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Sex-Dependent Sensory Phenotypes and Related Transcriptomic Expression Profiles Are Differentially Affected by Angelman Syndrome

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Abstract

Angelman syndrome (AS) is a genetic disorder which entails autism, intellectual disability, lack of speech, motor deficits, and seizure susceptibility. It is caused by the lack of UBE3A protein expression, which is an E3-ubiquitin ligase. Despite AS equal prevalence in males and females, not much data on how sex affects the syndrome was reported. In the herein study, we thoroughly characterized many behavioral phenotypes of AS mice. The behavioral data acquired was analyzed with respect to sex. In addition, we generated a new mRNA sequencing dataset. We analyzed the coding transcriptome expression profiles with respect to the effects of genotype and sex observed in the behavioral phenotypes. We identified several neurobehavioral aspects, especially sensory perception, where AS mice either lack the male-to-female differences observed in wild-type littermates or even show opposed differences. However, motor phenotypes did not show male-to-female variation between wild-type (WT) and AS mice. In addition, by utilizing the mRNA sequencing, we identified genes and isoforms with expression profiles that mirror the sensory perception results. These genes are differentially regulated in the two sexes with inverse expression profiles in AS mice compared to WT littermates. Some of these are known pain-related and estrogen-dependent genes. The observed differences in sex-dependent neurobehavioral phenotypes and the differential transcriptome expression profiles in AS mice strengthen the evidence for molecular cross talk between Ube3a protein and sex hormone receptors or their elicited pathways. These interactions are essential for understanding *Ube3a* deletion effects, beyond its E3-ligase activity.

Keywords Angelman syndrome · Ube3a · Sex · Behavioral phenotypes · Transcriptome · Bioinformatics analyses

Lee Koyavski and Julia Panov are of equal authorship.

Highlights
- AS mice exhibit aversion towards novelty while healthy mice are attracted to novelty.
- AS mice exhibit olfactory sensitivity deficits compared to WT mice littermates.
- AS mice exhibit motor deficits especially when coordination or proprioception is required.
- AS mice show altered repetitive behavior and enhanced anxiety.
- AS mice exhibit aberrations in male-to-female variations, mainly in sensory phenotypes.
- RNA sequencing yielded genes with opposite sex–dependent expression profiles between WT and AS mice that reflect the pain perception phenotype.
- Some of these genes with opposite sex–dependent expression profiles are related to pain perception.

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Introduction

Angelman syndrome (AS) is a genetic neurodevelopmental disorder, characterized by cognitive and motor impairments, developmental delay, motor deficits such as ataxia of gait or tremor, intellectual disability, absence of speech, and easily excitable personality with frequent laughter and happy demeanor [1]. Most AS individuals also have seizures, and repetitive or stereotyped behaviors [1]. The cause for AS is the loss of function of UBE3A protein in the brain. UBE3A plays a critical role in processes of learning and memory through synaptic dependent plasticity [2–4]. In the brain, the paternal *UBE3A* gene is imprinted and only the maternal copy is expressed. The majority of AS cases are due to deletion of small portions of the maternal chromosome 15 (q11–13) that contains the *UBE3A* gene [5, 6]. Many features of AS, such as impaired motor function and cognitive deficits in learning and memory as well as the imprinting itself, have been replicated in

animal models, mainly in mice [2, 3], making these models an efficient tool for investigating AS. In general, the life expectancy of most AS subjects appears to be normal, with relatively good health, though subjects with this disorder are not able to function independently at any stage of their life [7]. The prevalence of AS in the general population is estimated between 1:10,000 and 1:40,000 [8], while AS incidence is approximately 1:10,000 to 1:20,000 live births [1]. AS patients have an equal sex distribution between males and females [1]. However, despite a quite comprehensive behavioral characterization, most behavioral studies with AS animal models were either performed exclusively in males [9] or did not report sex dependent differences [10, 11]. Hence, it is imperative to explore the possible sex differences in AS phenotypes, especially given that sex hormones, mainly estrogens, play a vital developmental role in cognition, sensory processing, and motor performance [12, 13]. To the best of our knowledge, only three studies explored sex differences in AS mouse model; two of them addressed only body weight differences [4, 10], and the third recent study examined a few mainly motor and depressive-anxiety behaviors [14].

In the herein study, we show that sex affects various cognitive, behavioral, and sensory phenotypes in AS mouse model while other phenotypes, such as motor performance, are mostly unaffected. In addition, mRNA sequencing analyses with emphasis on sex differences show many genes with opposite sex–dependent mRNA expression profiles between wild-type (WT) and AS littermates, which reflect the behavioral, sensory phenotypes, especially pain perception. Some of these genes with opposite sex–dependent expression profiles are known to be pain related, and some are estrogen dependent.

Materials and Methods

AS mice and wild-type littermates on C57BL/6 background strain were generated and genotyped as described previously [2]. All along the manuscript, the term "group" refers to the combination of genotype and sex, so the four groups compared are WT females, WT males, AS females, and AS males.

Supplementary Methods

For a detailed description of the animals, behavioral experiments, statistical analyses, tissue preparation for RNA sequencing, RNA sequencing, and bioinformatics analyses, see the Additional Material.

Results

Hippocampal-Dependent Memory

Previous studies reported that AS mice showed severe deficits in contextual fear conditioning (CFC) and Morris water maze (MWM), which are hippocampal-dependent memory tasks [2–4, 15–17]. However, none of the studies examined whether sex plays a role in hippocampal deficits in AS mice. Hence, we performed CFC followed by testing memory retention at 24 h and 1 week after conditioning. At first, we analyzed the freezing response during the conditioning phase, which showed no significant difference between WT and AS littermates ($F(4,184) = 0.54$, $p = 0.71$ for interaction of genotype × time in two-way repeated measures ANOVA (RM-ANOVA)) (Fig. 1A). However, further focusing on sex differences within the genotypes showed a very surprising effect; WT females exhibited significantly enhanced freezing response to the shocks compared to the WT males, while AS mice showed the exact opposite effect ($F(12,176) = 34.57$, $p < 0.0001$ for interaction of group (sex and genotype) × time in two-way RM-ANOVA, post hoc comparisons: WT males to WT females $F(1,44) = 6.55$, $p < 0.0001$, and AS males to AS females $F(1,44) = 7.26$, $p < 0.0001$) (Fig. 1B). In fact, AS males had an enhanced freezing response just like WT females while AS females showed reduced freezing just like WT males during acquisition (post hoc comparisons: WT males to AS females—$F(1,44) = 0.23$, $p = 1.0$; AS males to WT females—$F(1,44) = 0.47$, $p = 1.0$) (Fig. 1B). The average percentage of freezing response from the start of electrical shock stimulation (last 3 min) was 65.3 ± 2.4 for WT females compared to 36.5 ± 2.5 for AS females, and 37.0 ± 2.4 for WT males compared to 66.0 ± 3.6 for AS males. Lower response threshold for electric shocks in females compared to males was previously reported in studies on rats [18, 19]. Despite these differences, during conditioning, we observed that sex did not affect the contextual fear memory retrieval at shorter time periods. The average freezing response at 24 h after conditioning was similar between the two sexes within each genotype ($F(1,44) = 2.1$, $p = 0.154$ for interaction of genotype × sex, $F(1,44) = 184.8$, $p < 0.0001$ for genotype, and $F(1,44) = 0.22$, $p = 0.64$ for sex, in two-way ANOVA; post hoc comparisons between sexes within each genotype $p = 0.98$ and $p = 0.36$ for WT and AS respectively) (Fig. 1C, D). However, coinciding with previous findings in rats [20, 21], at 1 week after conditioning, WT males showed a stronger retention of long-term contextual fear memory compared to WT females, while AS group did not show any sex-dependent differences which might be attributed to "floor effect" ($F(1,44) = 5.2$, $p < 0.05$ for interaction of genotype × sex, $F(1,44) = 428.8$, $p < 0.0001$ for genotype, and $F(1,44) = 22.5$, $p < 0.0001$ for sex, in two-way ANOVA; post hoc comparisons within each genotype show $t(44) = 5.0$, $p < 0.0001$, and $t(44) = 1.74$, $p = 0.53$ for WT and

