The COL6A5-p.Glu2272* mutation induces chronic itch in mice

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Abstract

Pruritus is a common irritating sensation that provokes the desire to scratch. Environmental and genetic factors, altering barrier skin dysfunction, or hypersensitivity of sensory nerves, contribute to the onset of pruritus. However, the itch can become a major burden when it becomes chronic, like in neuropathic itch. The rare Collagen VI alpha 5 (*COL6A5*) gene variant p.Glu2272* was recently identified in two families and an independent patient with chronic neuropathic itch. These patients showed reduced COL6A5 expression in the skin and normal skin morphology. However, little progress has been made until now toward understanding the relationships between this mutation and chronic itch. Therefore, we developed the first mouse model that recapitulates *COL6A5*-p.Glu2272* mutation using the CRISPR-Cas technology and characterized this new mouse model. The mutant mRNA, measured by RT-ddPCR, was expressed at normal levels in dorsal root ganglia and decreased in skin. The functional exploration showed changes in the behavior of control individuals kept with mutant carriers and confirmed the effect in the mutant mice with some sex dysmorphology. Spontaneous scratching was detected in male and female mutants, with increased anxiety-like behavior in female mutants and despair-like behavior in sex-grouped mutants. These results suggest that the *COL6A5*-p.Glu2272* mutation found in patients contributes to chronic itch and probably induces additional behavioral changes. The *COL6A5*-p.Glu2272* mouse model could elucidate the pathophysiological mechanisms underlying *COL6A5* role in neuropathic itch and help identify potential new therapeutic targets.

Introduction

Itch (pruritus) is a common, irritating, unpleasant sensation that elicits the desire or reflex to scratch (Weisshaar et al. 2019). Clinically, the itch can be characterized as acute or chronic when it lasts more than 6 weeks (Yosipovitch et al. 2018). A European study revealed that the prevalence of acute itch in the general population is 8%, while it is 13% for chronic itch. Moreover, chronic itch with lifetime prevalence is 22% in the general population, indicating that more than 1 in 5 people experience chronic itch once in their life (Weisshaar et al. 2019). Moreover, the chronic itch greatly impacts the quality of life. Chronic itch is considered neuropathic when neuronal damage is responsible for the symptom (Stumpf and Ständer 2013). Damages at any site of the somatosensory system, including peripheral nerve fibers and ganglia, and the central nervous system, including the spinal cord, brainstem, thalamus, and cortex, may lead to neuropathic itch (Misery et al. 2014). Several conditions affecting the nervous system are associated with neuropathic itch. These include small fiber neuropathy (SFN), metabolic (e.g., diabetes), infections, and autoimmune and genetic diseases (Oaklander, 2011). The epidemiological studies investigating the prevalence and incidence of neuropathic itch have estimated that 8%-19% of chronic itch cases have a neuropathic origin (Meixiong et al. 2020; Pereira et al. 2021).

In recent years new insights have been gained. In particular, rare collagen VI alpha 5 (*COL6A5*, previously known as *COL29A1*) gene variants were identified in patients suffering from neuropathic itch (Martinelli-Boneschi et al. 2017). *COL6A5* is a member of the collagen protein superfamily. Collagens contain domains with VWA motifs that form filaments and are mainly associated with protein-ligand interactions for organizing tissue architecture and cell adhesion (Söderhäll et al. 2007). COL6A5 is expressed in epithelial tissues, including the lungs and gastrointestinal tract (Fitzgerald et al. 2008), with a high expression at the dermal-epidermal junction (Philippeos et al. 2018) and around the vessels of the reticular dermis (Sabatelli et al. 2011). Previously, Martinelli-Boneschi and colleagues identified a heterozygous c.6814G > T transversion in the *COL6A5* gene of chronic neuropathic itch patients, resulting in a p.Glu2272-to-Ter (E2272X) substitution. Autosomal dominant transmission of chronic neuropathic itch was reported in 5 patients from 2 unrelated families (families 1 and 2) using whole-exome sequencing (Martinelli-Boneschi et al. 2017). These patients carrying the p.Glu2272* nonsense mutation also showed reduced COL6A5 expression in the skin. However, due to the complex nature of neuropathic itch, progress in understanding the mechanisms leading to chronic itch in these patients has not been made yet.

In order to investigate the consequence of this mutation, we developed a new mouse model for the *COL6A5*-p.Glu2272* mutation using the CRISPR-Cas9 technology. Our data provide evidence for augmented spontaneous scratching behavior and increased anxiety and despair-like behaviors in mutant mice. This report demonstrates that the *COL6A5*-p.Glu2272* mutation contributes to itching, with anxio-depressive consequences.

Material and method

Animal research

Animal research was performed in agreement with the EC directive 2010/63/UE86/609/CEE, in compliance with the animal welfare policies of the French Ministry of Agriculture (Art. R. 214 – 107 and 214 – 122). Animal were bred and maintained in our animal facility which is accredited by the French Ministry for Superior Education and Research and the French Ministry of Agriculture (agreement #C67-218-40) following the French Law (Decree n° 2013 – 118 01 and its supporting annexes entered into legislation on 01 February 2013) relative with the protection of animals used in scientific experimentation. All experiments were done in agreement with the local ethical committee. Studies are reported in accordance with the ARRIVE 2.0 guidelines. Mouse breeding and behavior experiments were conducted in SPF (Specific Pathogen Free) conditions in our animal facility at PHENOMIN-ICS, following the 3R principle. Mice were kept grouped in two to five animals in each cage. Mice were kept in the controlled light cycle at 12 h light and 12 h dark (light turned on at 7 AM and off at 7 PM through an automated control system). Mice were kept under a controlled temperature of 21 ± 1°C and humidity of 55 ± 10% and had free access to food (standard chow) and autoclaved tap water. For all experiments, mice were transferred to the experimental facility of ICS two weeks prior to behavior tests so that they have some time for habituation to the experimental facility of ICS. Animals were transferred to the experimental room 30 min before each experimental test.

Generating the *COL6A5*-p.Glu2272* mice model using CRISPR/Cas9

The mutant mouse line *Col6a5*^{em1YahICS}, also named *Col6a5*^{E2302*}, for the human *COL6A5*-p.Glu2272* mutation, was generated in ICS using CRISPR-Cas9 technology. In the mouse, the mutant codon is located in exon 38 (Ensembl ID ENSMUSE00000891350.1; **figure S1**). The guide RNA had to drive the double strand break generated by the Cas9 protein very close to the site of insertion of the selection mutation (leading to a STOP codon). We used the CRISPOR online software (http://crispor.tefor.net/crispor.py) to select high specificity-score sgRNAs with a low number of predicted off-target sequences. This guide RNA, AGTCATGGCAGAGAAGAACT, had a specificity score of 52 (internal name gR52). The guide RNA was validated *in vitro* for its ability to drive DSB (Double Strand Break) on a PCR fragment containing the targeted sequence in the presence of Cas9 protein. A donor single-stranded oligonucleotide (donor ssODN) was designed that bares the expected mutation (G > T, corresponding to the rs11537567 human variant) as well as 2 silent mutations (A > G and A > C). Its sequence was AGCCACGTCCATGTAATATTCTTGAAGAGAAGCATCCCCAGGCT-CATGAGCAAGACCCAGCTCTTATCGGCCATGACTTTCATGTTCTGCAACAGAATAACTTTAGTCACTAGTGGTGAAAGGGTTAACTTG. Together, these 2 mutations generate a new HaeIII restriction site. In addition, a third A > G mutation was introduced after the new STOP codon. These 3 mutations generated 3 mismatches in the donor DNA that allowed to avoid new double strand breaks after the occurrence of homology directed repair.

