Premorbid Characteristics of the SAPAP3-Mouse Model of Obsessive-Compulsive Disorder: Behavior, Neuroplasticity, and Psilocybin Treatment.

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Abstract

Deletion of the SAPAP3 gene in mice results in a characteristic phenotype that manifests from the age of 4-6 months and consists of repetitive bouts of self-grooming, head-body twitches, and anxiety-related behaviors. The phenotype is attenuated by sub-chronic fluoxetine and by single injections of ketamine and psilocybin and is considered a model for OCD. We investigated the premorbid characteristics of SAPAP3 knockout (SAPAP3-KO) mice. Two cohorts of juvenile SAPAP3-KO mice (aged 10-13 weeks) were assessed for anxiety and other behavioral phenotypes. Compared to wild-type (WT) mice, male and female homozygous SAPAP3-KO mice manifested significant anxiety-like behaviors in the open field and elevated plus maze tests, reduced marble burying, and altered performance on the buried Oreo test. These behaviors were not alleviated by psilocybin treatment. Adult male SAPAP3-KO mice showed increased levels of synaptic proteins (GAP43, synaptophysin, and SV2A) in the frontal cortex, hippocampus, and amygdala, while adult female SAPAP3-KO mice showed increased SV2A in the frontal cortex. These findings suggest enhanced synaptic growth and vesicle-associated plasticity in adult SAPAP3-KO mice that may reflect a compensatory mechanism. Increased synaptic proteins were not observed in juvenile mice, suggesting age-dependent alterations in neuroplasticity. Our finding that SAPAP3-KO mice exhibit anxiety-like behaviors before the onset of compulsive grooming is analogous to prodromal anxiety observed in patients with OCD. The study provides a basis for further research into the development of OCD-like behaviors and associated neuroplasticity changes and for studying potential treatments.

Introduction

Mice that carry a homozygous deletion of the SAPAP3 gene manifest, from the age of 4-6 months, a characteristic phenotype consisting of repetitive bouts of self-grooming, head-body twitches, and anxiety-related behaviors [1-3]. The SAPAP3 knockout mouse (SAPAP3-KO) is regarded by many investigators as a relevant preclinical model of obsessive-compulsive disorder (OCD) because of the resemblance of compulsive self-grooming to compulsive behaviors manifested by patients with OCD and OCD-related phenotypes such as trichotillomania and skin picking. The model thus has considerable face validity [4]. The self-grooming behavior and anxiety of SAPAP3-KO mice are reduced by sub-chronic administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine [1], which is used to treat OCD, reflecting predictive validity. Moreover, there is evidence of impaired cortico-striatal connectivity in SAPAP3-KO mice, which was alleviated by ketamine administration [5]. In patients with OCD, reduction of cortico-striatal hyperconnectivity was reported in patients who responded to treatment with dorsomedial prefrontal repetitive transcranial magnetic stimulation (rTMS) [6], suggesting that the SAPAP3-KO model of OCD also has construct validity [4].

SAPAP3 KO mice do not manifest an excessive self-grooming phenotype before the age of 4-6 months [1,2] . A key question is whether there are characteristic phenotypic manifestations in SAPAP3-KO mice prior to the emergence of the adult phenotype. Few studies have addressed this question directly. Tesdhahl et al. [7] recorded ultrasonic vocalizations from 5-day-old SAPAP3—KO mice and found an increase in the number and duration of these vocalizations compared to wild-type mice. Assessments of other phenotypic manifestations in SAPAP5-KO mice under 4 months old are currently lacking. Thus, it is not clear whether anxiety-like manifestations observed in adult SAPAP3 KO mice are present before the emergence of the full phenotype. This is an important question because it is well-established that there is a significant comorbidity between anxiety and OCD [8]. In this context, a recent study of 206 children and adolescents showed that generalized anxiety disorder was a significant predictor of obsessive-compulsive symptoms as well as risk for OCD [9]. Early diagnosis of OCD is important because of the possibility of early intervention, which is a focus of considerable interest [10]. Thus, phenotypic manifestations in SAPAP3 KO mice under 4 months old are a key unexplored question.

From the perspective of treatment, other than fluoxetine [1], there are reports that excessive self-grooming in SAPAP3-KO mice is reduced by a single administration of ketamine, the effect lasting for 3 days [5]. The atypical antipsychotic, aripiprazole, was found to reduce head-body twitches and short grooming bouts in SAPAP3-KO mice but not long grooming bouts [2]. Finally, it was recently demonstrated by Brownstien et al. [3] that a single administration of psilocybin or psilocybin-containing psychedelic mushroom extract significantly reduced excessive self-grooming and also head-body twitches and anxiety in male and female SAPAP3-KO mice >6 months old. The effect on self-grooming persisted for up to 42 days following a single administration.

In the current study, we examined male and female SAPAP3-KO mice aged 10-13 weeks for anxiety-related features and compared them to mice heterozygous for the mutation and wild type mice. We then examined the effect of treatment with a single dose of psilocybin on anxiety-related measures in SAPAP3 KO mice. In addition, we examined the levels of four proteins that play a key role in synaptic plasticity in four different brain areas in juvenile (≤3 months) and adult (>6 months) SAPAP3-KO mice. Our findings indicate that SAPAP3-KO mice manifest significant anxiety-related behavioral features before the emergence of the excessive self-grooming phenotype, but these manifestations are not responsive to treatment with psilocybin. Significant increases in brain levels of neuroplasticity-related synaptic proteins were observed in adult but not juvenile SAPAP3 -KO mice.

Material and Methods

Animals and Experimental Design

As previously described [3], a SAPAP3-KO breeding colony was established using 5 heterozygous SAPAP3-KO male and female mice kindly provided by Dr. Guoping Feng (Massachusetts Institute of Technology). Mice were housed up to 8 per cage under standardized conditions with a 12-h light/dark cycle, stable temperature (22 ± 1 °C), controlled humidity (55 ± 10%), and free access to mouse colony chow and water. Both female and male experimenters

handled the mice and performed the studies. The cages included all genotypes under study. Mice from the same cage received the same treatment. Throughout the experiment, male and female SAPAP3-KO mice were assessed separately in order to identify any significant difference in the behavioral phenotype between the genders. Experiments were approved by the Authority for Biological and Biomedical Models, Hebrew University of Jerusalem, Israel (Animal Care and Use Committee Approval Number: MD-21-16596-4). All efforts are made to minimize animal suffering and the number of animals used.

44 mice homozygous (HOM) for SAPAP3-KO were used for Study 1 (20 male, 24 female), 42 heterozygous (HET) mice (21 male, 21 female), and 55 wild-type (WT) mice (26 male, 29 female). Mice were aged 11.03±0.84 (mean ± SD) weeks at the time of evaluation (Supplementary Table 1). No treatments were administered to the mice in this study.

For Study 2, 32 mice HOM for SAPAP3-KO were used (16 male, 16 female) and 32 WT mice (16 male, 16 female). Mice were 11.85±0.87 (mean ±S D) weeks at the time of entry into the study and treatment administration (Supplementary Table 1). Drugs were administered by intraperitoneal (i.p.) injection in a standard injection volume in the ratio of 10 µL/1g 48 hours before behavioral tests were initiated. Mice were randomly divided into the following treatment groups: Vehicle (VEH): 0.9% saline (Male, n=16; Female, N=16); Psilocybin (PSIL): 4.4 mg/kg dissolved in the saline vehicle (Male, n=16; Female, N=16).