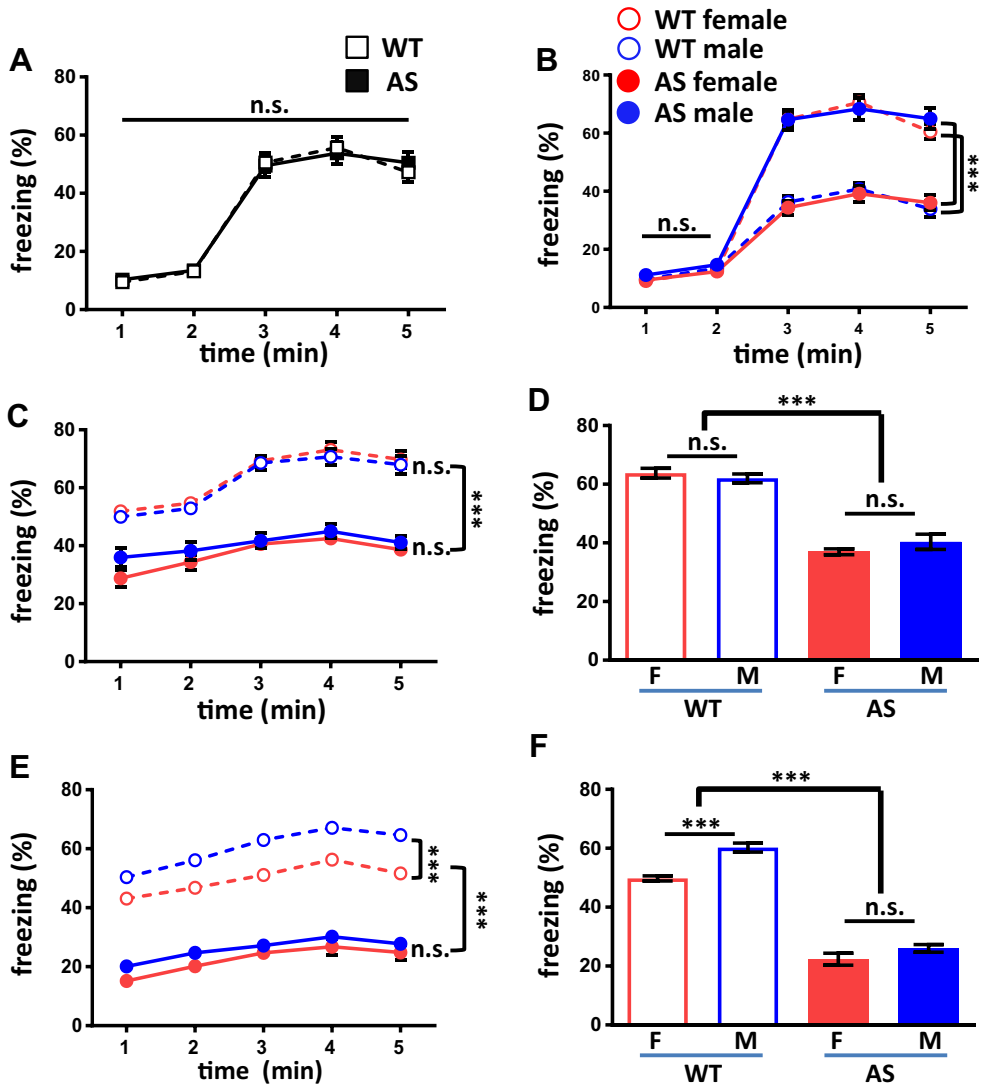


Fig. 1 Contextual fear memory paradigm shows that freezing response to electrical shock stimuli during conditioning is oppositely affected by sex in AS mice, but these effects are expunged in memory testing 24 h after conditioning. (A) Freezing response curves to the two shocks during conditioning to context are similar between WT and AS mice. (B) WT females respond with enhanced freezing to the two shocks compared to WT males, while the opposite occurs in AS mice; AS males respond with enhanced freezing comparable to WT females while AS females freeze less, similar to WT males. (C–D). Contextual fear memory 24 h after conditioning shows no sex differences within each genotype, but very strong genotype differences. Data is presented as averaged freezing percentage in 1-min blocks (C) and average freezing percentage of the entire 5-min exposure (D). (E–F) WT males show a better contextual fear memory than WT females 7 days after conditioning, while AS mice show comparable contextual fear memory deficits between males and females. Data is presented as the minute-by-minute freezing percentage (E) and as the average percent of freezing during the 5-min exposure (F). Data presented as mean ± SEM. ***$p < 0.001$ indicate significant differences between the groups. $N = 12$ mice per each group (genotype × sex)

AS respectively) (Fig. 1E, F). Comparison of only genotypes, by pooling both sexes together, replicated the classical literature results in which WT mice show a stronger contextual fear memory than AS littermates at both 24-h and 1-week time points (Additional File Fig. S1A-D).

Corresponding with previous studies [3, 4, 10], in the MWM, WT mice performed better than AS mice, showing shorter latencies to reach the platform, both in the learning sessions and in the reversal probe trials (learning phase: $F(5,190) = 50.1$, $p < 0.0001$; and reversal phase: $F(3,114) = 10.0$, $p < 0.0001$ for interaction of genotype × time in two-way-RM-ANOVA) (Fig. 2A). However, there were no significant sex differences within the genotypes (learning phase: $F(5,90) = 0.8$, $p = 0.54$; $F(5,90) = 0.0$, $p > 0.99$; and reversal phase: $F(3,54) = 0.5$, $p = 0.69$; $F(3,54) = 0.05$, $p = 0.99$, for WT and AS respectively in two-way RM-ANOVA) (Fig. 2B). These results are consistent with analyses of time spent in quadrants and counts of platform crossing. WT mice spent more time in the target quadrant and less time in all other quadrants ($F(3,152) = 172.4$, $p < 0.0001$ for interaction genotype × quadrant in two-way ANOVA; post hoc $t(152) = 19.44$, $p < 0.0001$ between WT and AS for the target quadrant) (Fig.

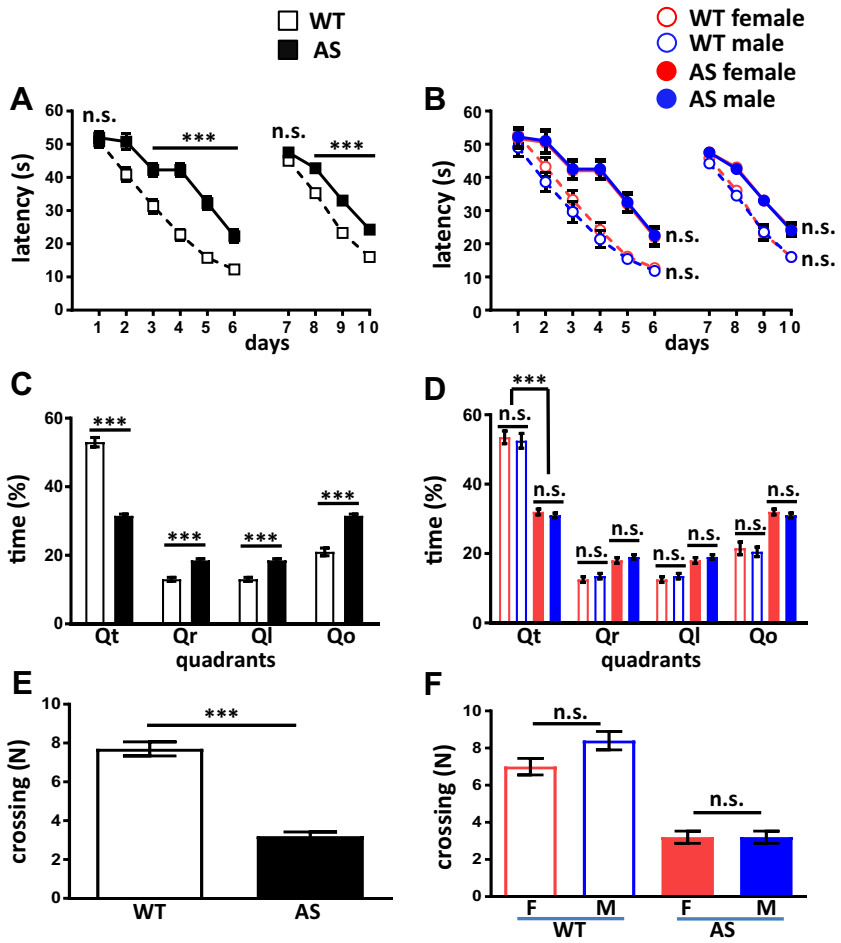


Fig. 2 Spatial learning and memory retention in the Morris water maze. AS mice show navigational memory deficits compared with WT littermates, independent of sex. (A–B) AS mice show learning deficits in latency to reach the hidden platform compared to WT littermates, both in the acquisition phase and in the reversal phase, without any sex differences within the genotypes. (C–D) When the platform is removed, WT mice show higher percentage of time spent in the target quadrant compared with AS littermates, without any sex differences in any of the genotypes. (E–F) WT mice show higher platform crossing frequency compared with AS littermates. Data is represented as the number of platform crossing during the trial. Post hoc comparisons show no differences between sexes within each genotype. Data presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between the groups. $N =$ 10 mice per each group (genotype × sex)

2C). WT mice also crossed the platform area more times than AS littermates ($t(38) = 10.53$, $p < 0.0001$ in unpaired t test between WT and AS) (Fig. 2E). Both tests had no significant sex differences within each genotype (post hoc time in quadrant $t(144) = 0.63$, $p > 0.99$ between males and females for both WT and AS (Fig. 2D); post hoc platform crossing $t(36) = 2.4$, $p = 0.12$ and $t(36) = 0$, $p > 0.99$ between males and females for WT and AS respectively (Fig. 2F)). Unfortunately, latency to reach the platform in MWM is motor dependent because it relies on the swimming speed and not only on spatial navigation memory. For that reason, we also performed a visible platform test which relies only on the motor and visual abilities. The visible platform test did not show any significant differences between any of the four groups (WT females, WT males, AS females, and AS males) (Additional File Fig. S2A). However, pooling the data of males and females together, thus increasing the sample sizes of the two genotype groups, showed a slight but significant advantage for the WT mice (Additional File Fig. S2B). These differences coincide with the slight motor favorability of WT mice compared to AS littermates as evident by the speed and distance differences in the MWM task (Additional File Fig. S2C-D). Nevertheless, these motor differences are subtle and cannot explain the full capacity of MWM performance deficits in AS mice. Moreover, unlike the latency to reach the platform in MWM, which is dependent on swimming speed, the number of platform crossings (Fig. 2E–F) and especially the time spent in quadrant (Fig. 2C–D) are less affected by motor performance and reflect mainly spatial memory.

Novelty Preference

Another facet of cognitive processing and behavior is novelty-seeking behavior. To determine the difference in this behavior

in WT compared to AS mice, we investigated two types of novelty: novel object location (spatial novelty) and novelty of objects themselves (novel object recognition). Both tests do not require the same high degree of motor skills as Morris water maze or sensing painful aversive stimuli such as in fear conditioning.

