University of Utah

UNDERSTANDING SEX DIFFERENCES IN EARLY PREDICTORS OF

PATHOLOGICAL AGGRESSION WITH A MOUSE MODEL

by

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1

ABSTRACT

Antisocial behavior (ASB) is characterized by aggression, violence, drug use, thrill-seeking behavior, and delinquent behavior. While childhood and adolescent maltreatment contribute to the ontogeny of this condition, converging evidence suggests that genetic factors play an equally important role. The best-characterized gene shaping ASB predisposition, *MAOA*, encodes the enzyme Monoamine oxidase A. Low-activity *MAOA* alleles (*MAOA-L*) eludes to an elevated risk for ASB development, particularly in males with a history of child abuse and neglect. Evidence on this gene x environment (GxE) interaction in females, however, remains more controversial. To study the biological factors underlying this GxE interaction, our lab established MAOA^{Neo} mice, a new line of mice harboring a low-activity MAOA genotype, and subjected them to an early-life stress (ES) regimen simulating child abuse and neglect. These animals develop high levels of aggression from their fourth week of postnatal life onward. Previous data showed that MAOA^{Neo} pups exposed to this early stress exhibit early phenotypic predictors of ASB also observed in humans, such as a reduction in resting heart rate. Here, we developed new behavioral paradigms to verify whether additional predictors (such as social deficits and anxiety-like responses) may be found in ES-exposed MAOA^{Neo} pups. After optimizing these models for behavioral assessment in pups, we began studying the effects of the interaction of MAOA genotypes, ES, and sex on social interaction and anxiety-like behaviors. Although our research progress was negatively impacted by COVID-19, our preliminary results led to the identification of several effects: first, we found that females exhibit higher anxiety-like responses than males from the end of the first week of life; second, we documented that early stress increased anxiety-like responses during the same developmental stage, irrespective of genotype or sex. Finally, our results confirmed that MAOA^{Neo} mice exhibit social deficits already from postnatal day 6. Further research will be needed to fully characterize the effects of the interactions between MAOA low-

activity genotype, early stress, and sex in early developmental stages; however, our newly-developed tools for behavioral assessment in mouse pups hold great promise to extend our current characterization of early predictors of ASB or other neurodevelopmental problems.

TABLE OF CONTENTS

Introduction:

Antisocial behavior (ASB) is a chronic display of aggression and hostility typically manifested during young adulthood, resulting in public violations, poor social encounters, and overall dysfunctional society. ASB results in delinquency, including weapon and homicidal offenses [1]. From a socioeconomic perspective, the repercussions of this condition seep into many facets of our community, including burdens to the mental health and criminal justice systems. Regardless of how communally stirring this issue has become [2], the treatment options remain limited [3]. The unsatisfactory intervention methods highlight our lack of knowledge about the mechanism behind this behavior from a pathophysiological standpoint.

Background:

Several studies show that ASB has a strong genetic basis. The best-characterized gene implicated in ASB is *MAOA*, encoding monoamine oxidase A, the enzyme that activates the degradation of monoamine neurotransmitters, such as dopamine (DA), norepinephrine, and serotonin [4].

The first studies that pointed to a contribution of this gene in ASB and aggression came from the discovery of Brunner syndrome. This genetic condition is caused by a rare nonsense mutation of the *MAOA* gene, leading to overt violence and antisocial conduct [5]. Two years after this discovery, MAOA knockout (KO) mice were serendipitously generated by the integration of an interferon β (IFN-β) transgene into the *Maoa* gene [6]. This insertion led to a mutation that shifted the frame in which the DNA was read, causing the protein to be cut off prematurely [6]. The resulting behavioral phenotype was characterized by intense intermale aggression, in a fashion strikingly similar to that observed in Brunner syndrome. MAOA deficiency may lead to high aggression by causing monoamine levels to be elevated (see Fig. 1). In fact, MAOA KO mice exhibited significantly higher brain levels of serotonin, norepinephrine, and dopamine than their wild type (WT) littermates.