Genotyping

Following the "HOTSHOT" method, genotype was determined by polymerase chain reaction (PCR) of mouse tail DNA or by mouse ear hole DNA. Three different SAPAP3 KO primers are used to distinguish the genotypes of wild type, heterozygous and Homozygous mice as follow: Primer F1 (5' ATTGGTAGGCAATACCAACAGG 3') and Primer R1 (5'GCAAAGGCTCTTCATATTGTTGG 3') identify wild-type allele (around 147 base pairs) , while Primer F1 and TK F2 (5' CTTTGTGGTTCTAAGTACTGTGG 3') identify KO allele (around 222 base pairs) [1].

Behavioral tests

For Study 1, behavioral tests were performed according to the schedule shown in Fig. 1a. Since all the tests have objective outcome measures that are not dependent on subjective assessment, the experimenter was aware of the genotypes of the mice. The same experimenter (MB) performed all the behavioral assessments. A detailed description of the tests is provided in the Supplementary Information section of this paper.

For Study 2, behavioral tests started 48 hours after drug administration, according to the schedule shown in Fig. 1b. As in Study 1, the outcome measures were objective, and the experimenter was not blind to genotype or treatment assignment. ML performed all the assessments with the assistance of BO. The methodology applied in the tests was the same as for Study 1, as described in Supplementary Information.

Synaptic protein assays

Study 1: Brain tissue samples (frontal cortex, hippocampus, amygdala, and striatum) were obtained, as previously described [11], from 60 adult SAPAP3-KO mice aged 7 months and 16 days ± 7.8 days (males) and 7 months and 26 days ± 7.5 days (females) (mean ± SD) (HOM: male n=15, female n=15; WT male n=15, female n=15;) that had not undergone behavioral testing. Samples were stored at −80˚C until assayed. Using Western blot analysis, we quantified 4 synaptic proteins (GAP43, PSD95, synaptophysin, and SV2A) in each of the 4 brain areas, as described in our previous report [11]. Briefly, the brain tissue samples were lysed in Pierce RIPA sample buffer (Thermo Scientific, USA), supplemented with protease inhibitor cocktail (Roche Diagnostics, Germany) and boiled for 10 min. Equivalent amounts of protein extracts (20 mg) were analyzed by SDS–12% PAGE, followed by transfer of the proteins to polyvinylidene fluoride membrane. Blots were blocked in 5% fat free milk in TBST buffer (Tris-Tween-buffered saline) and incubated in primary antibodies, one hour at room temperature. Primary antibodies

included rabbit anti-GAP43 (ab75810, 1:2000; Abcam, UK), rabbit anti-PSD95 (ab238135, 1:2000; Abcam, UK), rabbit anti-Synaptophysin (ab32127, 1:2000; Abcam, UK), rabbit anti-SV2A (ab54351, 1:100; Abcam, UK) and mouse anti-β-Actin (8H10D10, 1:5000, Cell Signaling Technology). Blots were washed 3 times and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, ABclonal, China) for 1 h, followed by repeated washing with TBST buffer. Proteins were visualized by using enhanced chemiluminescence (ChemiDoc Reader MP, Bio-Rad, USA). The amount of each phosphorylated protein was normalized to the amount of the corresponding total protein detected in the sample and measured by intensity of β-actin.

Study 2: On day 13 after drug administration, brain tissue samples (frontal cortex, hippocampus, amygdala and striatum) were obtained from 24 juvenile SAPAP3-KO mice aged 12.96±0.999 (mean ± SD) weeks (HOM: male n=5, female n=6; WT male n=7, female n=6;) SAPAP3-KO (n=11) and WT mice (n=13) that had been administered VEH, and were stored at −80˚C. Synaptic proteins (GAP43, PSD95, synaptophysin and SV2A) were quantified as described above.

Statistical analysis

The experimental data are presented in all figures as the mean ± standard error of the mean (SEM) and in the text as the mean ± standard (SD). To determine inter-group differences, student t-tests or one or two-way analysis of variance (ANOVA) were used as indicated. Sidak, Tukey, or Dunnett's Tests were used to analyze post-hoc comparisons. $p < 0.05$ (two-tailed) was the criterion for significance. Graph Pad Prism, version 9.3.1 software was used for all statistical analyses. A nested t-test was performed to compare the expression levels of all 4 synaptic proteins (GAP43, PSD95, synaptophysin, SV2A) in each brain area (frontal cortex, hippocampus, amygdala and striatum) between two genotypes (SAPAP3 KO and WT) and of each synaptic protein

over all 4 brain areas. Statistical significance was assessed using a t-test (two-tailed). $p < 0.05$ was considered statistically significant. Results are shown as mean ± SEM.

Results

Behavioral Tests

Study 1

In Study 1, SAPAP3 HOM, HET, and WT KO mice underwent a series of behavioral tests to evaluate anxiety and depression-like phenotypes, cognition, social interaction, and social dominance. The schedule for these tests is given in Fig 1a. The results of this study show significant effects of the SAPAP3-KO genotype on several behavioral tests.

On the OFT (Fig. 2-Ia. Fig. 2-Ib), one-way ANOVA of total distance traveled showed a significant effect of genotype in both males ($F=7.89$, $df=2,63$, $p=0.0009$) and females ($F=12.71$, $df=2,56$, $p<0.0001$). Male HOM SAPAP3-KO mice traveled a significantly lower distance ($p=0.0007$) than male WT mice (Fig2-Ia), and female HOM SAPAP3-KO mice traveled a significantly lower distance than female WT ($p=0.0007$) and HET mice ($p=0.04$) (Fig2-Ib). There was also a significant effect of genotype on time spent in the center of the open field for male ($F=5.74$, $df=2,64$, $p=0.005$) and female ($F=3.687$; $df=2,70$; $p=0.03$) SAPAP3-KO mice (Fig. 2-Ic, Fig. 2-1d). Male ($p=0.004$) and female ($p=0.026$) HOM SAPAP3-KO mice both spent significantly less time in the center of the OFT arena than their WT littermates (Fig. 2-Ic, Fig. 2-Id), indicating a greater level of anxiety. Regarding time spent in the periphery of the open field, one-way ANOVA showed a significant effect of genotype in male mice ($F=7.46$, $df=2,59$, $p=0.0013$). Male HOM SAPAP3-KO mice spent significantly more time in the periphery ($p=0.0009$) than male WT mice. For the female group, there were no significant differences in time spent in the periphery.

On the EPM, time spent in the unprotected open arms and frequency of entry into these arms are indicative of level of anxiety, with more anxious mice spending less time in these arms and entering less frequently. There was a significant effect of genotype on time spent in the open arms of the EPM for male ($F=4.874$, $df=2,58$, $p=0.001$) and female ($F=3.55$, $df=2,64$, $p=0.034$)

SAPAP3-KO mice (Fig. 2-IIa, Fig. 2-IIb). Male HOM SAPAP3-KO mice spent significantly less time in the open arms than HET SAPAP3-KO (p=0.03) and WT mice (p=0.01) (Fig. 2-IIa) and also female HOM SAPAP3-KO mice compared to female WT mice (p=0.04) (Fig. 2-IIb). One-way ANOVA also showed a significant effect of genotype on the number of times mice entered the open arms for male SAPAP3-KO mice (F=4.19, df=2,58, p=0.02). The total number of times entered into the open arm was significantly lower in male HOM SAPAP3-KO mice (p=0.03) compared to WT. (Fig2-IIc).

On the MB test (Fig 2-III), one-way ANOVA showed a significant genotype effect for both males and females (males: F=18.9, df=2,63, p<0.0001; females: F=53.74, df=2,71, p<0.0001). Male and female HOM SAPAP3-KO mice buried significantly fewer marbles than their WT littermates (p<0.0001) and female HOM significantly fewer than female HET mice (p<0.01) (Fig. 2-IIIa and b).