The CRISPR guide efficiency was tested *in vitro* using a Sureguide kit (Agilent Technologies 5190–7716). In the presence of the Cas9 protein, the PCR product, including the region of interest, should be cut. A guide is validated if it cuts the target PCR fragment. A mix containing the Cas9 mRNA, the guide RNA and the ssODN was microinjected in fertilized C57BL/6N oocytes, and PCR screened the newborn offspring. The microinjection of a mix of gR52 guide RNA (12 ng/µl), spCas9 mRNA (25 g/µl), and 10 ng/µl ssODN in the male pronuclei of 355 C57BL/6N fertilized oocytes led to 28 pups. PCR followed by HaeIII restriction digests, screened the pups. Primers F1 (AATGGAAATAATTCTGCACCAAGTG) and R1 (TAAGACAGAGGTCAGTGGAGCTGGG) were used to amplify a PCR fragment of expected size 426 bps. In the presence of the mutations of interest, 2 fragments of 245 bps and 181 bps were detected after HaeIII restriction digest. All undigested PCR products from pups showing HaeIII digests were subsequently sequenced by Sanger sequence. The insertion of the new STOP codon was confirmed by Sanger sequencing. Eleven F0 pups (> 39%) had the expected mutations. Five F0 animals were bred with wild type (wt) C57BL/6N mice to generate F1 founders. F1 mice were analyzed for germline transmission by Sanger Sequencing to establish the *Col6a5*$^{em1\ -\ E2302*}$ mouse line. All founders gave heterozygous pups. Two lines were fully established and cryopreserved, and one was further studied, as shown in the present paper.

Determination of genotype

Crude genomic DNA was extracted from mouse tail samples through Direct PCR Lysis Reagent-Tail (Viagen Biotech, Cat # 101-T) according to the manufacturer's instructions. Subsequently, the Phusion Hot Start II High-Fidelity DNA polymerase kit (Thermo Scientific) was used with primers specific for the wt (+) and mutant (*Col6a5*^{E2302*}) alleles. Moreover, PCR reaction containing the following: 500 ng genomic DNA extracted from a wt or mutant mouse, 4 µl 5×Phusion HF Buffer, 0.4 µl dNTP mix (dATP, dCTP, dGTP, dTTP at 10 mM, Thermo Scientific), 0.2 µl each primer at 0.5 µM and H2O in a total volume of 20 µl. Using a T100 thermocycler (Bio-Rad), PCR was performed with the following thermal condition: 96°C for 5min followed by 30 cycles of 96°C for 8s, 62°C for 10s and 68°C for 45s, and then annealing temperature as 68°C for 5min with final elongation step for 5min at 72°C. Next, 10 µl of PCR outcome was used to digest with enzyme HaeIII 0.2 µl, 10X Buffer of volume 2 µl, and remaining H2O to make a total volume of 20 µl for each reaction and kept at 37°C incubator for 15min. 2% agarose gel was run for both digested and non-digested PCR outcomes and differentiated between wt and mutant mice. Our point mutation (PM) containing sequence of DNA was digested by HaeIII restriction enzyme into further 236 bps and 190 bps *Col6a5*^{E2302*}allele along with 426 bps wt allele. In contrast, the non-digested = uncut PCR outcome gave only a wt allele of 426 bps. Mice genomic DNA showing only one band of the wt allele (+) of 426 bps in digested PCR outcomes were identified as wild-type littermate mice (wt lit). When both the wt allele band of 426 bps and the 236 bps & 190 bps PM alleles (mut) in digested PCR outcome, mice were heterozygous. Similarly, when mice genomic DNA showed no wt allele of 426 bps and only two bands of 236 bps and 190 bps of PM allele in digested PCR outcome they were genotyped as homozygous mice. Primer sequence information is shown in **Table S1**.

Analysis of transcript expression level by Droplet digital PCR (ddPCR)

We determined *Col6a5* mRNA expression using Real-Time droplet digital PCR (RT-ddPCR) on 42-week aged mice that underwent the previous behavioral characterization. Eight control (wt), 5 heterozygotes (hets) and 6 homozygotes (homs) males, plus 10 wt, 7 hets and 6 homs females were used. Dorsal root ganglions (DRGs) were collected from wt and mutant mice and frozen in liquid nitrogen. Later, samples were lysed in TRIzol Reagent through magnetic beads containing Precellys®CK14 tubes. Total RNA was collected and purified using RNeasy Mini Kit (Qiagen) according to the protocol of the manufacturer company. Afterward, cDNA was synthesized by using the cDNA synthesis Kit "SuperScript™VILO™cDA Synthesis Kit" (Invitrogen). Then, ddPCR was performed for mRNA amplification in the volume of 20µL reactions for each sample. According to the manufacturer's recommendation, 250 nM specific primers, 125 nM of each probe, 1× ddPCR Supermix for Probes, and 50 ng DNA were used. PCR machine was run with the thermal condition as following: 10 min at 95°C, 40 cycles of 20s at 95°C, 30s at 59.2°C, 2min at 72°C, and 10min at 98°C. Droplet Digital PCR System (Bio-Rad) was used for droplet generation and quantification. Data were further analyzed using QuantaSoft Software (Bio-Rad). The sequences of primers and probes are shown in **Table S2.**

Evans Blue dye penetration assay for skin permeability

To examine the skin barrier integrity of mice, the Evans blue dye assay was performed on 42-week aged mice that underwent the previous behavioral characterization. This assay allows to detect skin barrier impairment as described previously (Zhang et al. 2018). Ten wt, 9 hets and 10 homs males, with 14 wt, 12 hets and 11 homs females were used. Just after euthanasia with cervical dislocation, the back of each individual was shaved, and Evans blue dye (Sigma, Ref# E2129) was applied as 1% in PBS and 50 µl/mouse. After two hours, the skin was collected and homogenized in formamide (Sigma, Ref#7503-). Subsequently, Evans blue dye extracted from skin samples was quantified by recording each sample by optical density (OD) at 620nm with a microplate reader.

Functional exploration and assessment

The behavioral characterization was performed on two experimental cohorts of males and females, ages 6 to 32 weeks. We used 13 B6N wt from the commercial breeders, 10 wt littermates, 12 hets and 10 homs males to evaluate scratching and grooming behaviors. For the female, 13 B6N wt, 14 wt littermates, 12 hets and 11 homs females were used. The same cohorts or parts of these cohorts were used for recording anxiety- and despair-like behaviors and social behavior. The experimenter was blind to the mouse genotype.

Scratching and grooming behaviors

Scratching and grooming behaviors were recorded during different sessions at 6, 12, 18, 26 and 32 weeks of age. To assess behaviors, mice were given 2 weeks in the experimental facility for adaption before assessment. For each session, mice were transferred 30 min prior to the beginning of the observations in the experimental room. Further, one day before the scratching observation, mice were placed for 30 min in transparent plastic experimental boxes for habituation. On the day of the experiment, mice were given 10 min in transparent plastic boxes for habituation before the evaluation began. Behind the transparent plastic boxes, a mirror was also positioned to assess mice behavior from the front and back view, as mentioned previously (Shimada and LaMotte 2008). Subsequently, mice behavior was video-recorded in the plastic box for 30 min as previously reported (Sun and Chen 2007; Shimada and LaMotte 2008; Liu et al. 2016). While analyzing the videos, different parameters were scored, including scratching time (i.e. the time of lifting of the hind paw to the region of the body that is scratched (back and face) and returning to the cage floor) **(Figure S2)**. While grooming behavior was analyzed for whipping mice with forelimbs and licking their body and tail (Shimada and LaMotte 2008). All scratching and grooming tests were performed between 8:00 AM and 2:00 PM.