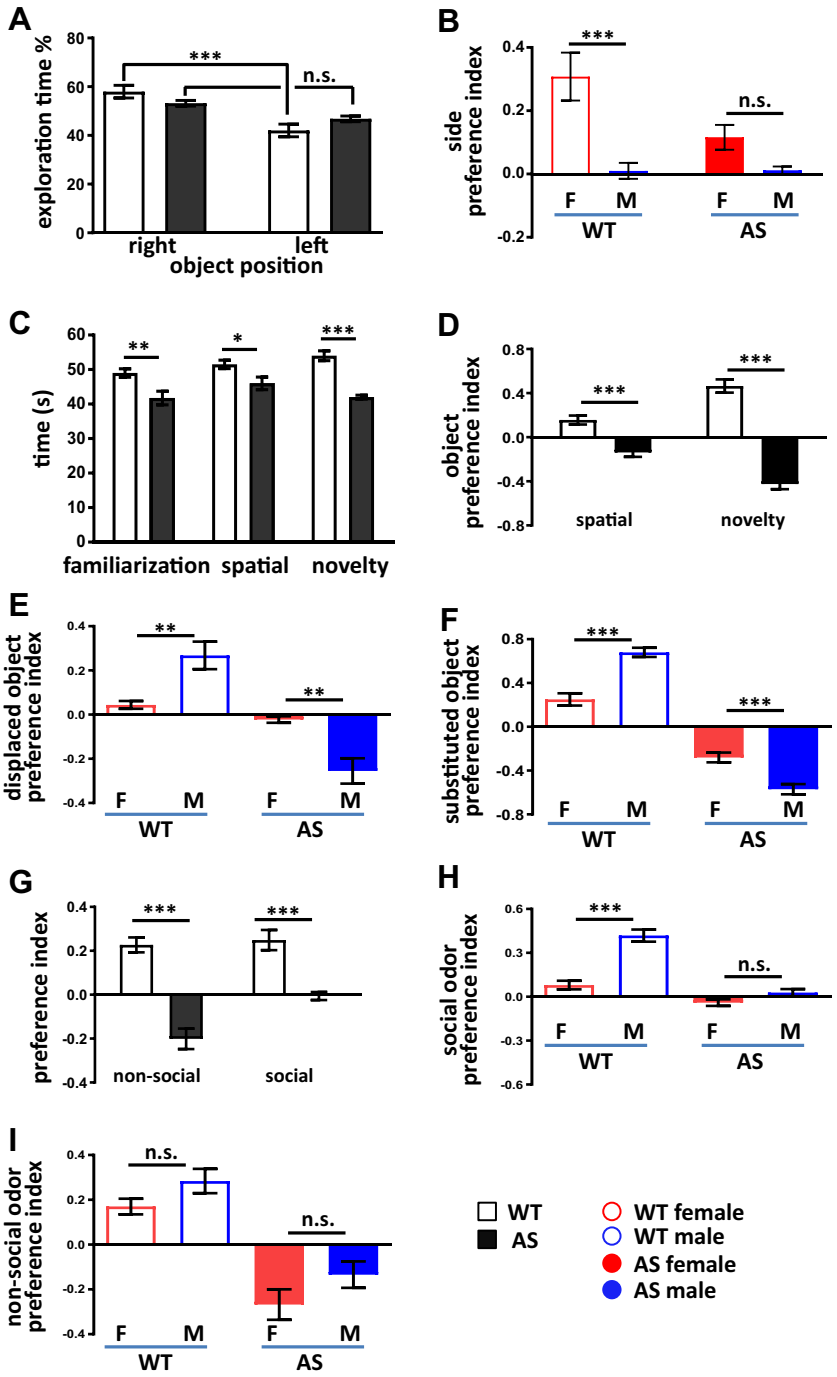
The spatial novelty test requires navigation and orientation and is classically considered hippocampal dependent [21–24]. The hippocampal involvement in novel object recognition test is controversial. Some studies report it as hippocampal dependent, while others as a prefrontal cortex dependent and hippocampal independent [22, 23, 25, 26]. Both types of novelty preference tests require an initial phase of familiarization to objects followed by a test phase. During the initial familiarization phase, WT mice showed right-side preference while AS mice showed no-side preference in exploration activity ($F(1,76) = 5.6$, $p = 0.02$ for interaction of genotype × side in two-way ANOVA; post hoc for right–left-side differences $t(76) = 5.6$ $p < 0.0001$ for WT and $t(76) = 2.3$ $p = 0.16$ for AS) (Fig. 3A). Looking at sex differences within the genotypes of right-side preference index showed that WT females have significant right-side preference compared to WT males, while AS males and females do not show any difference in right–left preference ($F(1,76) = 4.66$, $p = 0.04$ for interaction of genotype × sex in two-way ANOVA; post hoc of female-to-male difference $t(36) = 4.7$, $p = 0.0002$ for WT and $t(36) = 1.64$, $p = 0.66$ for AS) (Fig. 3B). These findings in WT mice coincide with the previous study [21]. WT females have significant right-side preference also compared to AS females and AS males (post hoc $t(36) = 3.0$, $p < 0.05$ and $t(36) = 4.7$, $p < 0.001$, respectively).

We further observed that generally, WT mice spent more time in exploration activity of all objects (old and new) compared to AS littermates. This observation was made in all three phases of the test: familiarization, displaced object test (spatial novelty), and the substituted object test (object novelty) ($t(38) = 3.47$, $p < 0.01$; $t(38) = 3.13$, $p < 0.01$; $t(38) = 2.5$, $p < 0.05$ respectively in t tests) (Fig. 3C). Separating the WT mice and AS mice by sex showed no sex-dependent differences in these tests (Additional File Fig. S3A). In accordance with previous work [24, 27], WT mice exhibited enhanced exploratory behavior in both spatial and object novelty (preference index of 0.16 ± 0.04 and 0.47 ± 0.06 respectively). AS mice preferred to explore the familiar location and object (preference index of -0.14 ± 0.04 and -0.43 ± 0.05 respectively), which might be interpreted as neophobia ($t(38) = 5.2$, $p < 0.0001$ and $t(38) = 11.8$, $p < 0.0001$ in t tests for discrimination indices of spatial and object novelty respectively) (Fig. 3D).

In consistence with previous studies on sex-dependent exploratory behavior of novelty [21, 28], we observed that our WT C57/Bl6 male mice exhibited increased exploration of spatial novelty and object novelty compared to WT females as indicated by the different preference indices. However, in the AS mice, sex entailed an opposite effect. AS males showed higher preference to explore the objects in familiar locations and the familiar objects compared to AS females (sex × genotype interaction: $F(1,36) = 27.0$, $p < 0.0001$ for spatial novelty and $F(1,36) = 57.0$, $p < 0.0001$ for object novelty in two-way ANOVA; post hoc comparisons of males to females discrimination indices for spatial novelty and novel object recognition respectively were $t(36) = 3.6$, $p < 0.01$, and $t(36) = 6.4$, $p < 0.0001$ for WT, and $t(36) = 3.8$, $p < 0.01$ and $t(36) = 4.3$, $p < 0.001$ for AS) (Fig. 3E–F). Analyzing the percent of time spent near the novel location or a novel object showed similar results (Additional File Fig. S3B-C). An additional paradigm of novelty preference is the novel odor preference test. Some studies showed this test to be hippocampal independent [28–32], while others implicated the involvement of the hippocampus in odor discrimination in both humans and rodents [33–35]. Like in spatial or object novelty paradigms, WT mice displayed significantly increased exploratory behavior for both novel social and non-social odors (preference indices of 0.23 ± 0.03 and 0.25 ± 0.05 respectively). However, AS mice presented indifference towards novel social odors (preference index of 0.006 ± 0.02), and even avoidant-like behavior towards novel non-social odors (preference index of -0.2 ± 0.05) (WT to AS comparison $t(38) = 5.16$, $p < 0.0001$ and $t(38) = 7.44$, $p < 0.0001$, in t tests for non-social and social odors respectively) (Fig. 3G). Surprisingly, WT males exhibited a stronger preference for novel social odor than WT females, whereas AS males and females did not exhibit any difference for such preference ($F(1,36) = 19.64$, $p < 0.0001$ in two-way ANOVA for interaction of sex × genotype; post hoc comparisons between males and females $t(36) = 7.85$, $p < 0.0001$ for WT and $t(36) = 1.58$, $p = 0.74$ for AS) (Fig. 3H). None of the genotypes exhibited any sex-dependent differences in non-social odor preference ($F(1,36) = 0.03$, $p = 0.86$ in two-way ANOVA for interaction of sex × genotype) (Fig. 3I). Our results of preference of novel social and non-social odors in WT females and males coincide with previous reports [28]. Data analyses of time spent sniffing the novel odors showed similar results between the genotypes and the sexes (Additional File Fig. S4A-C). These results suggest that memory tasks that involve novelty preference might not be suitable for AS mice because of their aversion towards novelty. In addition, AS mice aversion towards novelty shows that AS mice have intact memory and correctly recognize novelty. It further indicates that for this object spatial novelty task, either hippocampal functioning does not play a substantial role or that 4 h after exposure are not sufficient for memory decay in AS mice.

Thermal Pain Perception

The opposite sex–linked differences observed in AS mice response to the electrical shocks during CFC (Fig. 1B)



suggested a divergence in sex-related nociceptive perception compared to WT mice. To further determine the difference in nociception in the two genotypes, we utilized the hot-plate test. Hot-plate test measures the sensitivity to thermal pain and quantitatively assesses the latencies to paw-licking and jumping responses. Sex-matched and pooled, the response to the hot-plate was similar between WT and AS littermates ($t(94) = 0.12$, $p = 0.9$ and $t(94) = 0.44$, $p = 0.66$ in t tests between WT and AS for licking and jumping respectively) (Fig. 4a, b). Previous studies reported that human females, as well as WT female mice, are more sensitive to thermal pain than males [12, 36–39]. In concurrence with these reports, we observed that WT females showed shorter reaction latency times than WT males (Fig. 4a, b) suggesting that WT females have lower pain threshold. Opposed to WT mice, AS males showed significantly shorter latency times to licking and jumping responses compared to AS females. This suggests that AS males are more sensitive to pain than AS females

◀ **Fig. 3** WT mice show enhanced exploration of novel objects and odors compared to AS littermates. These exploratory behaviors are oppositely affected by sex in AS mice. (A) WT show right-side preference while AS mice show no-side preference during familiarization phase. Data represent exploration time percentage during familiarization phase, when both objects are novel. (B) Exploration time percentage of right-sided object during familiarization phase show that WT mice have sex differences; WT females have a right-sided preference compared to WT males, while AS mice do not show sex differences. (C) Time spent in general exploration of all objects. WT mice exhibit increased exploratory behavior compared with AS mice in every phase of the test: familiarization, displaced object test, and the substituted object test. (D) Novelty exploration discrimination index in both novel spatial (displaced object) and novel object recognition (substituted object). WT mice have preference to novelty in both paradigms, while AS mice prefer to explore the familiar. (E–F) Discrimination indices of spatial novelty (displaced object) and novel object (substituted object) show opposite sex effects between WT and AS littermates. WT males show enhanced exploration of spatial novelty (E) and object novelty (F) compared to WT females, while AS males have an enhanced exploration of the familiar than AS females in both paradigms (E–F). (G) Discrimination indices of novel odor exploration. WT mice show enhanced exploration of novel non-social and social odors, while AS are either avoidant (non-social) or indifferent (social) to novel odors. (H) Discrimination indices of novel social odor show sex differences in WT mice but not in AS mice. WT males show enhanced exploration of novel social odor compared to WT females, while AS males and AS females show a comparable indifference. (I) Discrimination indices of novel non-social odor are sex independent in WT and AS mice. WT males and females show enhanced exploration of novel non-social odor, while AS males and females show a comparable avoidance. Both genotypes show no sex-dependent variations. Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences between the groups. $N = 10$ mice per each group (genotype × sex)

($F(1,92) = 311.8$, $p < 0.0001$ and $F(1,92) = 378.3$, $p < 0.0001$ for interaction of sex and genotype in two-way ANOVA for licking and jumping respectively; post hoc male to female comparison for licking $t(92) = 12.16$, $p < 0.0001$ for WT and $t(92) = 12.81$, $p < 0.0001$ for AS; post hoc male-to-female comparison for jumping $t(92) = 12.36$, $p < 0.0001$ for WT and $t(92) = 15.14$, $p < 0.0001$ for AS) (Fig. 4a, b). These results further indicate that WT males are less sensitive to thermal pain than WT females and are comparable to AS females. AS males, on the other hand, are more sensitive to thermal pain than AS females and are similar in their thermal pain threshold to WT females (post hoc WT females to AS males $t(92) = 0.15$, $p = 1.0$ for both licking and jumping; post hoc WT males to AS females $t(92) = 0.5$, $p = 1.0$ and $t(92) = 2.1$, $p = 0.24$ for licking and jumping respectively) (Fig. 4a, b). These sex-linked differences in pain sensitivity between the two genotypes coincide with the results above of the freezing response during CFC acquisition phase (Fig. 1B).