Performance on the tube dominance test reflects social assertiveness [12]. There were no significant differences between genotypes in the female group (Fig. 2-IVb). In male mice, one-way ANOVA showed a significant effect of genotype (F=4.516, df=2,64; p=0.01) with a higher percent of winning (p=0.01) in HOM compared to WT littermates. (Fig. 2-IVa)

The Oreo test is based on the preference of mice for sweet objects [13]. The first assessment was whether previously habituated mice placed in a cage with a buried Oreo would find the cookie and dig it up. There was a significant effect of genotype on this parameter among male mice (Table 1). 88.96% of male WT mice found the Oreo and 81.82% of male HET mice but only 35.00% of male HOM mice (x^2=16.75, df 2, p=0.0002). Among female mice, the differences were not significant (WT 62.07% found; HET 61.90% found; HOM 58,33% found; x^2=0.10, df 2, p=0.93). There was no significant effect of genotype on Oreos eaten by those mice that found them among males or female mice (Table 1a).

Contrary to these positive findings, other tests did not show an effect of SAPAP3-KO genotype in male or female nice. On the forced swim test (FST), there was no significant effect of genotype on duration (Supplementary Fig 1a, 1b) or frequency (Supplementary Fig. 1c, Fig. 1d) of inactivity in male or female mice. The social exploration test (Supplementary Fig. 2a, 2b), the

NOR test (Supplementary Fig. 2c, 2d), and the Y maze (Supplementary Fig. 2d, 2e) also showed no significant differences between genotypes in both male and female groups.

Study 2

In Study 2, we examined the effect of treatment with psilocybin 4.4 mg/kg i.p. on the performance of juvenile SAPAP3-KO mice (mean age 11.85±0.86 weeks) on a series of tests that had shown significant differences between SAPAP3-KO genotypes in Study 1. The study timeline and test battery are summarized in Fig 1b.

There were no significant effects of psilocybin treatment on any of the behavioral measures, as indicated by the lack of a significant main effect of treatment on two-way ANOVA and the lack of significant interaction between genotype and treatment for any of the tests. However, there were significant effects of genotype over and above treatment and significant post-hoc effects in both saline and psilocybin-treated mice on a number of tests.

On the Open Field Test (Fig. 3-Ia and b), which examines activity and anxiety-like behaviors, there was a significant effect of genotype over and above treatment on distance traveled in male ($F=8.121$; df 1,28; $p=0.0081$) (Fig. 3 I-a) and female ($F=32.35$; df 1,28; $p<0.0001$) (Fig. 3 I-b) SAPAP-3 KO mice. Post-hoc tests showed significantly lower distance traveled for males ($p<0.05$, Psilocybin WT vs. Psilocybin HOM) (Fig. 3 1-a) and females ($p<0.01$, Saline WT vs. Saline HOM; $p=0.0014$, Psilocybin WT vs. Psilocybin HOM) (Fig. 3 I-b) SAPAP3-KO mice. The center duration of SAPAP3 KO mice was not significantly shorter than WT mice in males (Fig. 7e) but was significantly so in females ($F=9.12$; df 1,28; $p=0.0053$; $p<0.05$, Psilocybin WT vs. Psilocybin HOM) (Fig. 3 I-d). For periphery duration there was no significant difference between genotypes in males or females.

EPM results (Fig. 3 II) revealed a striking level of anxiety among HOM SAPAP3-KO mice of both genders compared to WT mice, as reflected by the effect of genotype on time spent in the open arms of the apparatus (males: F $F=27.76$, df 1,28, $p<0.0001$; females: $F=23.46$, df 1,28, $p<0.0001$). Vehicle-treated HOM mice of both genders spent significantly less time in the open arms of the EPM than vehicle-treated WT mice (males: $p=0.0005$; females: $p=0.004$), and also

psilocybin-treated HOM mice compared to psilocybin-treated WT mice (males: p=0.03; females: p=0.02) (Fig. 3 II-a, b). For time spent in the closed arms of the EPM, there was also a significant effect of genotype in both genders (males: F=7.74; df 1,28; p=0.01; females: F=19.17; df 1,28; p=0.0002); post hoc tests were significant only for vehicle-treated HOM female mice (p=0.048) that spent more time in the closed arms than vehicle-treated WT female mice. The effect of genotype on frequency of entrances to the open arms was significant in males (F=11.15; df 1,28; p=0.002; and females (F=14.19; df 1,28; p=0.0008; p<0.039, Psilocybin WT vs. Psilocybin HOM) (Fig. 3 II-d). The difference in frequency of entrances to closed arms was significant between genotypes in females (F=19.64; df 1,28; p=0.0001; p<0.01, Saline WT vs. Saline HOM; p<0.05, Psilocybin WT vs. Psilocybin HOM), but not in males.

As in Study 1, the marble burying test (Fig. 3-III) yielded striking results. There was a highly significant genotype effect in both genders (males: F=113.6, df 1, 28, p<0.0001; females: F=35.40, df 1, 28, p<0.0001). Vehicle-treated HOM mice of both genders buried significantly fewer marbles than WT mice (males p<0.0001; females p=0.0006), as was the case for psilocybin-treated HOM mice compared to psilocybin-treated WT mice (males: p<0.0001; females p=0.0028) (Fig. 3-IIIa, Fig. 3 III-b).

On the Tube Dominance test (Fig. 3 IV), male SAPAP3 KO mice were significantly more dominant than WT mice (F=13.28; df 1,28; p=0.001; ***p<0.001, Saline WT vs. Saline HOM) (Fig. 3. IV-a); the effect of genotype was also significant in females (F=9.86; df 1,28; p=0.003).

The Oreo test results in Study 2 (Table 1b) were similar to those in Study 1. Whether treated with saline or psilocybin, WT mice found the Oreo cookie significantly more frequently than HOM mice. The percentage of mice succeeding was >85% in all cases. For HOM mice, the percentage finding the Oreo was 50% or less. Comparisons between WT and HOM for Oreos were statistically significant for both genders. For Oreos eaten, there was a clear numerical trend for WT mice to eat more of the Oreos found but the number of observations was too small to permit statistical significance to be demonstrated.

On the Y Maze which measures short-term special memory [14], there were no significant effects of genotype on direct and indirect revisits (Supplementary Fig. 3a-d). 2-way ANOVA

showed a significant effect of genotype on max alternations in females (F=7.77; df 1,28; p=0.009) (Fig 4f) but not males (Supplementary Fig. 3e). Complete alternations showed significant difference between genotype in males (F=11.85, df 1,28, p=0.001; *p<0.05, Saline WT vs. Saline HOM) (Supplementary Fig. 3g) and females (F=10.09; df 1,28; p<0.003) (Supplementary Fig 3h). However, the complete alternation ratio did not show a significant difference between genotypes (Supplementary Fig. 3i).

In both of our studies, mouse activity on the open field was significantly lower in HOM than in WT mice, irrespective of psilocybin treatment in Study 2. To determine whether reduced time spent in the open arms of the EPM and lower number of marbles buried could be a consequence of reduced activity, we performed an analysis of covariance (SPSS), entering distance covered on the OFT as a covariate and examining genotype as the independent factor. The analysis was performed in male and female mice separately. It showed no significant effect of distance covered on the OFT on the dependent variable - time spent in the open arms of the EPM or number of marbles buried - under conditions of saline or psilocybin treatment in Study 2.