Exploration and anxiety-like behaviors

Anxiety-related behavior was evaluated at 36 weeks of age on the same animals as those used for scoring scratching and grooming behaviors through an elevated plus maze experiment as previously described (Dubos et al. 2018). For locomotor and exploration activity, we also performed an open field test (Dubos et al. 2018). In an open-field experiment, a square apparatus (Panlab Harvard apparatus IR ACTIMETER, Bioseb, Vitrolles, France) containing all required sensors was used, and a polypropylene sheet covered the arena. Light for the open-field experiment was measured and kept at 150 lux in the center of the arena. Mice were placed at the periphery of the open field apparatus and were allowed to explore the arena freely for 30 min. The experiment was performed in a closed room without any experimenter disturbance. The automated system measured the total distance, number of rearings, and time spent in center and peripheral regions with video tracking and infrared sensors. Mice activity was recorded with a video tracking system (Ethovision, Noldus, France) during this session of 30 min.

Further, anxiety-like behavior was assessed with the elevated plus maze. The elevated plus maze apparatus was placed at a height of 50 cm above the floor. It was made of PVC and completely automated (Imetronic, Pessac, France). It has two enclosed arms with dimensions (30 X 5 X 15 cm) and two open arms with dimensions (30 X 5 cm). The apparatus has infrared sensors to detect different parameters for anxiety, such as the number of entries in open arms, time spent in the open or closed arms, and the number of attempts made by mice etc. Mice were placed on a central platform and their exploration of the maze was recorded for 5 min.

Despair-like behavior

Despair-like behavior was evaluated at the age of 38 weeks on the same animals as those used for scoring scratching, grooming, and anxiety-like behaviors through the tail suspension test as described previously (Dubos et al. 2018). In this experiment, mice were suspended with the help of tape and hanged for 6min, and immobility time was monitored using video recording. An increased immobility time is indicative of a despair-like phenotype.

Social behavior

Social behavior was determined at the age of 38-weeks on the same cohorts and before scoring of despair-like behavior. The Stoelting system (Dublin, Ireland) was composed of three successive identical chambers (20 cm × 40 cm × 22 cm) with (5 cm × 8 cm) openings to allow access between the chambers. The protocol used was similar to the previously described (Duchon et al. 2011; Arbogast et al. 2016). We used adult C57BL/6N mice as stranger mice (unfamiliar mice). The age and sex of stranger mice and the mice tested for their social behavior were the same. Stranger mice were kept separately to avoid any olfactory or visual contact with test mice. Before the day of the experiment, stranger mice were habituated in wire cages for 10min for 3–5 days until they felt comfortable staying in wire cages. The experiment was divided into three phases. In the first

phase, test mice were placed in the middle chamber and allowed to habituate for 10min. In the second phase (social exploration), the test mouse was enclosed in the central box, an unfamiliar mouse (stranger 1 or S1) was placed randomly in one of the wire cages, and on another side, an empty wire cage was placed. The doors were re-opened, and the test mouse was allowed to explore the entire social test box for 10 min. Time spent in each chamber, the number of entries into each chamber and the time spent sniffing each wire cage were recorded. In the third phase (social discrimination), a new, unfamiliar mouse (stranger 2 or S2) was placed by replacing the empty wire cage, and the test mouse was allowed to explore for 10 more min. During this time, the test mouse could explore or sniff the already-investigated mouse (S1) and the novel unfamiliar mouse (S2). The entire social test box was washed with tap water and dried with absorbent paper between each test to remove odors.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM) for each experimental group. Student's t-test (two-tailed) was used to compare the two groups' differences. In addition, multiple groups were compared using one-way or two-way repeated measures analysis of variance (ANOVA) with a Tukey post-hoc test where appropriate. Data was analyzed by using GraphPad Prism 9 software. For all analyses, a p-value was considered significant as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Generation of the COL6A5-p.Glu2272* mice model

The *COL6A5*-p.Glu2272* mouse model for the human mutation c.6814G > T (ID rs115535867) p.Glu2272* was generated by homologous directed repair through a CRISPR/Cas9 approach in the exon 38 (Fig. 1A). The *Col6a5*^{em1} (E2302*) carrier mice line displayed the mutation as shown by both PCR analysis and Sanger sequencing (Fig. 1B-D). Mutant heterozygous mice were inter-crossed to obtain wt, heterozygous and homozygous littermates. The *Col6a5* transcript expression level was evaluated in DRGs isolated from individuals with different genotypes through RT-ddPCR, and the results are shown in Fig. 1E. *Col6a5* transcripts from the wt allele were detected in control littermate mice of both sexes. In homozygous mice, only the mutant transcripts were found expressed. In heterozygous mice, both wt and mutant alleles were expressed at comparable levels in both sexes. On the other hand, in skin, mRNA expression was decreased significantly in mutants as compared to controls (Fig. 1F). This observation was similar to that for patients with chronic neuropathic itch harboring the p.Glu2272* mutation: COL6A5 expression was reduced in the papillary dermis and around the dermal vessels compared to controls (Martinelli-Boneschi et al. 2017). Nevertheless, it is important to indicate that there was no significant sex difference in the reduction of *Col6a5* expression in the skin. *Col6a5* transcript expression levels were similarly decreased in both sexes. Yet, the expression of *Col6a5/Tbp* in the skin was noticeably lower in wt females vs males, with a value of 0.014 compared to 0.05 in wt males, implying that *Col6a5/Tbp* expression in wt females is approximately 3.57 times lower than in wt males. This baseline difference in *Col6a5* expression between wt males and females may result in a more pronounced impact of the mutation in females.

Increased skin permeability in COL6A5-p.Glu2272* mutant mice

To assess the impact of the mutation on the skin barrier, we investigated skin permeability in wt and mutant animals using the Evans blue dye assay. We observed that both heterozygous and homozygous females showed a significant increase in skin permeability compared to wt females (Fig. 2). However, no difference was found between mutant and wt male mice. Control wt females had lower skin permeability compared to wt males. Altogether, these results indicate a sexually dimorphic effect of the p.Glu2272* mutation on skin permeability in mice.

COL6A5-p.Glu2272* mice displayed spontaneous scratching behavior

The patients with the *COL6A5*-p.Glu2272* mutation complained of spontaneous chronic itch appearing at ages ranging from 5 to 40 years (Martinelli-Boneschi et al. 2017). To investigate spontaneous itch in mutant mice, we scored their scratching

behavior (time of scratching and number of bouts during 30 min) at different ages, starting from 6 weeks until 32 weeks (Fig. 3). Because scratching behavior was previously shown to be contagious and imitative in mice (Yu et al. 2017), we defined two different types of controls: a first set made of B6N unrelated wt aged-match controls (B6-Unr), that were kept in separate cages to prevent any physical or visual contact with the mice of the mutant line; and a second set of controls were the wt littermates (B6-Lit) of the mutant mice bred in the same cage with mutant carriers. The comparison of the B6-Unr and B6-Lit showed that B6-Unr female mice had an effect of age and an interaction between age and genotype but no genotype effect (two-way RM ANOVA, P = 0.01 and P = 0.006, respectively). Interestingly, B6-Lit males had increased scratching in littermates as compared to unrelated B6 (two-way RM ANOVA P = 0.032 for genotype). On sex-grouped animals, we observed a comparable scratching behavior in B6-Unr and B6-Lit (two-way RM ANOVA P = 0.015 for age). Altogether, we could not detect a strong effect between the two groups of control by analyzing at each time point.

