WT: 2.5 and *Col5a1*^{+/-}: 3.5, females: WT: 2.25 and *Col5a1*^{+/-}: 2.75) (Supplementary Fig. S3). Likewise, there was no damage in the lateral compartment of the knees (not shown).

DISCUSSION

Animal models are valuable tools in pain research that enable genetic and environmental standardization and in depth examination of pain-relevant tissues [27]. Pain is a major concern for patients with EDS, yet there is a substantial gap in the study of pain associated with EDS because no animal models for EDS pain have been described. Therefore, we explored whether *Col5a1*^{+/-} mice, a model that mimics cEDS clinically and molecularly

[53], exhibit pain-like behaviors [37,51]. We investigated these behaviors in both male and female mice since sexual dimorphism has been described in mice with chronic pain [26,46], but we did not aim to directly compare both sexes. Our study revealed that *Col5a1*^{+/-} mice of both sexes show signs of generalized mechanical, but not thermal, hypersensitivity. In addition, *Col5a1*^{+/-} mice vocalized more when they were scruffed. This may be indicative of increased sensitivity when being handled, but it could also be an anxiety- or stress-related behavior [17]. Spontaneous behaviors (distance travelled and rearing) were overall normal, but the time spent climbing was significantly lower in female *Col5a1*^{+/-} mice. Furthermore, both male and female *Col5a1*^{+/-} mice showed decreased grip strength, but after correction for body weight, this was maintained only in female and not in male mice. Decreased grip strength could be indicative of muscle weakness and/or painful movements. Grip strength has previously been used as a surrogate measure for deep tissue hyperalgesia and may thus indicate the presence of hyperalgesia in female *Col5a1*^{+/-} mice [18]. However, further evaluation is necessary to confirm if this is a true pain-related behavior or points to motor function impairment.

Since knee osteoarthritis is associated with pain-related behaviors, including mechanical hypersensitivity, in mice [23], we ascertained that *Col5a1*^{+/-} mice do not develop premature knee osteoarthritis up to the age 30 weeks. The low cartilage degradation scores in *Col5a1*^{+/-} mice suggest that the observed pain behaviors are not secondary to overt knee joint damage.

Collectively, these findings reveal for the first time that *Col5a1*^{+/-} mice show increased sensitivity to mechanical stimuli, which is indicative of sensitization of the nervous system in a mouse model for cEDS. Generalized hyperalgesia has been described in patients with hypermobile EDS, a molecularly unresolved EDS type, which clinically overlaps with cEDS [35,38] and hyperalgesia to pressure has also been documented in musculocontractural EDS due to *CHST14* defects [16,43]. To date, no reports have objectively assessed the occurrence of sensitization in cEDS patients, thereby representing a gap in the cEDS pain literature.

Our studies did not yet attempt to investigate the mechanisms underlying the observed hypersensitivity, but exploratory studies in Nav1.8 tdTomato-reporter mice revealed a strikingly abnormal nociceptive innervation of the glabrous skin in the footpad of *Col5a1*^{+/-} mice. Indeed, we found that, compared to WT mice, *Col5a1*^{+/-} mice show an aberrant innervation pattern of Nav1.8-positive nociceptors with a decreased number of nerves crossing from the dermis into the epidermis, despite an unaltered cutaneous nerve density. In addition, nerve endings in *Col5a1*^{+/-} mice tend to curve parallel to the epidermis-dermis junction more often compared to WT littermates (as clearly seen in the Supplementary Videos S1–S4). Decreased intra-epidermal nerve fiber density has been described in a study including 20 patients with joint hypermobility syndrome/hypermobile EDS and a single cEDS patient [7] and also in a hypermobile EDS case report [32]. Comprehensive cutaneous innervation data on cEDS patients are currently not available, but we plan to address this in the near future.