Odor Sensitivity and Simple Odor Discrimination Test

The sex-dependent alterations in pain sensitivity led us to further examine additional critical sensory modality in rodents, smell. For this, we utilized modified odor sensitivity and simple odor discrimination paradigms. The choice in olfaction modality stemmed from previous studies in humans that showed a deficit in this sensory modality in neurodevelopmental and autism spectrum disorders [31, 32, 40, 41]. When sex was matched and pooled within genotypes, WT mice showed more sensitivity to odor than AS littermates ($F(1,190) = 58.37$, $P < 0.0001$ for genotype in two-way ANOVA for genotype × odor concentration) (Fig. 5A). This enhanced odor sensitivity of WT mice was especially prominent at the lower odorant concentrations (post hoc WT to AS comparisons: $t(190) = 1.8$, $p = 0.36$; $t(190) = 2.3$, $p = 0.1$; $t(190) = 3.9$, $p < 0.001$; $t(190) = 4.9$, $p < 0.0001$; $t(190) = 4.2$, $p < 0.0005$ for concentrations of 75%, 50%, 25%, 10%, and

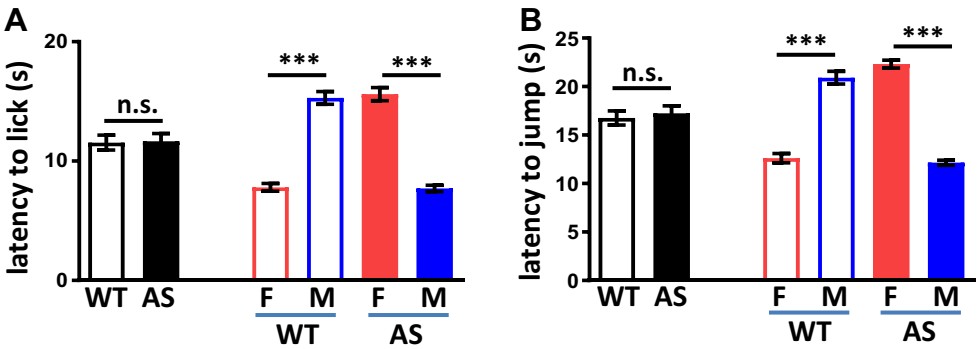


Fig. 4 Thermal pain perception as indicated by the hot-plate test show opposite sex effects between WT and AS mice littermates. **a** Latencies to licking the paws in the hot-plate test show that WT and AS mice in general have similar pain perception. However, while WT females show a higher sensitivity to thermal pain and begin to lick their paws earlier than WT males, AS mice show the exact opposite sex effect. Furthermore, post hoc comparisons show that WT females respond similar to AS males and WT males respond similar to AS females. **b** Latencies to jumping in the hot-plate test in order to avoid the painful stimulus show that WT and AS mice in general have similar pain perception. However, while WT females show a higher sensitivity to thermal pain and start jumping before WT males, AS mice show the exact opposite sex effect. Moreover, WT females respond similar to AS males and WT males respond similar to AS females. Data presented as mean ± SEM. ***$p < .001$ indicate significant differences in post hoc comparisons between the sexes in each group. $N = 24$ mice per each group (genotype × sex)

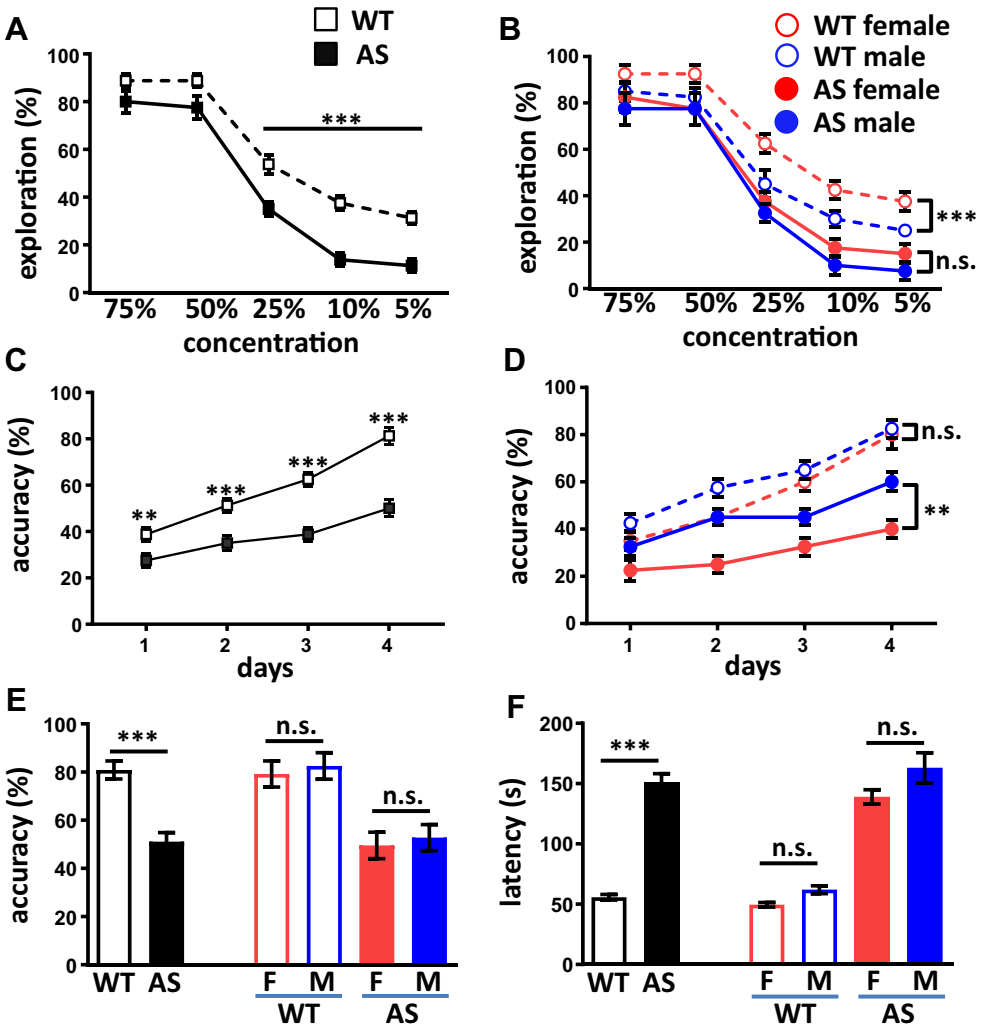


Fig. 5 AS mice exhibit sensory olfactory functioning deficits with a significantly different sex effects compared to WT littermates. (A) AS mice show less sensitivity to odor stimulation as demonstrated by reduced exploration of the scented cube. Differences are more pronounced at the lower odor concentrations. (B) In low concentrations, odor sensitivity is affected by sex in the WT mice but not in the AS littermates. Data is presented as percentage of exploration of the scented cube. Sex-dependent odor sensitivity is different between AS and WT mice. WT mice show sex differences (WT females are more sensitive than WT males) while AS littermates do not exhibit any sex differences. (C) Simple odor discrimination test shows that AS mice perform poorly in discriminating the rewarding odor and have a lower percentage of correct choices for finding the hidden chocolate cube under the associated odor. Data is presented as the percentage of correct choices. (D) Success in SOD test is sex dependent in AS mice but not so in WT mice. WT males and females are comparable, but AS males are superior to AS females. Data is presented as the percentage of correct choices. (E) Success percentage in finding the three hidden cookies is considerably higher in WT mice than in AS littermates. Data is presented as the percentage of finding and consuming the three hidden fruit loops. Segregation to sex shows no differences between sexes in any of the genotypes. (F) Latency to find the hidden cookie is considerably lower in WT mice than in AS littermates. Segregation to sex shows no differences between sexes in any of the genotypes. Data is presented as the time required for revealing the cookie. Data presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between the groups. $N = 10$ mice per each group (genotype × sex)

5% respectively) (Fig. 5A). However, a division of genotype groups by sex showed sex-linked differential effects in odor sensitivity within each genotype. WT females were significantly more sensitive to odor than WT males, whereas AS mice exhibited no such sex-linked difference ($F(3,180) = 25.8$, $p < 0.0001$ for group (sex and genotype) in two-way ANOVA; post hoc comparison between males and females in each genotype $t(180) = 4.0$, $p < 0.001$ and $t(180) = 1.7$, $p = 0.6$ for WT and AS respectively) (Fig. 5 B).

An additional test of non-spatial reference working memory that utilizes odor learning is the simple odor discrimination (SOD) task. In this task, mice learn to find a reward (chocolate cube) buried under an associated masking odor. Primarily, AS mice performance was poorer than that of the WT littermates and they exhibited a reduced learning curve compared to WT mice ($F(3,114) = 8.83$, $p < 0.0001$ for interaction (time × genotype) in two-way RM-ANOVA) (Fig. 5C). However, dividing the genotype groups according to sex revealed a significantly impaired odor discrimination learning curve in AS

females compared to AS males and comparable learning curves in WT females and WT males ($F(9,108) = 3.86$, $p < 0.001$ for interaction (time × group) in two-way RM-ANOVA; post hoc between males and females $t(36) = 1.54$, $p = 0.8$ and $t(36) = 3.5$, $p < 0.01$ for WT and AS respectively) (Fig. 5D). Interestingly, although AS males have a poor SOD curve compared to WT males (post hoc main effect $t(36) = 3.6$, $p < 0.01$), a day-by-day analysis showed that the initial odor discriminatory performance of AS males was similar to that of WT mice (both females and males), but their learning curve plateaued compared to the WT mice (post hoc comparison of AS males to WT males: $t(144) = 1.75$, $p = 0.5$, $t(144) = 2.2$, $p = 0.18$, $t(144) = 3.5$, $p < 0.01$, $t(144) = 3.9$, $p < 0.001$ for days 1–4 respectively) (Fig. 5D).