Synaptic Proteins

Study 1

Because of significant gender effects (see below), male and female mice were analyzed separately for synaptic protein levels in this study (See Supplementary Figures 4-7). The synaptic protein that showed the strongest effects of genotype was SV2A. Two-way ANOVA showed a significant effect of genotype on SV2A levels in the frontal cortex (F=17.22, df 1,55, p=0.001), with female HOM mice manifesting significantly higher SV2A levels than female WT mice (Tukey post-hoc, p=0.01). Genotype effects were also highly significant in the hippocampus (F=14.67, df 1,56, p=0.0003), amygdala (F=14.48, df 1,53, p=0.0004), and striatum (F=4.59, df 1,53, p=0.03). Post-hoc tests showed that SV2A levels were significantly higher in male HOM than male WT mice in all three areas (p<0.0001) (Supplementary Fig. 7). Significant effects of genotype were observed for GAP43 in the frontal cortex (F=7.30, df 1,56, p=0.009) and amygdala (F=8.24, df 1,54, p=0.005), post-hoc significance for male HOM vs. male WT was 0.02 and 0.04 for each area

respectively (Supplementary Fig. 4). For PSD95 there was a significant effect of genotype in the frontal cortex (F=6.70, df 1,54, p=0.01) (Supplementary Fig. 5), and for synaptophysin in the amygdala (F=7.34, d 1,56, p=0.008), but post-hoc tests were not significant in either gender separately (Supplementary Fig. 6).

There were significant gender effects on SV2A levels in the amygdala (F=12.32, df 1,53, p=0.0009) and striatum (F=7.48, df 1,53, p=0.008) (Supplementary Fig. 7); on GAP43 in the amygdala (F=6.15, df 1,54, p=0.01) (Supplementary Fig. 4); on PSD95 in the frontal cortex (F=5.43, df 1,54, p=0.02) and hippocampus (F=4.38, df 1,56, p=0.04) (Supplementary Fig. 5); and on synaptophysin in the striatum (F=11.77, df 1,55, p=0.001) (Supplementary Fig. 6). A significant gender x genotype interaction was observed for SV2A in the hippocampus (F=20,94, df 1,56, p=,0.0001), amygdala (F=26.75, df 1,53, p<0.0001) and striatum (F=15.08, df 1,53, p=0.0003) (Supplementary Fig. 7) and for synaptophysin the frontal cortex (F=13.51 df 1,55, p=0.005) (Supplementary Fig. 6).

To analyze synaptic proteins over all four brain areas, nested t-tests were performed (Fig. 4) comparing each synaptic protein in HOM and WT mice over all brain areas. Nested t-tests were performed separately for male and female mice. In males, the analysis showed significantly higher levels of GAP43 (p=0.001), synaptophysin (p=0.003) and SV2A (p<0.0001) but not of PSD95 across the 4 brain areas in HOM vs WT SAPAP3-KO mice (Fig. 4, Left Panel). None of the differences were significant for female SAPAP3-KO mice (Fig. 5, Right Panel). Examining all 4 synaptic proteins within each brain area separately, significantly higher synaptic protein levels were observed in male HOM SAPAP3-KO mice than in WT mice in the frontal cortex (p=0.0001), hippocampus (p=0.0002) and amygdala (p=0.0001) but not striatum (Supplementary Fig. 8, Left Panel). No significant differences were observed in any of the brain areas in female mice (Supplementary Fig. 8, Right Panel).

Study 2

We analyzed synaptic protein neuroplasticity markers on brain samples obtained from 11 HOM (5 males and 6 females for each genotype) and 13 WT (7 males and 6 females for each genotype) vehicle-treated mice on day 13 after treatment administration. We performed two-

way ANOVA for each of the 4 synaptic proteins in each of the 4 brain areas, with gender and genotype as factors (Supplementary Figures 8-11). This analysis revealed a significant gender effect for PSD95 in the amygdala (F=7.10, df 1,16, p=0.01), a significant gender by genotype interaction for PSD95 in the hippocampus (F=7.54, df 1,20, p=0.01) and a significant gender by genotype interaction for synaptophysin in the striatum (F=7.49, df 1,20, p=0.008). No post-hoc comparisons of HOM vs. WT genotype in males or females were significant for any of the significant gender effects or gender x genotype interactions. We performed a nested t-test comparing each synaptic protein (GAP43 – Fig. 13-Ia, PSD95-Fig. 13-Ib, synaptophysin – Fig. 13-Ic and SV2A – Fig. 13-Id) in HOM versus WT mice over all brain areas (frontal cortex, hippocampus, amygdala and striatum). This analysis did not show significant differences between genotypes for any of the synaptic proteins across all brain areas. For analysis of the synaptic protein neuroplasticity markers within each brain area, a nested t-test was performed comparing levels of all four synaptic proteins in HOM and WT mice in each brain area (frontal cortex – Fig. 13-IIa, hippocampus – Fig. 13-IIb, amygdala – Fig. 13-IIc and striatum – Fig. 13-IId). This analysis did not show significant differences over all synaptic proteins within any of the brain areas.

Discussion

Age of onset in OCD has been reported to be bimodal, with a mean of 12.8 (S.D. = 4.9) years in the early-onset group and 24.9 (S.D. = 9.3) years in the late-onset group [15]. Anxiety is a common comorbid feature of OCD [8], and prodromal anxiety symptoms are frequent in youth [9] and adults [16] who subsequently develop OCD. Since the excessive self-grooming observed in SAPAP3-KO mice is regarded as a rodent model of OCD-like behavior, it is of interest to know whether SAPAP3-KO mice below the age at which the characteristic phenotype is observed manifest behavioral and other abnormalities. This question was the focus of the current study in which SAPAP3-KO mice of both genders were assessed for a series of behavioral characteristics, including anxiety, depressive-like features, cognitive function, sociability, and social dominance. The effect of treatment with psilocybin on these behavioral features was evaluated. In addition, we measured levels of synaptic proteins in four brain regions in mice homozygous for the SAPAP3 deletion compared to wild-type mice as markers of neuroplasticity [11].

Our results in Study 1 for time spent in the center of the open field and time spent in the open arms of the EPM, show clear evidence in male and female mice for higher levels of anxiety in homozygous SAPAP3-KO mice compared to WT mice. These findings were supported by those of Study 2, for females in regard to center duration in the open field and for both genders in regard to time spent in the open arms of the EPM. This evidence for higher anxiety levels in juvenile SAPAP3-KO mice is in accordance with findings of [1] and [3] in adult SAPAP3 KO mice that manifest the full self-grooming and head-twitch phenotype. The anxiety-like effects observed on the EPM were not a result of reduced activity since when open field activity was entered as a covariate in the analysis, it had no significant effect. Welch et al [1] found that 6 days of treatment with fluoxetine significantly alleviated anxiety like features on the elevated plus maze. Brownstien et al [3] found that a single treatment with psychedelic mushroom extract (containing 4.4 mg/kg psilocybin) significantly improved anxiety-like behavior on the OFT and EPM; treatment with chemical psilocybin at the same dose was not as effective. In Study 2 of the current project, psilocybin 4.4 mg/kg was not effective in improving anxiety-like behavior in SAPAP3-KO mice. The effect of psychedelic mushroom extract remains to be studied in juvenile SAPAP3-KO mice.