Type V collagen shows a broad tissue distribution, and it occurs in a variety of type I collagen-containing tissues, including skin, tendon, bone, muscle, cornea, placenta and fetal

membranes [54]. The predominant heterotrimeric $[\alpha 1(V)]_2\alpha 2(V)$ isoform, consisting of two $\alpha 1(V)$ - and one $\alpha 2(V)$ -chains, assembles with type I collagen and regulates fibrillogenesis of heterotypic type I/V collagen fibrils. [4] The minor $[\alpha 1(V)]_3$ homotrimeric isoform was shown to contribute to the stabilization of the epidermis-dermis interface in skin, where it has been described to have a bridging role between the basal membrane and the dermis [5]. It is interesting to speculate that in the *Col5a1*^{+/-} mice, nociceptors fail to cross the dermis-epidermis junction due to a reduced amount of $\alpha 1(V)$ -collagen chains. As such, it is possible that $[\alpha 1(V)]_3$ homotrimers have a guiding function during nerve growth. Type V collagen is relatively abundant in peripheral nerve tissue [9]. In Schwann cells, type V collagen is expressed as an $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ heterotrimer [9]. Moreover, it is also found in the endo- and perineurium of sciatic nerves [41] and in the nodes of Ranvier [21]. This suggests that type V collagen defects may directly influence the peripheral nervous system.

Peripheral nerves are surrounded by supportive layers of connective tissue, including endoneurium, perineurium and epineurium, providing flexibility and robustness to the nerve and supporting and protecting peripheral axons from mechanical and chemical insults [15]. During neural development and regeneration, constituent ECM molecules, such as collagens, are known to play important roles by providing guiding cues for growing axons [28,36]. It is therefore possible that the aberrant ECM resulting from a decrease in type V collagen as seen in *Col5a1*^{+/-} mice provides insufficient support for axon growth during development, thereby affecting sensory innervation, which might contribute to the decreased intra-epidermal nerve fiber density.

A recent study analyzed the transcriptome of dorsal root ganglia, spinal cord, brain, and blood in mouse models of nerve injury- and inflammation-induced pain and human low-back pain. This work identified ECM organization as a central molecular pathway in the development of chronic pain [31]. Taken together, it is tempting to speculate that a general disturbance of the ECM due to decreased type V collagen levels, as seen in *Col5a1*^{+/-} mice, can affect the proper organization and/or function of peripheral nerves leading to the observed alterations in dermal nociceptors, which may contribute to the observed pain phenotype.

The study of pain and pain-related behaviors in murine models for heritable connective tissue disorders is only in its infancy. Pain-related behaviors have recently been described in *Colla1*^{Jrt/+} mice, a recently developed model of severe dominant osteogenesis imperfecta [8]. These mice have significant skeletal abnormalities and develop spontaneous fractures, joint dislocations and vertebral deformities, similar to severe osteogenesis imperfecta in humans. *Colla1*^{Jrt/+} mice show increased hypersensitivity to mechanical and thermal stimuli, as well as reduced motor activity. Innervation of the glabrous skin of the hind paw and expression of the pain-related neuropeptide calcitonin gene-related protein in sensory neurons did not show any alterations in *Colla1*^{Jrt/+} mice [1]. In addition, a recent study provided evidence that tenascin-X, a glycoprotein that is deficient in classical-like EDS (cEDS, Supplementary Table S1) modulates neuronal behavior and function [2]. In *Tnxb* knockout (*Tnxb*^{-/-}) mice, colonic nociceptive afferents are hypersensitive and show increased sensory neuron sprouting [2]. A recent paper evaluated pain-related behaviors in *Tnxb*^{-/-} mice, and reported increased sensitivity to mechanical and chemical, but not

thermal, stimuli. Moreover, hypersensitivity of myelinated A δ and A β fibers was apparent and increased neuronal activation in the dorsal horn was shown in these mice [30]. These two examples add to our findings that the nervous system and associated pain-related behaviors are compromised in animal models of heritable connective tissue disorders, and further highlight the potentially important role of an intact ECM for proper innervation and pain processing.

Limitations of this study include the descriptive nature of the experiments and more in depth functional and mechanistic analyses are necessary. The lack of a phenotype in the thermal hypersensitivity experiments might be limited by the sensitivity of the thermal testing protocol since most mice outperformed the cold test and no positive control was included. Since no analgesic drugs that provide adequate and sustained pain relief for EDS are currently available, rescue experiments were not performed yet, but future studies aim to evaluate the effect of several analgesics.