We tested whether the *Col6a5*^{em1}(E2302*) mutant mice showed altered scratching at 6, 12, 18, 26, and 32 weeks. For this, we observed the time spent scratching of the four groups, i.e. the 2 controls as well as heterozygotes and homozygotes. Because we observed some variation of scratching over the weeks, we also calculated the Area Under the Curve (AUC) for further statistical analysis. An increase in the time spent scratching over the weeks was unraveled when comparing heterozygote or homozygote females with both wt control groups (One-way ANOVA with Tukey's multiple comparisons test; ($F(3, 46) = 12.27$; $P < 0.0001$; Fig. 3 left panels). No difference was observed between Het and Hom mutant females. An effect on scratching time was also detected in Het males ($F(3,40) = 17.68$; $P < 0.0001$), with increased time of scratching, and the increase was slightly lower in male homozygotes compared to heterozygotes. When considering the two sexes together, the increase of scratching in mutants versus the two wt controls was more significant ($F(3,90) = 23.36$; $P < 0.0001$), that may be due to the increased number of individuals as compared to single-sex analysis.

Further, we compared the number of bouts per 30 min with a similar analysis and calculation of the AUC (Fig. 3, right panel). Briefly, the number of bouts was increased in heterozygotes and homozygote mutant females as compared to the 2 wt controls ($F (3,46) = 14.36$; $P < 0.0001$). The genotype effect was also observed in the males ($F(3,41) = 15.57$; $P < 0.0001$) and when both sexes were analyzed together (sex-grouped, ($F(3,91) = 25.37$; $P < 0.0001$). Overall, despite the lack of patients described with homozygous mutation, homozygous mice showed similar scratching behavior as heterozygous mice. These results obtained on the *Col6a5*^{em1}(E2302*) mouse model confirmed the dominant effect of the mutation. A relation of causality can now be drawn with one copy of the *COL6A5*-p.Glu2272* mutation being enough to produce the scratching phenotype.

COL6A5-p.Glu2272* mice showed increased grooming behavior

Induction of chronic itch in mice also led to increased grooming behavior (Wang et al. 2018). Therefore, we decided to investigate the spontaneous self-grooming behavior without administering any external inducer. Grooming behavior was recorded for 30min through video recording (Fig. 4). Increased grooming was detected in homozygous females (One-way ANOVA; $F(3, 46) = 3.773$; $P = 0.0167$) and males (One-way ANOVA; $F(3, 41) = 3,300$; $P = 0.0297$). Combining the two sexes showed more significant differences (One-way ANOVA; $F(3, 91) = 4.820$; $P = 0.004$). Overall, the data demonstrate that the p.Glu2272* mutation leads to augmented grooming.

Increased anxiety and despair-like behaviors in *COL6A5*-p.Glu2272* mice

Chronic itch is clinically correlated with mood disorders such as anxiety and depression (Wang et al. 2018; Long et al. 2022). Such itch-associated mood disorders have already been studied to characterize the affective consequences of chronic itch in mice (Zhao et al. 2018). Therefore, we also investigated these disorders in the mutant mice and performed anxiety and despair-like behavioral tests. We tested the mutant mice in the elevated plus maze (EPM) to assess anxiety-like behavior. We evaluated three different parameters of the EPM, 1) % of open arms entries, 2) % of the time in open arms, and 3) the number of attempts (Fig. 5). We observed that male mutant mice showed no difference in all three parameters. On the other hand, female homozygous mice showed significant anxiety-like behaviors by reduced entries into open arms and less time spent in open arms than their wild-type counterparts.

We also tested the mutant mice for their behavior in the open field test measured to assess the basic status of mice. There was no difference between mutant and wt mice for any of the parameters studied which were the time spent in the center, rearing number, and total distance traveled (Figure S3). Overall, these results indicate that the p.Glu2272* mutation induced more anxiety in females as measured in the EPM.

In chronic pruritus, the “itch-scratch-itch” cycles may lead to a depressive state which was also studied in mice previously (Wang et al. 2018). To investigate the despair-like behavior in < p.Glu2272* mutant mice, we tested them in the tail suspension test (Figure S4). No difference was found between genotypes (One-way ANOVA).

COL6A5-p.Glu2272* mice show no deficit in social preference but male mutant displayed altered social discrimination

We have also investigated the social behavior of mutant mice by using the three-chamber test. In the first experiment, it was found that mice of all three genotypes, wt, heterozygous and homozygous mice, had a higher sniffing time toward a stranger mouse (S1) than in the empty compartment (E) (Figure S5). This showed that mutant animals had a social preference behavior comparable to their wt counterparts. In the second part of the test, the preference for social novelty was measured by placing another novel stranger mouse (S2) in the empty compartment and comparing the interactions of the test mouse with the familiar stranger (S1) mouse and a novel stranger (S2) mouse. We observed that wt male mice showed more sniffing time towards the novel stranger mouse. In contrast, mutant males did not show any preference for a novel stranger (Fig. 6), suggesting that the mutant males have a deficit in social discrimination. For females, none of the genotypes preferred the novel stranger (S2) vs the familiar stranger (S1), precluding any conclusion. Overall, the results from the two phases of the three-chamber test indicate no alteration of social exploration in the mutant males and females and a deficit in social discrimination in the mutant males.

Discussion

We successfully generated the *Col6a5*^{E2302*} mutant mouse model corresponding to the human *COL6A5*-p.Glu2272* mutation using the CRISPR/Cas9 technology. We used this genetic model we confirmed the genotype-phenotype association of this mutation with chronic itching in mice. We explored molecular aspects of this mutation and its consequences on other behaviors. We found that the wt and mutant *Col6a5* transcripts were expressed at similar levels in DRGs. However, mutant transcripts were expressed at lower levels in the skin, in accordance with the decreased level of COL6A5 expression found in patients’ fibroblasts of the affected families in the clinical study (Martinelli-Boneschi et al. 2017). We also found enhanced skin permeability in mutant female mice. Further, we observed spontaneous itch in adult p.Glu2272* mutant mice, providing evidence of long-lasting itch with consequences on emotional behaviors.

Mutant female mice showed enhanced skin permeability compared to their wt littermates, which could be due to several factors. For instance, a study in Human reported that in skin dermis higher concentration of collagen in the skin was correlated with the thickness of the skin. Another study reported the differences in skin structure in male and female mice (Azzi et al. 2005). These observations were also confirmed in other studies (Calabro et al. 2011; Arai et al. 2017). Interestingly, this article (Arai et al. 2017) demonstrated that the expression of *Col1a1, Col1a2*, and *Col3a1* was higher in the skin of male mice than in female mice, while *Col5a1* and *Col4a1* were expressed similarly in both sexes. Our results show a similar expression of wt *Col6a5* transcripts in male and female skin. We can hypothesize that being expressed close to the skin barrier, a change in COL6A5 structure may affect the permeability of the skin. This is an interesting hypothesis to follow, considering the association of COL6A5 polymorphisms with atopic dermatitis (Szalus et al. 2023) and an additional hypothesis based on COL6A5 + dermis fibroblasts contributing to skin inflammation (Li et al. 2023).

The clinical study by Martinelli-Boneschi and colleagues showed that all individuals in the two families with the p.Glu2272* mutation showed chronic itch at various ages. Family 1 had three patients, including one female and two males, and family 2 had two female patients. In all five patients, the onset age for the appearance of spontaneous chronic itch was different but shared similar characteristics. In family 1, two males and one female patients reported onset age for chronic itch as 30, 33, and 40 years respectively. In family 2, the two patients reported age onsets for itch 5 and 8 years (Martinelli-Boneschi et al.