The MBT showed intriguing and highly significant effects of genotype in both genders. In Study 1 and Study 2, juvenile SAPAP3-KO mice homozygous for the deletion, buried significantly fewer marbles over 30 minutes than WT mice. Similar findings were reported by [3] in adult SAPAP3-KO mice. Reduced marble burying was not a result of reduced activity since when open field activity was entered as a covariate in the analysis, it had no significant effect. Digging, burrowing, and burying are natural behaviors in rodents and not necessarily indicative of pathology [17]. The MBT is widely used as a predictive test for the potential efficacy of compounds such as psilocybin in treating OCD [18,19] and as a measure of anxiolytic effects [20]. However, the relevance of the test has been questioned on the grounds of face and predictive validity [21,22] . If, indeed, marble burying is a natural behavior in rodents, reduction of this behavior by SAPAP3 gene deletion could reflect pathological consequences of the deletion. In this context, it is of great interest that in adult SAPAP-3 KO mice treated with a single injection of psilocybin (4.4 mg/kg) (or psychedelic mushroom extract containing the same dose of psilocybin), marble burying was significantly increased by the treatment [3]. The MBT was performed by [3] 3 days after the administration of the mushroom extract. At this time, the

pathological self-grooming and head-body twitches characteristic of adult HOM SAPAP3-KO mice were also alleviated by psychedelic mushroom extract and by psilocybin. In contrast, marble burying was reduced by the same dose of psilocybin in ICR mice; this effect was observed acutely, 30 minutes after drug administration. These observations support the conclusion that effects of psilocybin and psychedelic mushroom extract on behavior and pathological grooming in SAPAP3-KO mice and effects of psilocybin on marble burying are likely mediated by different mechanisms. In contrast to the effect of psilocybin on marble burying in adult SAPAP3-KO mice, in Study 2 of the current project, there was no effect of psilocybin on the same phenotype in juvenile mice. This suggests that the effect of psilocybin on behavioral manifestations of SAPAP3 deletion may be related to the maturation of brain systems.

The results of the tube dominance test in Study 1 revealed greater assertiveness of male HOM mice than male WT mice but no significant effect in females. In Study 2, male and female HOM mice were both more assertive than WT mice. Greater assertiveness of HOM mice on the tube dominance test is unexpected in view of the reported association of SAPAP3 deletion with lower sociability [23], although it should be noted that in Study 1 of the current project, genotype-associated impairment of social interaction was not observed in SAPAP3-KO mice. It could be due to dissociation between social interaction and social dominance, anxiety-driven assertiveness or developmental compensation (it is possible that the loss of SAPAP3 from early development leads to compensatory changes in other neural circuits that influence social dominance behaviors).

Buried food seeking is a frequently used test in olfaction research in mice [24] [25]. The Buried Oreo test used in our studies combines the predilection of mice to unearth buried food substances with the preference of mice for sweet taste. This preference is a basis for the sucrose preference test that is widely used as a measure of anhedonia in mice [13]. In both studies reported here, HOM juvenile SAPAP3-KO mice of both genders unearthed significantly fewer buried Oreo cookies than WT mice. There was no difference between genotypes in either study in number of unearthed Oreos that were eaten although there was a trend in this direction in Study 2. Thus, it is not entirely clear whether the lower tendency to unearth buried Oreos in the HOM mice is a manifestation of anhedonia or lack of motivation, Psilocybin treatment in Study 2 did not alter number of Oreos unearthed or eaten.

Contrary to these positive findings, there was no effect of SAPAP3-KO genotype on the FST reflecting absence of depressive-like features (Study 1), on social exploration (Study 1) or on two cognitive measures, the Novel Object Recognition test (Study 1) and Y-Maze (Studies 1 and 2). Lack of difference on the FST, which is sensitive to activity, supports the finding that in other tests (such as EPM and MBT) the significant effect of genotype was not a consequence of reduced activity in HOM SAPAP3-KO mice.

Our findings reveal significant effects of genotype on synaptic plasticity markers in adult SAPAP3-KO mice in the frontal cortex, amygdala, hippocampus, and striatum. In post-hoc analyses by gender, the synaptic proteins showing significantly higher levels in male HOM mice were GAP43 (in frontal cortex and amygdala), synaptophysin (in frontal cortex), and SV2A (in hippocampus, amygdala, and striatum). In female HOM mice, SV2A was significantly elevated in the frontal cortex compared to female WT mice. Although there was a significant effect of genotype on PSD95 in the frontal cortex, levels of this synaptic protein were not significantly different by post-hoc testing in either gender separately in any of the four brain areas. Minimal effects of genotype or gender on synaptic protein levels were observed in juvenile SAPAP3 KO mice aged 12 weeks. Our findings regarding genotype are partially in accordance with those of Glorie et al. [26], who used positron emission tomography (PET) to examine SV2A levels in the cortex, hippocampus, thalamus, and striatum of female SAPAP3-KO and WT mice. Glorie et al. [26] found that at the age of 3 months, the female SAPAP3-KO mice they studied manifested significantly lower SV2A binding compared to WT littermates in the cortex and hippocampus. thalamus and striatum. Aging in WT mice was associated with a significant ($p < 0.001$) decline in SV2A binding throughout the brain, whereas in SAPAP3 KO mice, this decline was confined to the corticostriatal level.

The increase in synaptic proteins in SAPAP3-KO mice points to enhanced synaptic growth and vesicle-associated plasticity in the affected regions. GAP43 is a marker of axonal growth and synaptic remodeling, while synaptophysin and SV2A are essential components of synaptic vesicle regulation and neurotransmitter release [27-29]. SAPAP3 is a scaffold protein that interacts directly with PSD95, as its name - SAP90/PSD95-associated protein 3 - suggests. Importantly, SAPAP3 is a post-synaptic protein located in the post-synaptic density and is highly

expressed in the striatum. Despite this, our findings showed less striking changes in PSD95 levels in male or female adult KO mice. This suggests that the alterations we observed may be secondary to disrupted signaling pathways involving the cortex-thalamus-striatum circuit. The increased synaptic proteins in the cortex, hippocampus, and amygdala of male SAPAP3-KO mice could reflect compensatory changes in response to dysregulated inputs from the striatum [30]. Thus, plasticity alterations in SAPAP3-KO mice may be presynaptic, affecting synaptic transmission and connectivity rather than postsynaptic structures. This is intriguing given that synaptic pruning, a normal developmental process that refines neural circuits during adolescence, involves the selective removal of unnecessary synapses, typically reducing plasticity in adult brain regions [31]. In this context, it is of interest that Glorie et al. [26] found a decrease in SV2A binding in adult compared to juvenile SAPAP3-WT mice. This decrease was less consistent in SAPAP3-KO mice.

As noted, the observed increases in synaptic proteins occurred only in adult SAPAP3-KO mice. The fact that these changes were absent in younger mice supports the idea of an age-dependent progression of pathology, potentially related to abnormal synaptic pruning. In healthy humans, synaptic pruning during adolescence serves to eliminate excess synapses, optimizing neural networks for efficient processing [31]. Disruptions in this process have been implicated in various neuropsychiatric disorders [32,33]. Specifically, delayed or abnormal pruning has been associated with atypical neural development, as seen in humans with higher neuropsychopathological (NP) factor scores, where a reduction in gray matter volume is inhibited during adolescence [34]. These findings suggest that similar processes may occur in male SAPAP3-KO mice, with altered synaptic pruning contributing to the pathological increase in synaptic proteins in adulthood. The increase in plasticity markers in older SAPAP3-KO mice might reflect a compensatory or maladaptive response to impaired pruning, particularly in regions involved in emotional and cognitive regulation. This may explain why the obsessive grooming behaviors, a hallmark of this model, emerge in adulthood rather than earlier in life.

Despite molecular differences, male and female mice showed similar OCD-like behaviors [3], indicating that behavioral phenotypes were not strongly influenced by gender. The less striking synaptic protein increases in females, despite comparable behaviors, may be due to sex-specific neurodevelopmental processes. Studies show distinct patterns of synaptic pruning and plasticity

between sexes, influenced by hormones like testosterone and estrogen, which shape brain plasticity differently [35]. Males undergo more robust synaptic pruning in regions like the nucleus accumbens during adolescence [31], possibly explaining the observed increase in plasticity markers in older male mice.