MAOA Levels vs. Agressive Behavior

Legend:
- $*$ = anxiety disorder
- $\bullet$ = humans; Brunner Syndrome mice; enhanced aggressivity

Y-axis: Agressive Behavior Instances
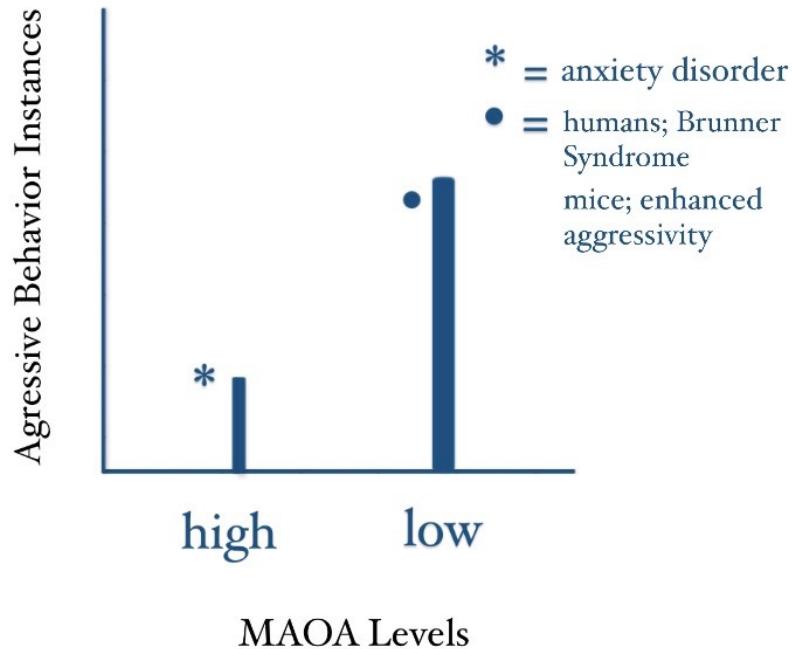X-axis: MAOA Levels (high, low)

Figure 1. Neurochemical and behavioral outcomes of low and high levels of MAOA in humans and mice. The lower the MAOA content, the more aggressive both mice and humans tend to become. Modified from: Human Gene Project (http://allaboutgenes.weebly.com/maoa.html)

Aside from the rare cases of total *MAOA* deficiency, a higher propensity for aggression has also been shown in relation to functional polymorphic variants of this gene associated with low activity. The best-characterized *MAOA* polymorphism is a 30-bp variable number of repeats (VNTR) in the promoter region. Some of the variants of this VNTR are associated with lower transcriptional efficiency (*MAOA-L*), in contrast with the other high-activity alleles (*MAOA-H*). *MAOA-L* alleles robustly influence the severity of aggression and violence in males. In 2002, Avshalom Caspi and

collaborators found that male carriers of *MAOA-L* variants with a history of child maltreatment (abuse and neglect) had a significantly higher risk of developing ASB [7]. This gene × environment (G×E) interaction was confirmed by many studies and three separate meta-analyses [8]. To study the mechanisms underlying this interaction, our laboratory developed we will use MAOA^{Neo} mice, a transgenic line that harbors a hypomorphic mutation of the *Maoa* gene [9]. When compared to wild-type (WT) littermates, male MAOA^{Neo} mice display lower propensity to engage in social interactions [10]. Furthermore, our lab recently documented that when these mice are exposed to an early-life stress (ES) regimen simulating child abuse and neglect, they develop aggression from the fourth week of life onward [11]. Most importantly, ES-exposed male MAOA^{Neo} pups exhibited early phenotypic predictors of aggression and ASB reminiscent of those observed in at-risk children and adolescents, such as low resting heart rate [12]. This evidence indicates that these animal models may be a valuable model to study early predictors of ASB, which may allow clinicians and practitioners to identify individuals at risk and initiate early preventative treatments. This evidence, however, was only limited to males, considering the high male predominance of ASB (with a male to female ratio estimated at 3:1 [13]). Another critical problem is that the assessment of early behavioral phenotypes in mouse pups is limited by the scarcity of tools and behavioral paradigms adapted to pups. Building on this background, we performed the following study with two main goals: 1) developing novel paradigms for behavioral testing in pups, to extending our phenotyping of early predictors of aggression in ES-exposed characterization of pup behavior in ES-exposed MAOA^{Neo} mice; 2) extending this characterization to female WT and MAOA^{Neo} pups, in order to study the biological causes of potential sex differences.