2017). Considering these early and adult ages for the onset of itch, in this study, we observed the scratching behavior of mutant mice over an extended period from early age to late age (6 weeks to 32 weeks). When the analysis started, at six weeks of age, the mice were still considered at a young age. Though many of the physiological systems were mature, the immune and nervous systems continued to be settled within the next 2 weeks. Indeed, before 8 and 11 weeks of age, the mouse systems were under ongoing maturation, marking by the end of rapid changes in mass and cell number in the mouse central nervous system and getting the various brain structures to reach their adult-like states during this age (Fu et al. 2013). Therefore, in this study, mice scratching behavior was observed not at a specific age but for an extended period. In our study, we have monitored for extended and continuous period as compared to other chronic itch model studies where often the tests are only done for a few weeks. For example, Ueda and colleagues produced a chronic itch mice model and observed scratching behavior till 8 weeks (Ueda et al. 2006). To gain a deeper understanding of spontaneous chronic itch, we argue that it is crucial to conduct analysis over an extended period of time. For instance, another study was performed for spontaneous chronic itch till 14 to 20 weeks of age (Zhao et al. 2013). For this reason, our study's approach of conducting a more prolonged and continuous assessment of genetically induced spontaneous chronic itch is more valuable in this context, enabling a more comprehensive exploration of chronic itch.

Recently, mood impairment was reported for the first time in male mice with chronic itch induced by a repetitive treatment with acetone and diethyl ether followed by water which models dry skin, the AEW model (Zhao et al. 2018). Also, anxiety-like behaviors were detected in mice with histamine or serotonin-induced acute itch (Sanders et al. 2019). When analyzed for mood alterations, only the female *Col6a5*^{E2302*} homozygotes showed anxiety in the elevated plus maze but no despair-like phenotypes, while males were not affected. Interestingly, both *Col6a5*^{E2302*} heterozygous and homozygous male mutants showed a deficit in social preference in the three-chamber test. This defect in discrimination may be due to attention deficit as a result of increased itch. Further exploration of the emotional and social behaviors of *Col6a5*^{E2302*} mutant mice is needed to decipher whether these altered behaviors are a direct or indirect effect of increased itch or a secondary effect of the *Col6a5* mutation itself.

This is the first study to report a mouse model of chronic itch developed from a mutation in a collagen gene identified in chronic neuropathic itch patients. Previously, another mutation, the L811P mutation in the *SCN11A* gene, has been identified in patients with chronic itch and shown to cause chronic itch in the *Scn11a*^{L811P} mouse model (Salvatierra et al. 2018; Ebbinghaus et al. 2020). Our mouse model provided useful insight for investigating the pathophysiology of human mutation *COL6A5*-p.Glu2272* as well as chronic neuropathic itch. Further, this study provided evidence that chronic neuropathic itch can be associated with other behavioral phenotypes, affecting anxiety and sociability. Together, these findings may open novel avenues for the study of chronic neuropathic itch and its underlying mechanisms and help identify new therapeutic targets for treating chronic itch.

Declarations

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Figures

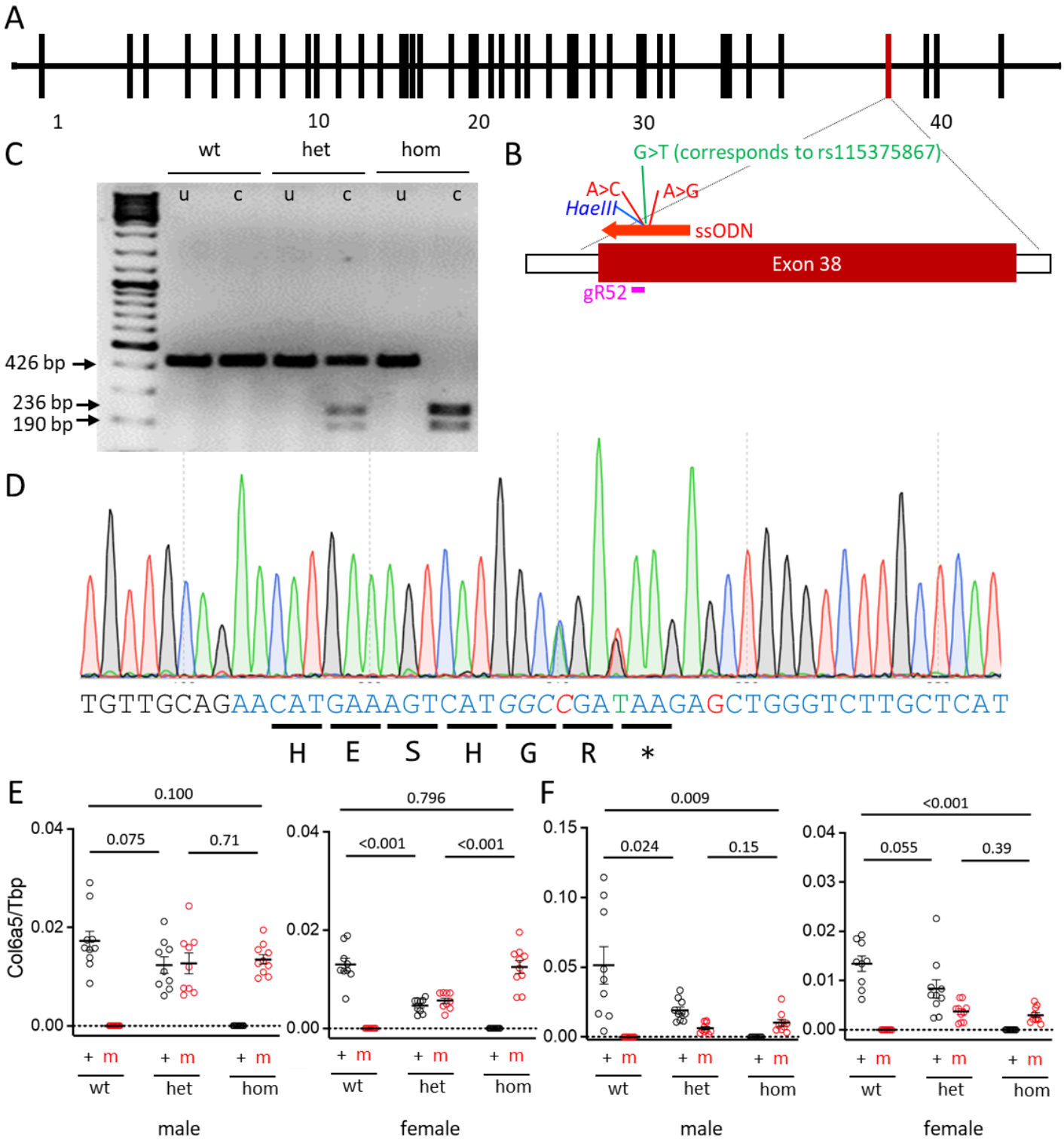

Figure 1

Generation and validation of the *COL6A5*-p.Glu2272* mouse model. A) Organization of the murine *Col6a5* gene. The numbers indicate the position of exons. The target exon (exon 38) is shown in red color. B) A donor single stranded oligonucleotide (ssODN) was designed. This donor bares the expected mutation (G>T, corresponding to the rs11537567 human variant) as well as 2 silent mutations (A>G and A>C). These 3 mutations also create 3 mismatches so that no new DSBs (double-stranded breaks) could occur after. Using CRISPOR online software, high specificity-score sgRNAs (gR52) were selected with low predicted off-target sequences. C) The DNA sequence containing point mutation (PM) was digested by HaeIII enzyme “c (cut)”

into further 236 bps and 190 bps PM allele along with 426 bps wt allele. In comparison, the non-digested “u(un-cut)” PCR outcome only gave wt allele of 426 bps. D) Through Sanger sequencing, PM mutation was confirmed and is shown with the stop codon (*). The wild-type (+) and mutant allele (mut) mRNA expression in DRG (E) or skin (F) isolated from *Col6a5* littermates (from 5 to 10 individuals per group) with the three genotypes: wt, heterozygous (het), and homozygous (hom) mice. *Col6a5* mRNA expression was normalized to *Tbp* expression as a control. In the DRG, Homozygous mutant mice expressed *Col6a5* mutant transcripts at levels comparable to “+” transcripts in wt mice, and heterozygous mice expressed comparable levels of each allele. F) The wt and mutant allele (Mut) mRNA expression in the skin of wt littermate, heterozygous (Het), and homozygous (Homo) mice for *Col6a5*^{em1}(E2302*). Mutant mice have significantly decreased *Col6a5* mRNA expression in the skin. Males, n=9-10/group; females, n=9-10/group. Student’s t-test.