Conclusions

Our findings demonstrate, in two separate mouse cohorts, high levels of anxiety-like behavior in juvenile SAPAP3-KO mice that are homozygous for deletion of the gene, as well as other behavioral differences. The high levels of anxiety that we observed were not responsive to treatment with psilocybin. The excessive self-grooming observed in adult SAPAP3-KO mice is regarded as modeling OCD; our current findings are analogous to the prodromal anxiety observed in patients with OCD and can provide an important basis for further studies. Our intriguing findings regarding increased neuroplasticity in adult, but not juvenile, male SAPAP3-mice are a basis for further research to elucidate the mechanisms underlying these changes and their implications for OCD.

Data Availability Statement

Data is available to qualified investigators on request from the corresponding authors.

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Author Contributions

Conception and design of the work: TL, BL

Acquisition, analysis, or interpretation of data for the work: ML, MB, AB, CS, OS, TL, BL

Drafting the work or revising it critically for important intellectual content: ML, OS, BL

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing Interests

BL is a consultant to Back of the Yards algae sciences (BYAS) and Parow Entheobiosciences (ParowBio). ParowBio has submitted a patent application (WO 2023\164092 A1) on the use of psilocybin-containing psychedelic mushroom extract to treat psychiatric disorders including OCD on which BL is a co-inventor. None of the other authors have any competing interests.

Table 1: Effect of SAPAP3-KO genotype on finding and eating Oreo Cookies (No./% of mice that found cookies in the test and number of those which found cookies that ate them)

a. Study 1

	No.	Percent		x^2 (df)	Significance
Male					
Found Oreos WT	20/23	88.96			
HET	18/22	81.82			
HOM	7/20	35.00		16.75 (2)	p=-.0002
Ate Oreos WT	8/20	40.00			
HET	6/18	33.33			
HOM	4/7	57.14		1.25 (2)	p=0.54
Females					
Found Oreos WT	18/29	62.07			
HET	13/21	61.90			
HOM	14/24	58.33		0.10 (2)	p=0.93
Ate Oreos WT	9/18	50.00			
HET	10/13	76.92			
HOM	9/14	64.29		2.49 (2)	p=0.28

b. Study 2

	No.	Percent	x^2 (df)	Significance
SALINE TREATED Males				
Found Oreos WT	8/8	100.00		
HOM	4/8	50.00	5.33 (1)	P<0.05

Ate Oreos WT	8/8	100.00		
HOM	1/4	25.00		p>0.1 (Fisher)
Females				
Found Oreos WT	7/8	87.50		
HOM	2/8	25.00	7.27 (1)	P<0.01
Ate Oreos WT	6/7	85.71		
HOM	1/3	33.33		p>0.1 (Fisher)
PSILOCYBIN TREATED Male				
Found Oreos WT	7/8	87.5		
HOM	2/8	25.00	7.27 (1)	P<0.01
Ate Oreos WT	6/7	85.71		
HOM	1/2	50.00		p>0.1 (Fisher)
Females				
Found Oreos WT	8/8	100.00		
HOM	3/8	37.50	8.73 (1)	P<0.01
Ate Oreos WT	7/8	88.89		
HOM	1/3	33.33		p>0.1 (Fisher)

Figures and Legends

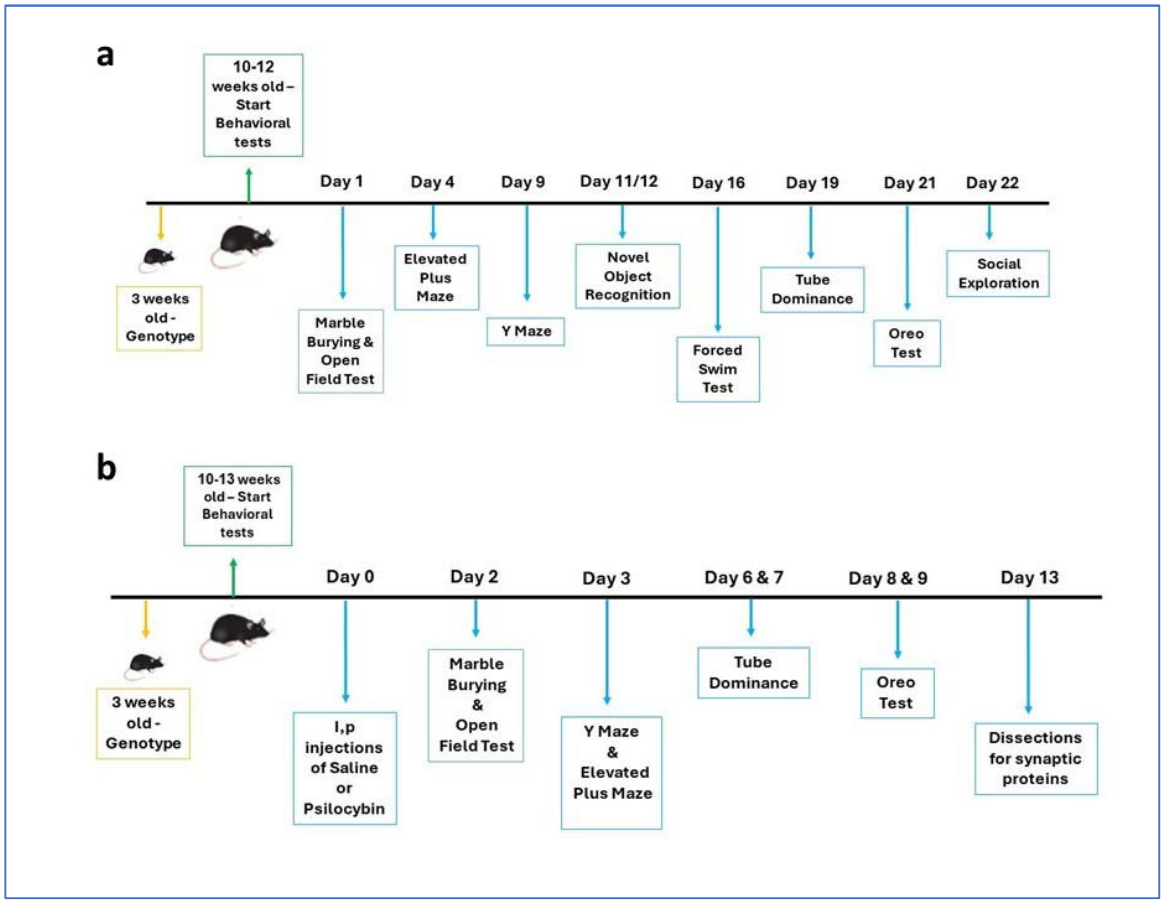
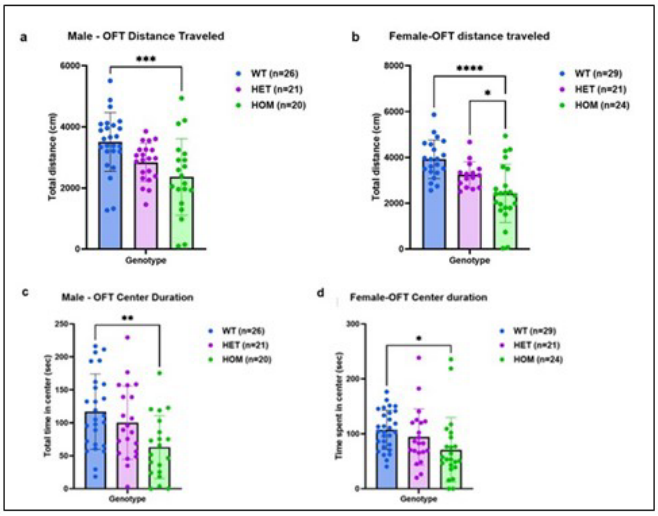