The presence of a pain phenotype in *Col5a1*^{+/-} mice, in combination with the abnormal innervation, provide a unique and clinically relevant animal model for more detailed investigation of the mechanisms contributing to pain. Against the backdrop of record-high opioid overdose deaths in the United States [44], this is of utmost importance, since a recent study in 4294 EDS patients from the United States showed an almost double opioid use in the EDS cohort compared to the control group [40]. Therefore there is an urgent need for the development of safer, more efficacious, and potentially disease-tailored therapeutic strategies. Ultimately, better understanding of the mechanisms of pain in EDS may lead to the discovery of new targets for the development of effective therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Mechanical sensitivity. (A–B) Withdrawal threshold (in grams [g]) tested in the hind paw of (A) male (WT: $n = 9$, Col5a1^{+/-}: $n = 12$) and (B) female (WT: $n = 13$, Col5a1^{+/-}: $n = 10$) mice. (C–D) Response frequency (in %) tested in the abdominal area for (C) male (WT: $n = 10$, Col5a1^{+/-}: $n = 10$) and (D) female (WT: $n = 11$, Col5a1^{+/-}: $n = 18$) mice. (E–F) Area under the curve (AUC) calculated from C and D for (E) male (WT: $n = 10$, Col5a1^{+/-}: $n = 10$) and (F) female (WT: $n = 11$, Col5a1^{+/-}: $n = 18$) mice. Male and female Col5a1^{+/-} mice are depicted in black and gray, respectively, and their wild-type (WT) littermates with open circles. * $P < 0.05$ by unpaired t test.

Figure 2: Thermal sensitivity. (A and B) Withdrawal latency (in seconds [s]) at 0°C for (A) male (WT: n = 14, *Col5a1*^{+/-}: n = 17) and (B) female (WT: n = 10, *Col5a1*^{+/-}: n = 16) mice. (C and D) Withdrawal latency (in seconds [s]) at 55°C for (C) male (WT: n = 14, *Col5a1*^{+/-}: n = 17) and (D) female (WT: n = 10, *Col5a1*^{+/-}: n = 16) mice. Male and female *Col5a1*^{+/-} mice are depicted in black and gray, respectively, and their wild-type (WT) littermates with open circles.

Figure 3:

Spontaneous behavior and grip strength analysis. Spontaneous behavior assessed over 15 hours on the LABORAS platform in male (WT: $n = 12$, *Col5a1*^{+/-}: $n = 13$) and female (WT: $n = 14$, *Col5a1*^{+/-}: $n = 11$) mice. (A and B) Distance travelled (in meters [m]) over 15 hours. (C and D) Total time rearing (in seconds [s]) over 15 hours. (E and F) Total time spent climbing (in seconds [s]) over 15 hours. (G–J) Grip strength analysis is depicted either as absolute values (G and I, in grams [g]) or values corrected for body weight (H and J) for male (WT: $n = 19$, *Col5a1*^{+/-}: $n = 20$) and female (WT: $n = 14$, *Col5a1*^{+/-}: $n = 18$) mice. Male and female *Col5a1*^{+/-} mice are depicted in black and gray, respectively, and their wild-type (WT) littermates with open circles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ by unpaired t test.

Figure 5:

Dermal innervation. (A–D) Representative images of the glabrous skin of the footpad from male (B) and female (D) *Col5a1*^{+/-} mice crossed with *Nav1.8-tdTomato* mice and their wild-type (WT) littermates (A and C). Dermal *Nav1.8*-positive nerve fibers genetically labeled with tdTomato are red. Sections are counterstained with DAPI (blue). (E–H) The same images without the DAPI counterstain. Scale bar = 50 μm. e: epidermis, d: dermis. (I–L) Higher-magnification images of the regions boxed in (E–H). The dashed white line indicates the junction between dermis and epidermis. Scale bar = 50 μm.