To further examine the olfactory function, we utilized the hidden cookie test, in which food-deprived mice search for a hidden familiar fruit loop (cookie) in established period of time using odor [42, 43]. In conjunction with our previous findings of odor sensitivity, WT mice showed a higher success rate of finding the cookie than AS mice and did it much faster ($t(38) = 5.6$, $p < 0.0001$ and $t(38) = 12.4$, $p < 0.0001$ for accuracy and latency respectively) (Fig. 5E, F). However, in this task, interaction analysis of sex and genotype, and post hoc multiple comparisons, did not show any sex-linked differences between and within WT and AS mice ($F(1,36) = 0$, $p > 0.99$ and $F(1,36) = 0.6$, $p = 0.43$ for interaction (sex x genotype) in two-way ANOVA for accuracy and latency respectively; post hoc between sexes in each genotype: WT—$t(36) = 0.4$, $p = 1.0$; AS—$t(36) = 0.4$, $p = 1.0$, (Fig. 5E, F) for accuracy, and WT—$t(36) = 1.2$, $p = 1.0$; AS—$t(36) = 2.3$, $p = 0.15$ for latency).

Motor Functioning

Previously, it has been reported that AS mice have cerebellar- and striatal-linked motor deficits [44–46]. Because AS mice displayed distinct sex effects in some sensory paradigms, we expanded our investigation to motor performance. Analysis of motor performance by comparing speed and moving distance in open-field arena and simple muscular strength measured by grip strength test showed no differences between WT and AS mice ($t(38) = 0.96$, $p = 0.34$ for both speed and moving distance and $t(38) = 1.54$, $p = 0.13$ for gross motor force in unpaired t tests), and no sex-linked differences between and within genotypes ($F(1,36) = 0.04$, $p = 0.84$, $F(1,36) = 0.03$, $p = 0.88$, $F(1,36) = 0.44$, $p = 0.51$ for interaction (sex × genotype), and $F(1,36) = 0.02$, $p = 0.89$, $F(1,36) = 0.01$, $p = 0.93$, $F(1,36) = 1.75$, $p = 0.2$ for sex effect alone, in two-way ANOVA for speed, distance, and gross force respectively) (Fig. 6a–c). However, AS mice exhibited significantly less rearing movements than WT littermates ($t(38) = 3.5$, $p < 0.01$ in unpaired t tests), but also with no sex differences within any of the genotypes ($F(1,36) = 1.07$, $p = 0.31$ for sex and $F(1,36) = 0$, $p = 1.0$ for interaction (sex × genotype) in two-way ANOVA; post hoc between sexes $t(36) = 0.73$, $p > 0.99$ for both WT and AS) (Fig. 6d). Considering the mice genetic background and ages, these results concurred with previous findings [10]. These results suggest that reduced rearing activity in AS mice is not due to a gross motor deficit but reflects altered anxiety levels associated with hippocampal functioning as part of navigational map registration process [47]; however, reduced hind limb strength cannot be excluded.

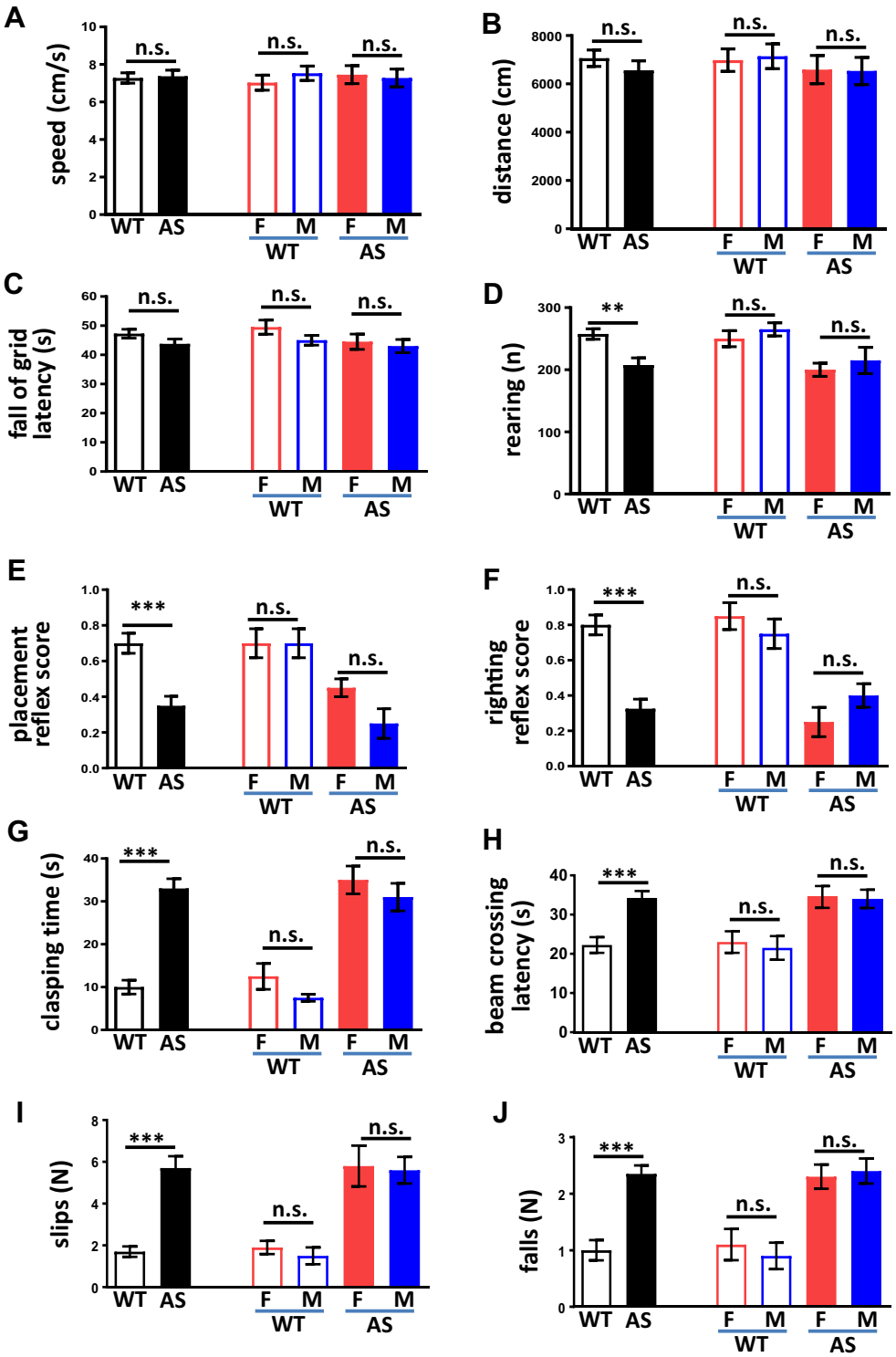
We further examined three basic motor reflex responses of mice: placing, righting, and clasping reflexes. These reflexes comprise activity regulation in several brain regions such as the cerebellum, proprioceptive system, visual system, and primary motor system. AS mice exhibited deficits compared to WT littermates in all three reflexes ($t(38) = 6.91$, $p < 0.0001$; $t(38) = 8.71$, $p < 0.0001$; $t(38) = 8.23$, $p < 0.0001$; unpaired t tests for placing, righting, and clasping reflexes respectively), but none of the genotypes showed any sex differences for any of these reflexes ($F(1,36) = 0.53$, $p = 0.47$; $F(1,36) = 1.53$, $p = 0.22$; $F(1,36) = 0.03$, $p = 0.86$ interaction (sex × genotype) in two-way ANOVA for placing, righting, and clasping respectively) (Fig. 6e–g).

Also, in the balance beam test, which examines the proprioceptive and motor performance, AS mice showed a poorer performance compared to WT littermates. This was manifested in an increased tendency to slip and fall off of the 10-mm round beam and increased time required to accomplish the beam crossing ($F(1,36) = 19.2$, $p < 0.0001$; $F(1,36) = 39.6$, $p < 0.0001$; $F(1,36) = 32.3$, $p < 0.0001$; differences between genotypes in two-way ANOVA for beam crossing latencies, slips, and falls, respectively) (Fig. 6h–j). Easier beam crossing tasks, the 10-mm square, and 20-mm beams showed smaller but still significant differences in latencies. Falls were not significantly different at 20-mm square beam (Additional File Fig. S5). Again, no sex differences were observed in these tests within each genotype ($F(1,36) = 0.33$, $p = 0.86$; $F(1,36) = 0.03$, $p = 0.88$; $F(1,36) = 0.4$, $p = 0.53$ for interaction (sex × genotype) and $F(1,36) = 0.13$, $p = 0.72$; $F(1,36) = 0.22$, $p = 0.64$; $F(1,36) = 0.04$, $p = 0.83$ for sex, in two-way ANOVA for beam crossing latencies, slips, and falls, respectively) (Fig. 6h–j).

Altogether, we conclude that AS mice show impairments in motor performance that require coordinated movement and proprioception but do not exhibit deficits in simple motor strength (Fig. 6a–j). Unlike in the abovementioned sensory paradigms, we did not observe sex-linked differences in motor functioning in any of the genotypes.