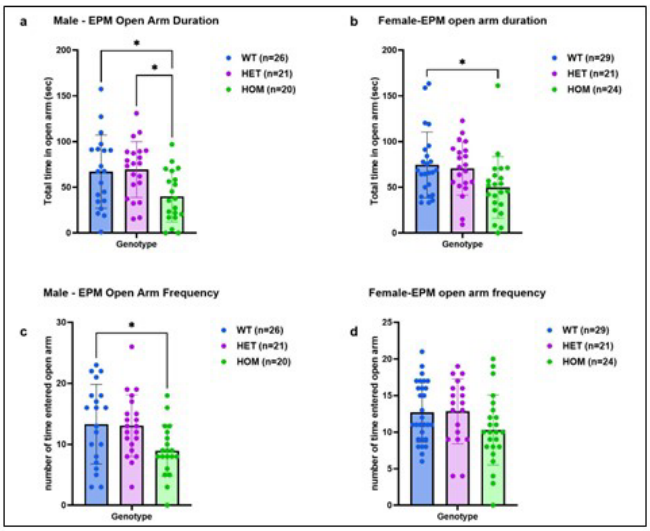
Fig. 1:

a. Timeline of the behavioral test in Study 1. **b:** Timeline of treatment and behavioral tests in Study 2.

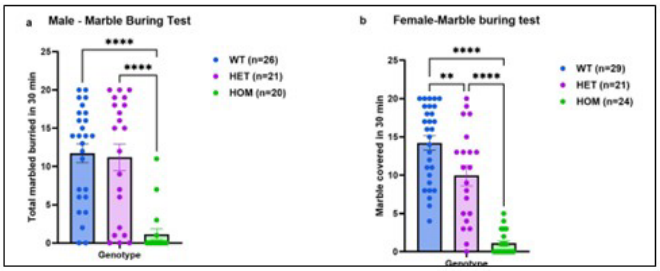
I


a
Male - OFT Distance Traveled
Total distance (cm)
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
b
Female-OFT distance traveled
Total distance (cm)
Genotype
WT (n=29)
HET (n=21)
HOM (n=24)
c
Male - OFT Center Duration
Total time in center (sec)
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
d
Female-OFT Center duration
Time spent in center (sec)
Genotype
WT (n=29)
HET (n=21)
HOM (n=24)

II


a
Male - EPM Open Arm Duration
Total time in open arm (sec)
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
b
Female-EPM open arm duration
Total time in open arm (sec)
Genotype
WT (n=29)
HET (n=21)
HOM (n=24)
c
Male - EPM Open Arm Frequency
number of time entered open arm
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
d
Female-EPM open arm frequency
number of time entered open arm
Genotype
WT (n=29)
HET (n=21)
HOM (n=24)

III


a
Male - Marble Buring Test
Total marbled burried in 30 min
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
b
Female-Marble buring test
Marble covered in 30 min
WT (n=29)
HET (n=21)
HOM (n=24)

IV

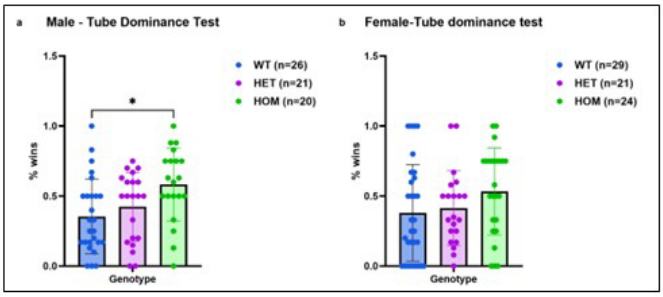

a
Male - Tube Dominance Test
% wins
Genotype
WT (n=26)
HET (n=21)
HOM (n=20)
b
Female-Tube dominance test
% wins
Genotype
WT (n=29)
HET (n=21)
HOM (n=24)

Fig. 2:

I. Open field Test comparing WT, HET, and HOM SAPAP3-KO mice:

a, b - Distance traveled by males (a) and females (b). Significant effect of genotype in a) males (F=7.89; df 2,63, p=0.0009) and b) females (F=12.71; df 2,56, p<0.0001).

c, d. - Center duration of males (c) and females (d) Significant effect of genotype in c) males (F=5.749, 2,64, p=0.0051) and d) females (F=3.687; df 2,70, p=0.030).

Sidak post-hoc test: *p<0.05, **p<.01, ***p<.001,****p<.0001

II. Elevated Plus Maze comparing WT, HET and HOM SAPAP3-KO mice:

a, b – Time spent in open arms in males (a) and females (b). Significant effect of genotype in a) males (F=4.874, df 2,58 p=0.001) and b) females (F=3.556, df 2,54, p=03).

c, d. – Number of times entered open arms in males (c) and females (d). Significant effect of genotype in c) males (F=4.19, df 2,58, p=0.02)

Sidak post hoc tests: *p<.05, **p<.02, ***p<.001

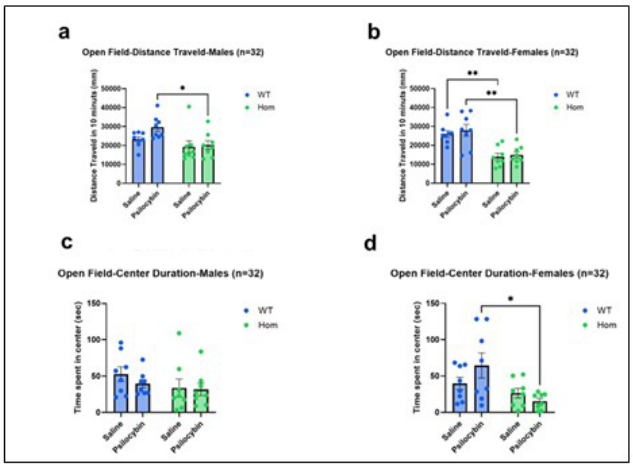
III. Marble burying test in males (a) and females (b), comparing WT, HET, and HOM SAPAP3-KO mice. Significant effect of genotype in a) males (F=18.90; df 2,63, p<0.0001) and b) females (F=53.74; df 2,71, p<0.0001).