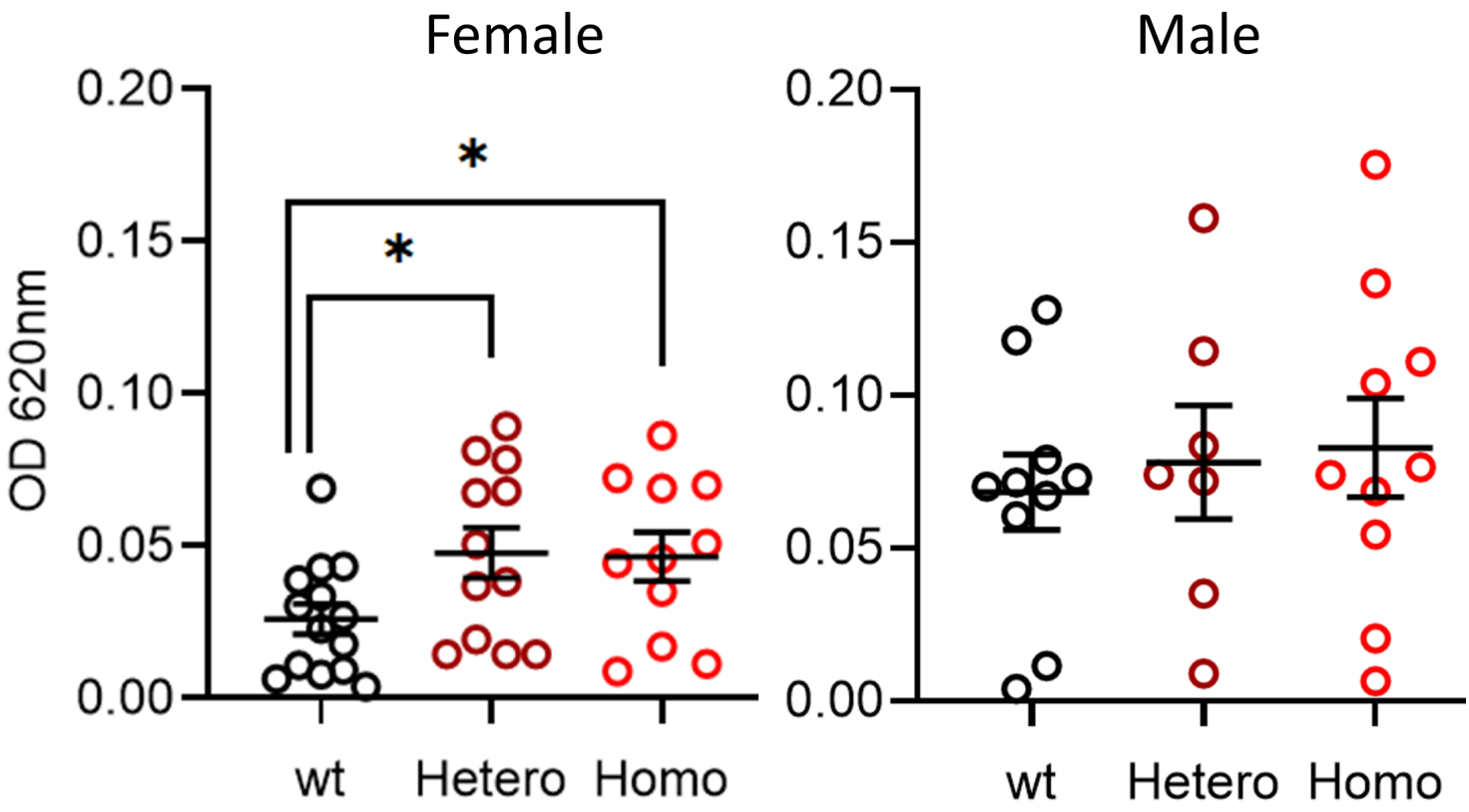

Figure 2

Increased skin permeability in COL6A5-p.Glu2272* mice. The permeabilization assay done on skin, on heterozygous (Hetero) and homozygous (Homo) skin mice showed significant differences in female mutants compared to wt littermates (wt) (n=11-14 /group; unpaired t test,*P < 0.05). No genotype difference was found in males (n=7-10 /group). Wt females had lower skin permeability compared to wt males (unpaired t test,P=0.002).