Anxiety-Like and Repetitive Behavior Measures

Another facet for characterizing behavioral differences between WT and AS mice is the element of anxiety. It is apparent from the aforementioned results that AS mice seemed to avoid



novelty (Fig. 3C–F), which might reflect high anxiety levels. To establish differences in anxiety levels, we examined the thigmotaxis phenomenon. Thigmotaxis is the tendency of anxious mice to move in the periphery closer to the walls than in the center of the open-field arena (OFA) test [48, 49]. All thigmotaxis anxiety markers were significantly enhanced in AS mice ($t(38) = 3.43$, $p < 0.01$; $t(38) = 4.0$, $p < 0.001$; $t(38) = 5.58$ $p < 0.0001$; $t(38) = 6.97$ $p < 0.0001$ in unpaired t tests between WT and AS, for time spent in the center, latency to the center, entries to the center, and thigmotaxis percentage respectively). However, no sex differences were found between any of the genotypes ($F(1,36) = 3.66$, $p = 0.064$;

◀ **Fig. 6** OFA and other motor performance tests show that simple locomotion and motor strength parameters are comparable between WT and AS mice. However, motor behaviors that reflect anxiety and stereotypy are more pronounced in AS mice, and motor tasks that require coordination and/or proprioceptive processing show significant deficits in AS mice. All of these tests show that various motor tasks and parameters are sex independent for both WT and AS mice. **a** Speed and **b** moving distance, as measured in the OFA, and **c** gross motor force, as measured by the latency to leave the grip and fall from grid, are comparable between WT and AS mice. No sex differences are shown within any of the genotypes. **d** AS mice have less rearing movements compared with WT littermates, but both genotypes show that rearing is sex independent. **e**–**g** Motor reflexes that require proprioception and coordination are impaired in AS mice compared to WT littermates. Data shown in **e** and **f** are scores of placement and righting reflexes, respectively, and data shown in **g** is the time spent in clasping within 60 s of tail suspension. All three tests show no sex differences between the genotypes. **h**–**j**. Voluntary motor performance that requires coordination and proprioception as shown in the balanced beam test shows that WT mice perform better than AS mice. Data represent the averages of trials crossing the 10-mm round beam. Data presented in **h** is the time required to cross the beam, and **i** and **j** show the number of slips or falls during the task. No sex-dependent differences were apparent for any of the genotypes, and there was no interaction between genotype and sex. Data presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between the groups. $N = 10$ mice per each group (genotype × sex)

$F(1,36) = 1.87$, $p = 0.18$; $F(1,36) = 1.27$, $p = 0.27$; $F(1,36) = 0.67$, $p = 0.42$ in two-way ANOVA for interaction (sex × genotype); post hoc comparisons between males to females for WT—$t(36) = 2.27$, $p = 0.18$, $t(36) = 2.48$, $p = 0.11$, $t(36) = 2.66$, $p = 0.07$, $t(36) = 1.73$, $p = 0.55$; and for AS—$t(36) = 0.44$, $p > 0.99$, $t(36) = 0.55$, $p > 0.99$, $t(36) = 1.06$, $p > 0.99$, $t(36) = 0.57$, $p > 0.99$, for time spent in the center, latency to the center, entries to the center, and thigmotaxis percentage, respectively) (Fig. 7a–d). Other corresponding markers of anxiety during OFA were enhanced defecation, in which AS mice exhibited a doubled amount of defecation ($t(38) = 6.46$, $p < 0.0001$ in unpaired t test) (Fig. 7e), and freezing behavior, which was enhanced in AS mice compared to WT littermates (Additional File Fig. S6A), with no sex differences between and within the genotypes ($F(1,36) = 2.8$, $p = 0.1$ in two-way ANOVA for interaction (sex × genotype); post hoc comparison of defecation between sexes $t(36) = 0.17$, $p = 1.0$ and $t(36) = 2.19$, $p = 0.21$ for WT and AS respectively) (Fig. 7e). Additionally, we performed the marble-burying test to check for anxiety levels [50–55]. Although it has been challenged whether this test reflects anxiety levels or repetitive and perseverative behavior [56], one study has shown that hippocampal lesions significantly reduce marble-burying behavior [57]. AS mice showed a significant decrease in parameters of marble-burying behavior compared to WT littermates ($t(46) = 10.61$, $p < 0.0001$ and $t(46) = 9.46$, $p < 0.0001$ in unpaired t tests between genotypes for time invested in burying activity and for average number of marbles buried, respectively). No sex-dependent differences within genotypes were found ($F(1,44) = 0.38$, $p = 0.54$; $F(1,44) = 1.19$, $p = 0.28$ interaction (sex × genotype) in two-way ANOVA for digging duration and number of buried marbles, respectively; post hoc comparisons between the sexes for burying time: $t(44) = 0.0$, $p > 0.99$, and $t(44) = 0.87$, $p > 0.99$; and buried marble numbers: $t(44) = 0.26$, $p > 0.99$, and $t(44) = 1.28$, $p > 0.99$; for WT and AS respectively) (Fig. 7f–g). These results concur with the reduced rearing activity observed in AS mice (Fig. 6d). Finally, we utilized the nest-building test to assess a motivated behavior which reflects good health and ability to self-care [58, 59]. AS mice showed a significant impairment in nest-building capability compared to WT littermates ($t(94) = 13.49$, $p < 0.0001$ in unpaired t test), again with no sex-dependent differences in any of the genotypes ($F(1,92) = 0.19$, $p = 0.66$ interaction (sex × genotype) in two-way ANOVA; post hoc comparisons between the sexes: $t(92) = 1.04$, $p > 0.99$, and $t(92) = 1.66$, $p > 0.99$; for WT and AS respectively) (Fig. 7h). It is important to note that most of the behavioral paradigms for AS which entail exploration contain an innate bias due to their dependence on motor functioning. However, not all of these paradigms require a high degree of motor skills like MWM [10]. Moreover, despite AS motor deficits, many of these paradigms were suggested as the appropriate behavioral testing battery for AS [14].

Sex-Dependent RNA-Seq Analysis

Given the sex-dependent differential manifestation of mainly sensory- and stress-related behavioral phenotypes in AS mice, we chose to examine the transcriptomic expression profiles in the ventral hippocampi of AS and WT littermates, males, and females. The hippocampus, a central brain structure which serves as a hub for cognitive-, emotional-, and stress-related learning processes, is known to be impaired in AS mice [2, 15, 60]. While the dorsal hippocampus is generally associated with spatial navigation, the ventral hippocampus is mainly implicated in the processing of stress- or pain-related memory [61, 62] and odor perception learning processes [63, 64]. We generated mRNA sequencing data from three WT females, three WT males, three AS females, and three AS male mice.

Alignment of raw reads on reference transcriptome utilizing Bowtie2 with the following up RSEM expression count analysis yielded 13,952 expressed genes and 29,739 expressed isoforms. Additionally, we aligned raw reads to reference genome utilizing HiSat2 and assembled isoforms (transcripts) with CuffLinks [65]. RSEM expression count in this analysis yielded 19,736 gene annotations and 42,290 isoform annotations.

Several of our behavioral studies showed interesting differential sex-dependent variation within each genotype. Amongst, one of the most prominent differences that showed an opposite sex–dependent interaction effect was related to pain perception as observed in the response to the electrical shocks in the CFC and in the hot-plate test (Figs. 1B and 4), to the extent that WT females were comparable to AS males and

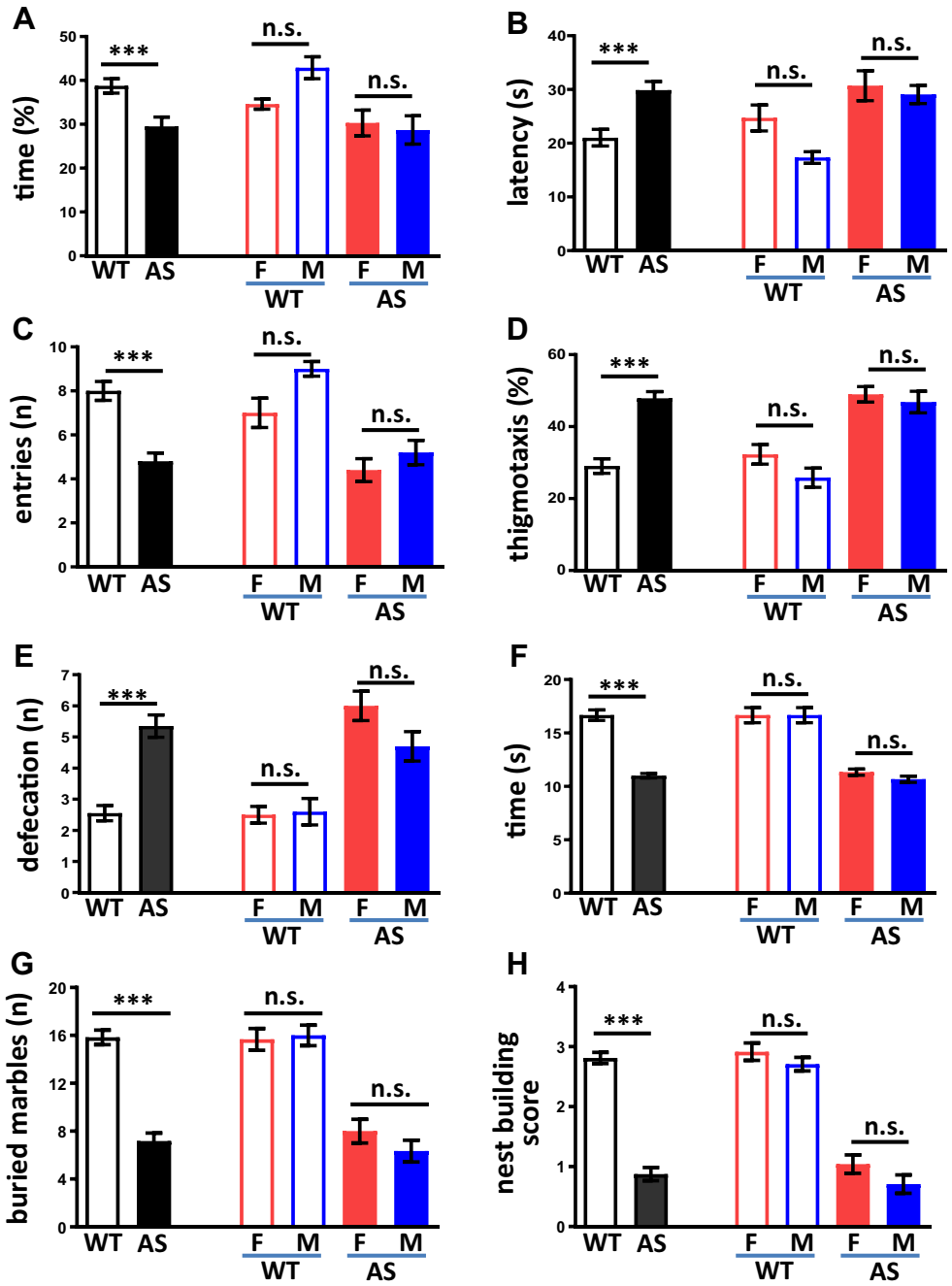


Fig. 7 Anxiety indicative behavioral parameters show sex-independent enhanced anxiety and enhanced stereotypic behavior in AS mice. **a**–**d** Various thigmotaxis measurements indicate enhanced thigmotaxis in AS mice. **a** Data is represented as the average time spent in the center of the arena. AS mice spent less time in the center. **b** Data represents the average latency to enter the center, which reflects the hesitation of AS mice to enter the center zone. **c** Data represents the average number of entries to the center zone. **d** Data represents the average of overall thigmotaxis percentage, which indicates the increased thigmotaxis in AS mice. Thigmotaxis is comparable between the sexes in both genotypes in all measurements. **e** Defecation during OFA test indicates enhanced levels of anxiety in the AS mice with no sex-dependent differences in any of the genotypes. Data represents the average number of droppings during the test. **f**–**g** Marble-burying test shows a decreased burying behavior in AS mice compared with WT littermates. Data represent the average time invested in burying activity (**f**) and average number of marbles buried (**g**). No sex-dependent differences were apparent in any of the genotypes. **h** AS mice exhibit poorer performance of nest building compared to WT littermates. Data represents the average nest-building scores in 4-point scoring system after 48 h. No sex differences were apparent in nest building. Data presented as mean ± SEM. ***$p < 0.001$ indicates significant differences between the groups. Numbers of mice per each group (genotype × sex) for data acquired in OFA (panels **a**–**e**), $N = 10$; for marble-burying data (panels **f**, **g**), $N = 12$; and for nest-building data (panel **h**), $N = 24$