Sidak post-hoc test: **p<.01, ****p<.0001

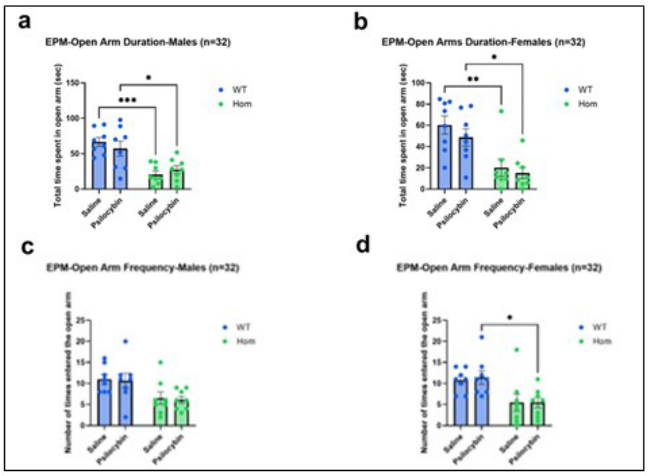
IV. Tube Dominance test in males (a) and females (b), comparing WT, HET and HOM SAPAP3-KO mice percentage of wins. Significant effect of genotype in a) males (F=4.516, df 2,64, p=0.01)

Sidak post hoc test: *p<.05

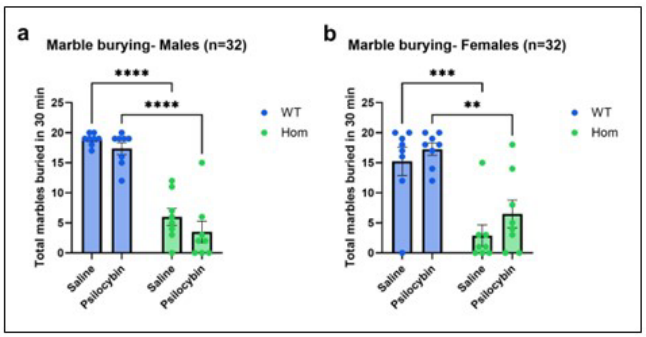
I


a
Open Field-Distance Traveld-Males (n=32)
Distance Traveld in 10 minuts (mm)
WT
Hom
Saline
Psilocybin
b
Open Field-Distance Traveld-Females (n=32)
c
Open Field-Center Duration-Males (n=32)
Time spent in center (sec)
d
Open Field-Center Duration-Females (n=32)

II


a
EPM-Open Arm Duration-Males (n=32)
Total time spent in open arm (sec)
WT
Hom
Saline
Psilocybin
b
EPM-Open Arms Duration-Females (n=32)
c
EPM-Open Arm Frequency-Males (n=32)
Number of times entered the open arm
d
EPM-Open Arm Frequency-Females (n=32)

III


a
Marble burying- Males (n=32)
Total marbles buried in 30 min
****
****
WT
Hom
Saline
Psilocybin
b
Marble burying- Females (n=32)
***
**

IV

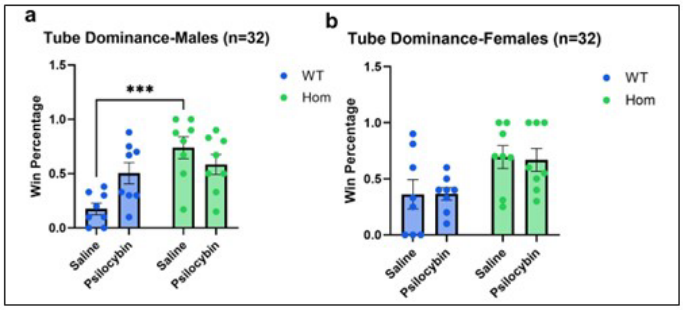

a
Tube Dominance-Males (n=32)
Win Percentage
***
WT
Hom
Saline
Psilocybin
b
Tube Dominance-Females (n=32)

Fig. 3:

I. Open field Test comparing WT and HOM SAPAP3-KO mice with and without treatment with psilocybin:

a, b -Distance traveled: Significant effect of genotype in a) males ($F=8.121$; df 1,28; $p=0.0081$) and b) females ($F=32.35$; df 1,28; $p<0.0001$).

c, d -Center Duration: No significant effects in a) males. Significant effect of genotype in b) females ($F=9.12$; df 1,28; $p=0.0053$).

*p<0.05, **p<0.01 (Dunnet's test)

II. Elevated Plus Maze Test comparing WT and HOM SAPAP3-KO mice with and without treatment with psilocybin:

a,b - Open arms duration of a) Males ($F=27.76$; df 1,28; $p<0.0001$; $p=0.0005$), b) Females ($F=23.46$; df 1,28; $p<0.0001$).

c,d - Open arm frequency of c) males ($F=11.15$; df 1,28; $p=0.002$); d) females ($F=14.19$; df 1,28; $p=0.0008$).

*p<0.05, **p<0.01, ***p<0.001 (Dunnet's test)

III. Marbles buried in 30 minutes comparing WT and HOM SAPAP3-KO mice with and without treatment with psilocybin:

a- Marbles buried in 30 minutes by males ($F=113.6$, df 1, 28, <0.0001).

b- Marbles buried in 30 minutes by females ($F=35.40$, df 1, 28, $p<0.0001$).

**p<0.01, ***p<0.001, ****p<0.0001 (Dunnet's test)

IV. Tube Dominance Test in males (a) and females (b) in WT and SAPAP3 KO mice, with our without treatment with psilocybin:

a. - Males ($F=13.28$; df 1,28; $p=0.001$)

b. - Females ($F=9.86$; df 1,28; $p=0.003$)

****p<0.0001 (Dunnet's test)

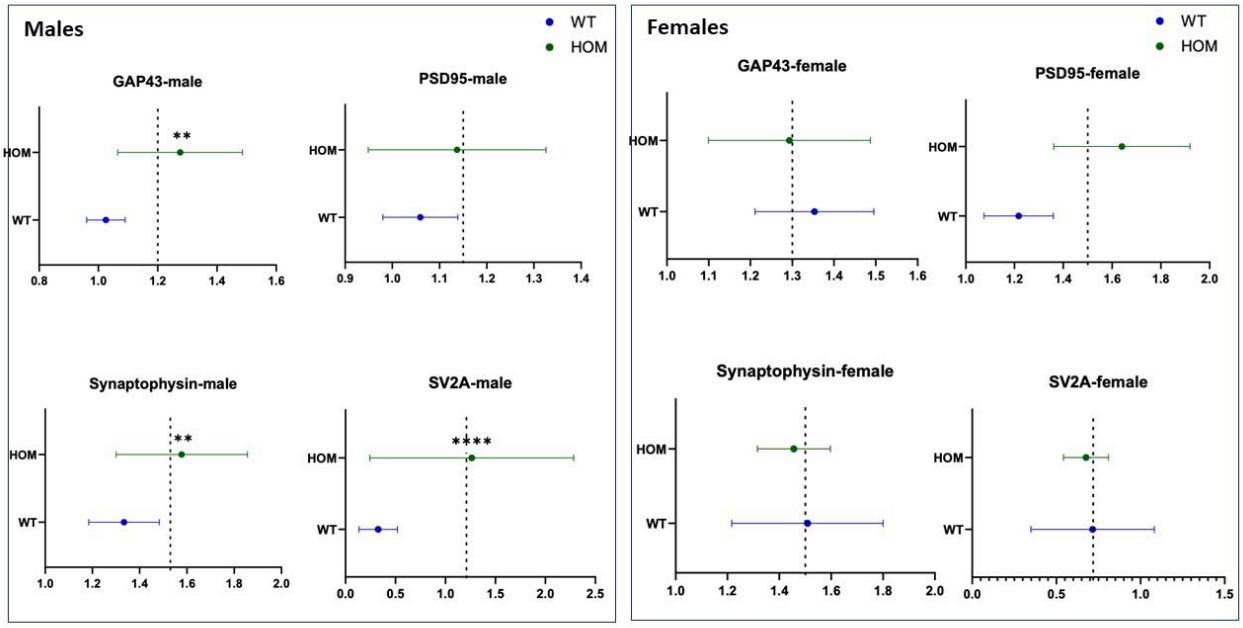


Fig. 4

Nested analysis of synaptic proteins (GAP43, PSD95, synaptophysin, and SV2A) by genotype (SAPAP3-KO and WT) across 4 brain areas (frontal cortex, hippocampus, amygdala, and striatum) in male (left) and female (right) mice. Statistical significance was assessed using t-test (two tailed). Results are shown as mean ± SEM.

**p<0.01, ****p<0.0001