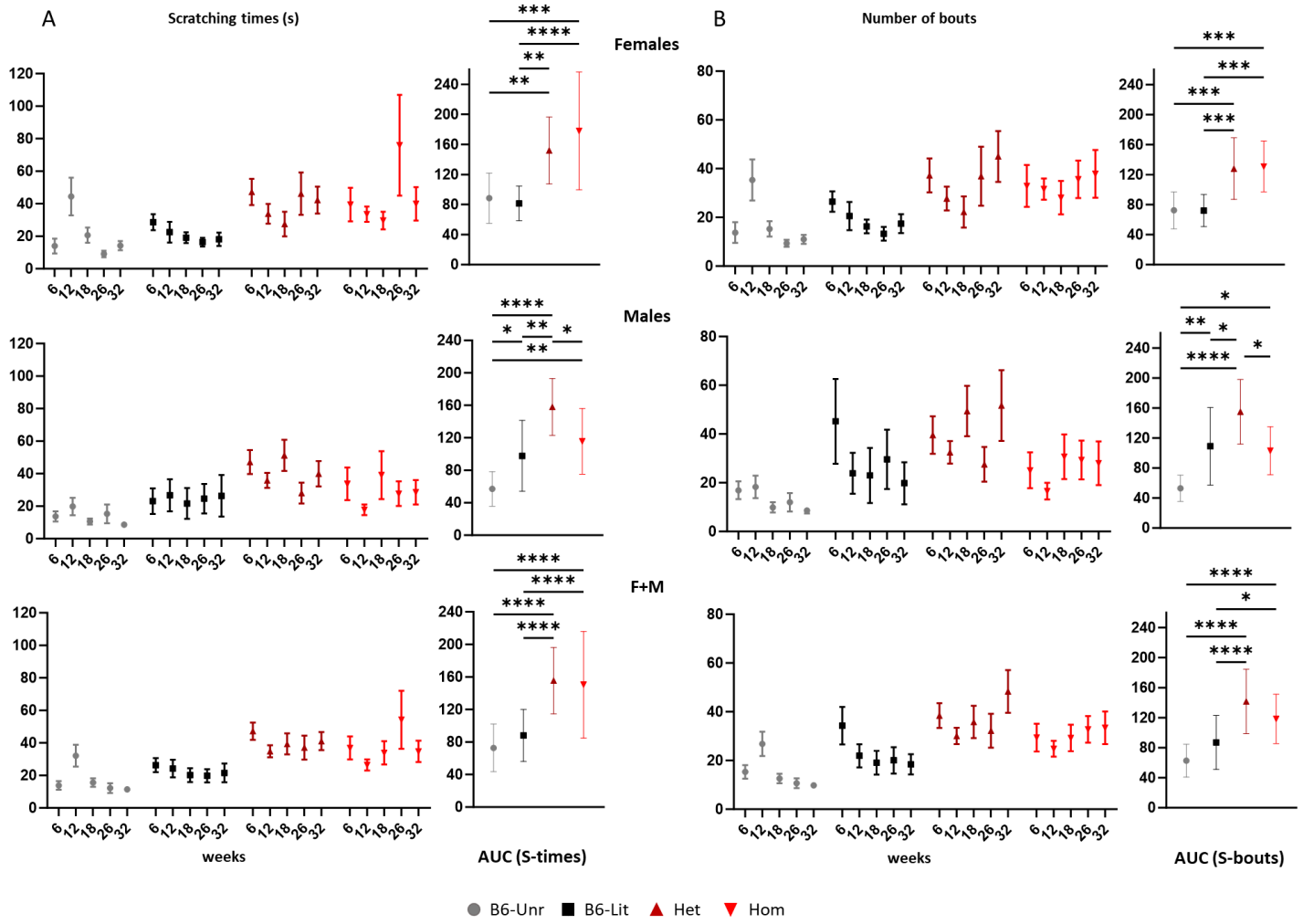

Figure 3

***COL6A5*-p.Glu2272* mice mice showed increased spontaneous scratching behavior.** Scratching times (s) (A) and number of bouts (B) were scored at 6, 12, 18, 26 and 32 weeks of age on individuals, from both sexes of unrelated B6N wt (B6-Unr) mice and of the different littermates, either wt (B6-Lit), heterozygous (Het) or homozygous littermate (Hom) genotypes, derived from *Col6a5*^{em1}(E2302*) heterozygous crosses. Overall, the mutant mice, either het or hom, showed significantly increased scratching, as evaluated by calculating the Area Under the Curve (AUC) for the scratching times (left) or the number of bouts (right) and determined by One-way ANOVA with Tukey's multiple comparisons test. (Male, n=9-13 /group; Female, n=11-14 /group). Data presented as mean +/- sem. P-value as indicated with * P < 0.05, ** P < 0.01, and *** P< 0.001 and **** P<0.00001.

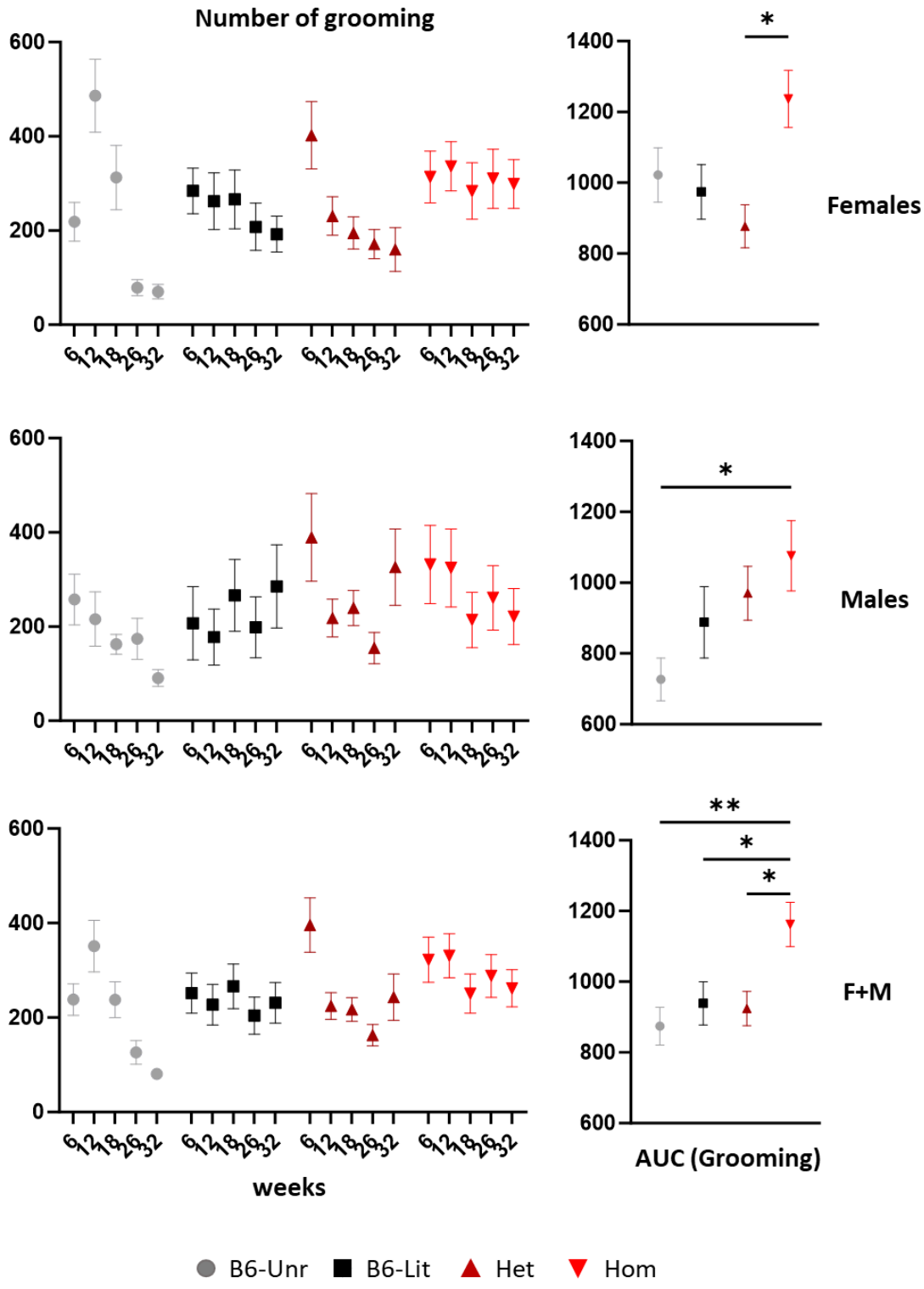

Figure 4

Increased grooming behavior in *COL6A5*-p.Glu2272* homozygote mice. Spontaneous self-grooming behavior was evaluated in B6N unrelated wt (B6-Unr), wt littermate (B6-Lit), heterozygote (het) and homozygote mutant mice over 6 weeks to 32 weeks of age by session of 30min (left panel). The Area Under the Curve (AUC) of grooming along the period showed that homozygotes displayed a significant increase, compared to het littermate in females and B6-Unr in males. When comparing males and females together the significance was observed between homozygotes and all the other genotypes. Male, n=9-13 /group; Female, n=11-13 /group. A p-value was considered as significant as *p < 0.05 and **p < 0.01.