WT males were similar to AS females. Based on these results, we wanted to identify genes with expression patterns that mirror these behavioral patterns, namely, genes and isoforms that their differential expression profile between sexes in WT mice is opposite to the differential expression profile between sexes in AS littermates. In order to identify such genes, we analyzed the RNA-seq data in a two-step manner. First, we identified all genes and isoforms with inverse expression ratios larger than 1.3 differences between averages of the two sexes within the two genotypes (4887 genes and isoforms). The 1.3 expression ratio threshold was chosen because we wanted, on one hand, to limit the analysis to highly differentiating genes/isoforms, while on the other hand to avoid excluding possibly important genes. Next, in order to extract from this list, only those genes/isoforms with significant interaction effect of sex and genotype, we applied a factorial regression model with finite orthogonal Chebyshev polynomials for estimating main effects and interaction effect [66]. This robust strategy was chosen because the interaction effect is the major parameter of our filtering and has to be estimated accurately. The factorial regression model is more suited than two-way ANOVA in estimating the interaction effect significance in bioinformatics studies with a limited number of replicates [67]. Thus, we identified altogether 548 sex-related opposite interaction (SROI) genes and isoforms ($p < 0.01$ and false discovery rate = 0.11) (Fig. 8).

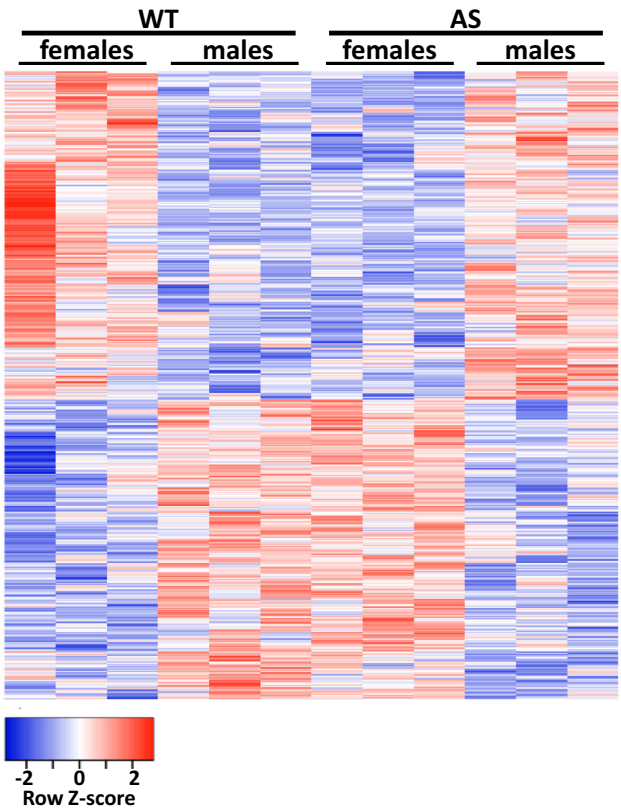


Fig. 8 Bioinformatics analysis of mRNA sequencing of ventral hippocampi, utilizing the factorial regression model with two factors, genotype and sex, yielded genes and isoforms that their sex-dependent expression profiles are opposite between WT and AS littermates. Displayed is an unsupervised hierarchical clustering heat map of 548 sex-related opposite interaction (SROI) genes and isoforms using Pearson correlation and average linkage. Criteria for SROI genes are (1) the expression in males and females is opposite between WT and AS, (2) the threshold difference of the means between the sexes in each of the genotypes is at least 30%, and (3) significant interaction effect for genotype and sex ($p < 0.01$). Roughly half of the genes are highly expressed in WT females and AS males and lowly expressed in WT males and AS females (upper half of the heat map), and half of the genes are highly expressed in WT males and AS females and lowly expressed in WT females and AS males (bottom half of the heat map). The expression level is color-coded: red for overexpressed, white for unchanged expression, and blue for underexpressed genes. Numbers of mice per each group (genotype × sex), $N = 3$

Crossing RNA-Seq Results with Known Pain-Related Genes

Because of our choice to focus the analysis on pain perception, we generated a list of pain-related genes utilizing three different databases: (1) Gene ontology consortium database [68] using the terms "behavioral response to pain" (125 genes); "response to pain" (240 genes); "detection of temperature stimulus involved in sensory perception of pain" (102 genes); "regulation of sensory perception of pain" (286 genes); "negative regulation of sensory perception of pain" (9 genes); "positive regulation of sensory perception of pain" (41 genes); "sensory perception of pain" (1102 genes). Combining all of these categories together and eliminating all duplicates resulted in 319 genes from the gene ontology database. (2) The Comparative Toxicogenomics Database (CTD) [69], using the term "acute pain" (1099 genes). (3) Ultsch et al.'s [70] publication of candidate genes associated with pain as a process of learning and neural plasticity (535 genes). All three databases combined excluding duplicates yielded a total of 740 genes. Crossing these 740 pain-related genes with the 548 SROI genes and isoforms by their official gene symbol yielded a list of 43 SROI pain-associated genes (Additional File Table S1) (Fig. 9A–B).

Because of the known Ube3a interactions with estrogen and androgen receptors [71–73], we further identified genes known to be estrogen or androgen dependent. For that, we crossed the 548 SROI genes, with a list of genes that contain estrogen response elements in their vicinity [74], and a list of androgen-dependent genes [75]. The crossing of the SROI genes with estrogen-dependent genes yielded a list of 66 genes (Fig. 9A, C and Supp. Table 2). Further crossing of the list of estrogen-dependent genes with the 43 pain-related genes yielded 7 genes: Asl, Entpd5, Gabbr1, Gfra1, Map2k1, Map3k7, Slc29a1 (Fig. 9A, B, and Additional File Table S1). The crossing of the SROI genes with the androgen-dependent genes yielded a list of 8 genes (Fig. 9A and Additional File Table S3). However, crossing the

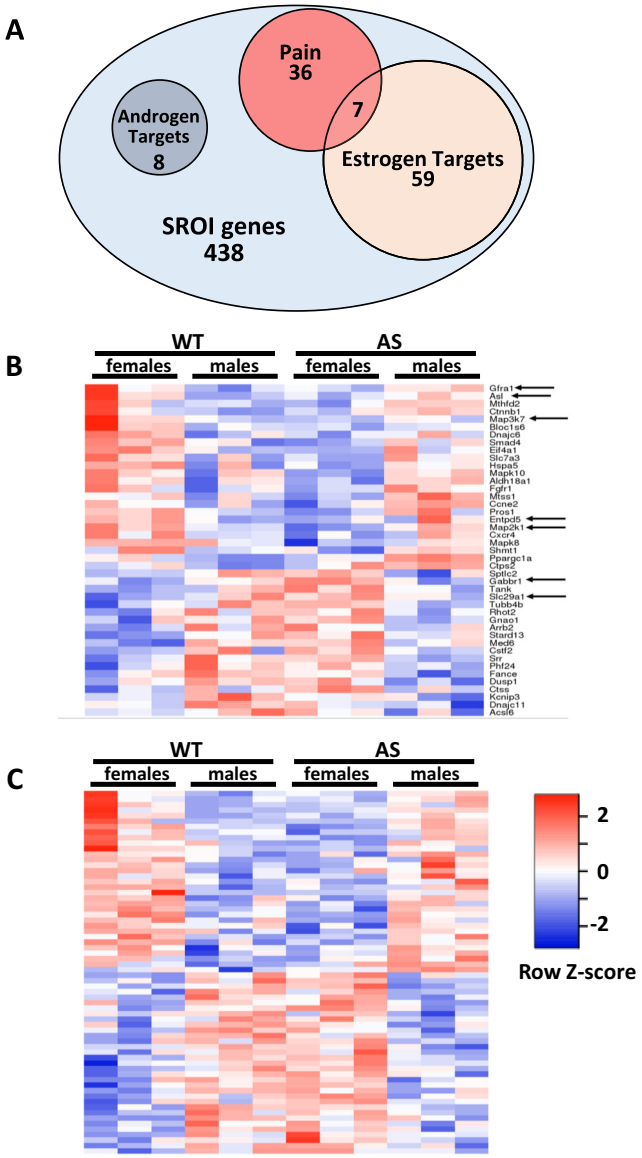


Fig. 9 SROI genes related to pain, estrogen, or androgen target genes. Crossing the list of SROI genes with lists of pain-related genes or with estrogen or androgen target genes yields lists of genes probably implicated in the altered pain perception of AS mice. (A) Venn diagram of the aforementioned 548 SROI genes. Crossing the 548 SROI genes with pain-related genes, extracted from GO consortium database, CTD database and Ultch et al. publication identified 43 genes. We also crossed SROI genes with lists of known estrogen and androgen target genes, which identified 66 estrogen-dependent genes and 8 androgen-dependent genes. Seven genes are pain-associated and estrogen-dependent SROI genes. (B) Unsupervised hierarchical clustering heat map of 43 genes from crossing the SROI with pain-associated lists using Pearson correlation and average linkage. The genes marked by arrows are known estrogen target genes. (C) Unsupervised hierarchical clustering heat map of 66 genes from crossing the SROI genes with estrogen target genes using Pearson correlation and average linkage. Heat maps of expression levels are color-coded: red for overexpressed, white for unchanged expression, and blue for underexpressed genes. Numbers of mice per each group (genotype × sex), $N = 3$

androgen-dependent genes with the 43 pain-related genes did not yield any result (Fig. 9A).

Discussion

It was previously shown that some of the various sensory perception modalities and motor capabilities are phenotypically different between males and females while others are similar between the two sexes [13, 21]. Angelman syndrome is a neurodevelopmental disorder with an equal prevalence in males and females [1], and yet, a thorough investigation of how sex affects the AS phenomenology was never performed. The AS mouse model was previously proven to represent well the human disorder and thus is a suitable model to study some facets of this genetic syndrome [2, 3, 44]. In the herein study, we utilized the AS mouse model for the sake of scrutinizing AS neurobehavioral phenotypes with regard to sex differences.

Our analyses show that when WT and AS groups are sex-matched with a 50% prevalence for each sex and the data of both sexes are pooled, the previously reported AS deficits are replicated. These AS deficits include impaired hippocampal functioning, as shown by the CFC, MWM [2, 16], novelty recognition of object [24], and object placement (Figs. 1–2) tests, and impaired motor performance as shown in several paradigms such as rearing motions, reflexes assessments, and beam balance test (Fig. 6) [10, 45, 46]. Furthermore, AS mice demonstrate anomalies in complex repetitive behaviors such as marble burying [10] and nesting (Fig. 7f–h). Moreover, we demonstrated for the first time additional behavioral and sensory deficits in AS mice. For example, unlike their WT littermates, AS mice do not show right-side preference. In addition, AS mice exhibit an impaired odor perception as evidenced by odor sensitivity tests, simple odor discrimination, and hidden cookie test (Fig. 5). These deficits are common in neurodevelopmental and autism spectrum disorders and were not previously reported in AS mice [40, 41].

Nevertheless, the overarching aim of the herein study was to determine whether sex affects the various pathological phenotypes of Angelman syndrome. Hence, we examined several brain functions by utilizing behavioral paradigms: two sensory perception modalities (pain and odor), three hippocampal-dependent learning paradigms, and aspects of simple motor performance up to more complex behavior. We analyzed the outcomes for each set of experiments in relation to sex and genotype. In order to work in batches of equal amounts of male and female littermates, we did not further divide the female mice to the three cycling phases (proestrous, estrous, and metestrous). We are aware that such pooling could have increased the variability within females, which might have concealed other possible differences. Nevertheless, this study replicated many of the sex-dependent differences reported in the literature for WT mice [12, 13, 36, 39].

The most remarkable finding of the herein study is that several AS phenotypes were differentially affected by sex compared to the WT littermates. The main sex-dependent

modifications were observed in the sensory phenotypes. A more defined tenet is that when a certain neurobehavioral phenotype in WT mice does not show any sex-dependent modification, there are usually no sex-dependent differences in the AS mice as well. Such examples are exhibited in Morris water maze memory test (Fig. 2), simple motor performance tasks such as motor reflexes, locomotion in the open field, beam balance test, grip strength test (Fig. 6), and complex behavior studies such as nesting and marble-burying tests (Fig. 7f–h).

However, when WT mice show a phenotype with a significant sex-dependent difference, AS mice either do not exhibit this sex-dependent variation, for example in enhanced right-sided exploration (Fig. 3A, B), social odor preference (Fig. 3I), and odor sensitivity (Fig. 5B, D), or even show an opposite sex–dependent variation such as in pain perception, as observed by freezing response due to the nociceptive stimulus in CFC during acquisition (i.e., electrical shocks) (Fig. 1B), and in response to thermal-induced pain as determined by the hot-plate test (Fig. 4). Another phenotype that has sex-dependent inversion is the attitude (attraction-aversion) of AS mice towards object spatial novelty (displaced object) and object novelty (substituted object) (Fig. 3E–F). Interestingly, a recent study reported that there is enhanced nociception in AS model mice [76]. However, the groups in that study were composed mainly of males (~ 80%), probably leading to a male-biased phenotype, and thus actually supporting our findings regarding the enhanced pain perception in AS males.

In addition to the sex-dependent variation of behavioral phenotypes between WT and AS mice, we performed transcriptome-wide expression analysis. This analysis identified many genes with a differential sex-dependent expression between the genotypes. Analysis of this differential expression with set thresholds narrowed the gene lists to what are probably the more significant ones that mirrored the behavioral manifestation.

This intriguing inversion of male-female phenotype raises questions about the role of sex hormones in neurodevelopment and why these are reversed in AS mice. A possible answer could be that Ube3a interacts directly or indirectly with sex hormones functioning and thus affects the neurodevelopment of specific pathways, in which this sex-dependent phenotype inversion is recognized. Few studies, mostly in non-neuronal cell lines, have shown some evidence that Ube3a affects androgen receptor (AR)–, progesterone receptor (PR)–, and estrogen receptor (ER)–induced transcriptional activity. These specific Ube3a effects can vary and either promote ER-induced transcription or inhibit it [71–73]. We believe that our data concurs with a previous study that suggested the importance of Ube3a activity as a coactivator in ER-induced transcriptional activity, and proposed this facet of Ube3a as a key mechanism in the development of AS [72]. To conclude, in addition to two distinct molecular pathways, sex hormones and Ube3a, each on its own, play a role in brain development; also, as the herein study shows for the first time, there is a phenotypic intersection between these two pathways, which suggests their interactions on the molecular level and its significance in brain development.

The bioinformatics analysis of coding transcriptomes of the ventral hippocampi of WT and AS littermates yielded lists of interesting molecular targets, in which of special interest are the lists of androgen- and estrogen-dependent genes. From those estrogen-dependent genes, the seven genes that are also pain related are expected to play a role in the inverse behavioral response to pain as shown by the males and females of WT and AS mice. Though their role in determining the phenotype is not currently understood, these reported genes are important and feasible targets for further investigation in determining how pain perception is affected by sex hormones, and why AS mice differ from WT littermates. Especially of interest is the role of Gfra1 which is a receptor to glial-derived neurotrophic factor (Gdnf) [77]. Gdnf promotes effects on the dopaminergic system [78], which is known to be affected in AS mice. Also interesting is the altered expression of Gabbr1 gene, which encodes the Gaba(b) receptor subunit-1. Enhanced expression of this receptor should reduce excitability by enhancing inhibition due to enhanced potassium conductance [79]. Gaba(b) is implicated in pain perception regulation, and its positive modulation is considered to entail an anti-nociceptive effect [80]. The differences in the transcriptome profiles between the two sexes call for a further in-depth investigation of these and other candidate genes on both the protein and mRNA levels, including their subcellular localization. Moreover, we are aware that the important molecular differences responsible for the sex-dependent variability between WT and AS might not be accountable to those found in the ventral hippocampus. However, it is possible that other brain regions that are involved in pain perception and transmission contain similar molecular expression profiles as the ventral hippocampus. On top of all of these particular findings, the fact that many estrogen-dependent genes are differentially regulated in AS mice further emphasizes the role of Ube3a in shaping the molecular response to sex hormones, especially estrogens, besides its role as an E3-ligase.

Additional clinical significance of the abovementioned findings is the contribution to understanding that non-pharmacological therapies such as occupational therapy (OT) should be tailored also in accordance with sex. OT is an essential tier in treating AS patients for alleviating some of the behavioral symptoms and motor deficits. Our data suggest that OT approach should be tuned according to sex-dependent alterations of distinct sensory aspects in AS, and that aberrant sensory modalities are not homogenous across sexes. The sex-dependent variations in different sensory modalities require more sophisticated tactics in the examination, assessment, and treatment of patients with neurodevelopmental disorders.

Another aspect relevant to AS behavioral therapy that should be carefully considered when treating AS patients is their possible negative attitude towards novelty and their aversion to novel experiences and interactions. Hence, therapeutic strategies should be assessed and tailored in a more sex-dependent manner. To the best of our knowledge, there are no similar reports in humans that address sex-dependent differences in AS patients. However, it might be that sex-dependent variation was overlooked because no focused systemic study was conducted.

Finally, our study goes beyond AS and further supports the notion of declaring sex as a biological variable (SABV) [81]. Here we show that scientific studies which either utilize only males or pool both sexes together are liable to experimental artifacts or miss significant findings due to this selection bias. For example, a study in mice that investigated the more frequent form of AS in humans, which is a large deletion of *UBE3A* and *GABRB3* genes, showed no differences between WT and AS mice in hot-plate test [17]. This study pooled both sexes, and therefore probably skipped the thermal pain sensitivity differences. Hence, we emphasize that translational studies aimed at developing therapeutic strategies should take into consideration the entire population that eventually will require these treatments and therefore should examine their effects on males and females separately.

Conclusions

The herein study is a first detailed report of sex-dependent behavioral phenotypes and coding transcriptome expression profiles in AS mice. Our findings show that male-to-female differences in some of the sensory phenotypes, but not the motor ones, are aberrant in AS compared to WT. In specifics, the male-to-female difference in pain perception is completely opposite in the AS mice, as is the attitude towards novelty of object or its displacement. In addition, we generated a new mRNA sequencing dataset which includes data regarding sex- and genotype-dependent expression. The bioinformatics analyses of this dataset yielded genes, known to be pain related, that have expression profiles that reflect this opposite pain perception phenotype.

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Author's contributions HK initiated the study. LK and HK designed the behavioral experiments. LK performed the experiments and supplied the data for analysis. PRR, LK, and HK analyzed the behavioral data. LS (Simchi) and LS (Sharvit) produced the biological material for RNA sequencing. JP, YF, and HK performed the bioinformatics analyses. LK, JP, YF, PRR, and HK wrote the manuscript.

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Data Availability The RNA-Seq data are available for download from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number PRJNA484226.

Compliance with Ethical Standards

Ethical Approval All procedures were performed in strict accordance with the University of Haifa regulations and the US National Institutes of Health guidelines (NIH publication number 8023). All experiments and breeding protocols were approved by the animal welfare committee of the University of Haifa and the Israeli ministry of health.

Conflict of Interest The authors declare that they have no conflict of interest.

Consent for Publication Not applicable.

Abbreviations AS, Angelman syndrome; WT, wild type; CFC, contextual fear conditioning; MWM, Morris water maze; OFA, open-field arena; SOD, simple odor discrimination; SROI, sex-related opposite interaction; CTD, Comparative Toxicogenomics Database

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