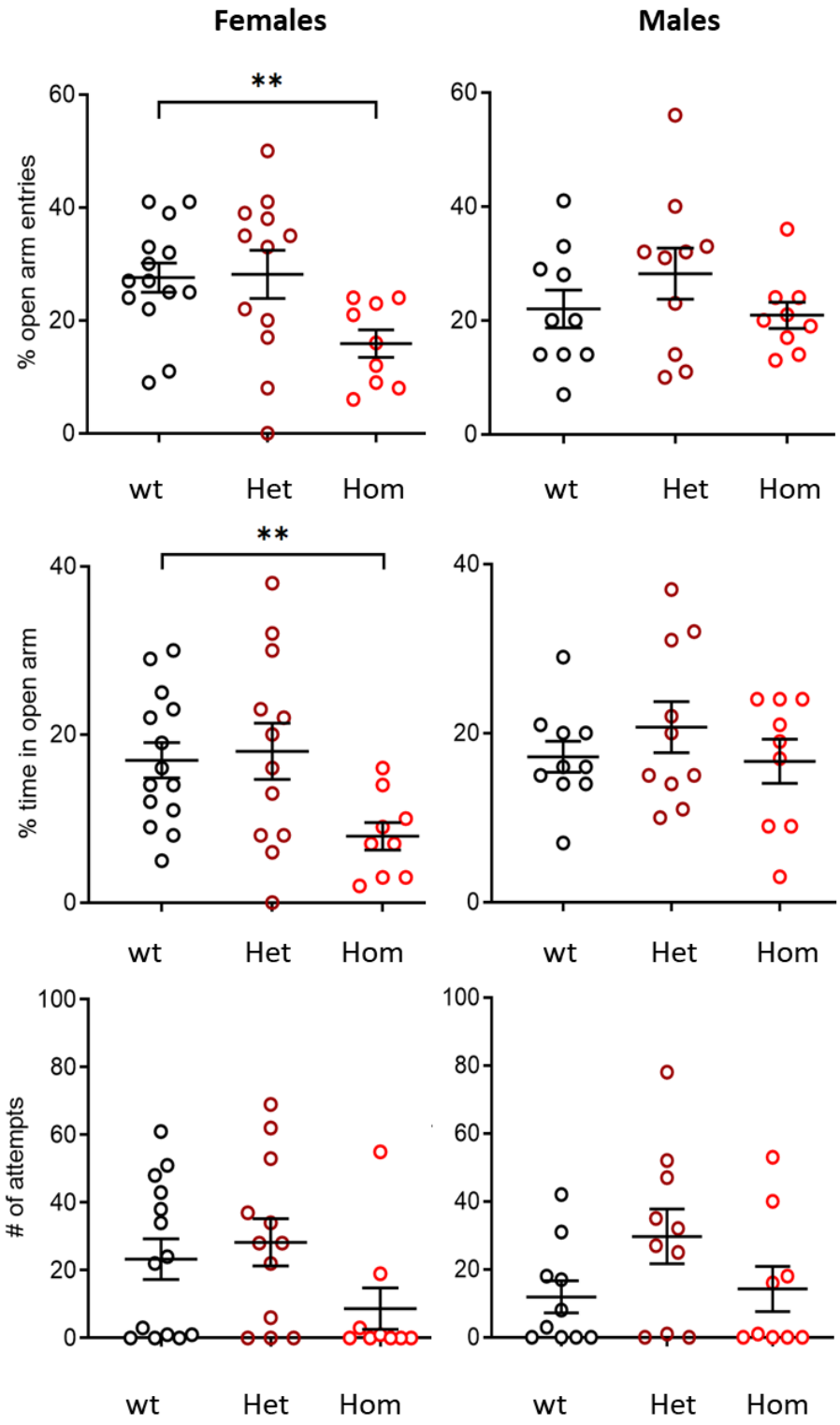

Figure 5

Increased anxiety-like behavior in *COL6A5*-p.Glu2272* homozygous females. Mutant and wt littermates control mice were assessed in the elevated plus maze test through three different parameters. **A)** % of open arms entries, **B**) % of the time in open arms, and **C)** the number of attempts. It was observed that female homozygous mice showed significant anxiety-like behavior by reduced entries into the open arm and spent less time in open arms as compared to wt mice. Overall results showed that homozygous mice have an anxiety-like behavior phenotype compared to wild-type mice. Male, n=9-10 /group;

Female, n=9-14 /group. wt littermate (wt), heterozygous (Hetero), and homozygous (Homo) mice were compared. A p-value was considered as significant as *p < 0.05 and **p < 0.01.

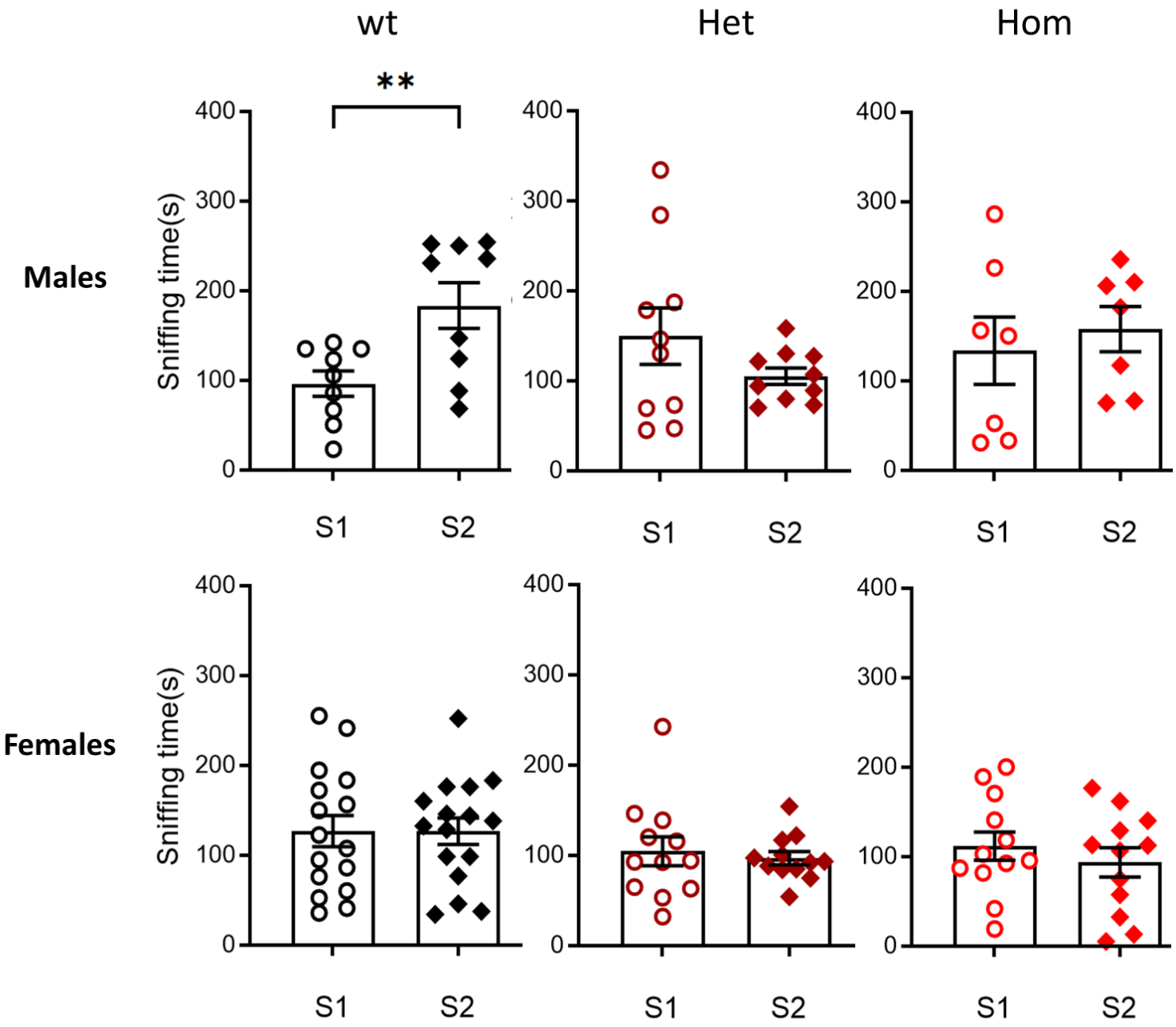

Figure 6

***COL6A5*-p.Glu2272* male mice showed a deficit in social discrimination.** In the three-chamber test, the preference for social novelty was measured by placing a new stranger mouse in the empty compartment and comparing the interactions of the test mice with the familiar stranger 1 (S1) mouse and the novel stranger 2 (S2) mouse. Male wt littermates (wt) mice showed more sniffing time towards the novel stranger S2, while mutant males did not. All of the female mice groups were impaired for discriminating social novelty. Heterozygous (Het) and homozygous (Hom) mice. Males, n=7-10 /group; females, n=12-16 /group. A p-value was considered as significant as **p < 0.01.

Supplementary Files

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