Materials & Methods:

Animals. Male and female MAOA^{Neo} were generated by mating MAOA^{Neo} heterozygous (HZ) females with wild-type (WT) or MAOA^{Neo} sires. *Maoa* is an X-linked gene; thus, male offspring of MAOA^{Neo} HZ dams were always either MAOA^{Neo} or WT. Conversely, depending on the genotype of the father, we could obtain either WT or MAOA^{Neo} homozygous females. Pregnant dams were single housed 3-5 days prior to parturition. Only litters with > 4 pups (and at least two males) were used. Bedding was changed in all cages at postnatal day (PND) 7 and PND 14, and mice were weaned at PND 21. Animals were housed in a room maintained at 22°C, on a 12 h:12 h light-dark cycle from 6 am to 6 pm. Food and water were available *ad libitum*. All experimental procedures were executed in compliance with the National Institute of Health guidelines and approved by the local Institutional Animal Use Committees. Throughout all studies, every effort was made to minimize the number and suffering of animals used.

Genotyping. For MAOA^{Neo} mice, specific primers for MAOA genomic DNA (intron region), In11F2 and In11R1, were used. DNA was amplified by a 4 min hot- start at 94°C, followed by 35 cycles of 30 sec at 94°C, 40 sec at 50°C and 45 sec at 72°C, and then a final 5 min extension at 72°C. The PCR product size is 220 bp for WT and 500 bp for MAOA^{Neo}.

ES procedure. To simulate child abuse and neglect, pups underwent a daily early-life stress (ES) regimen consisting of maternal separation (for 2–4 h/day), and saline intraperitoneal injections (SIs, executed with body restraint via dorsal pinch) from PND 1 through 7. MS and SI stressors were administered either alone or as a combination of both, with pups subjected to different durations of MS and at different times in a pseudorandom fashion. For each procedure, male and female pups were removed from their nest and placed into a novel cage in a separate temperature-controlled (25°C) room. SIs were performed using a microinjector connected to a micro-syringe; the stressfulness of each intraperitoneal injection was always ensured by verifying the response

from each pup (typically consisting of urination, vocalization, rapid limb and head movements after the puncture). Non-stressed control pups were briefly removed from their cages and returned to their home cage after brief gentle handling. All pups in each litter were subjected to the same manipulation; to control for litter effects, each experimental group included mice from at least five litters.

Behavioral procedures and testing paradigms. Behavioral experiments occurred between 11 AM and 5 PM. All experiments were conducted on groups comprised of subjects from at least five litters, and randomly assigned to each group. Group size was determined based on power analyses based on preliminary results. To ensure scientific rigor, all analyses were performed in blind. Behavioral testing was conducted on PND 6 and PND 8.

**Social interaction**. A protocol for social interaction was adapted from Godar's procedures in a 2019 study [11] to pups. Rather than testing the approaches to a single social counterpart, as done in juvenile and adult mice, we tested social propensity by measuring the latency to reach a huddle of foreign WT pups of the same age. The foreign huddle and the proband mouse were placed in an arena measuring 22 x 9.5 x 10 cm (Fig.2). The foreign huddle of fic WT subjects was placed at an extremity of the arena, while the test pup was positioned at 5 cm from the huddle, alongside the shortest axis of the arena, as shown in Fig. 2. The arena was specifically designed to promote movement of the test mouse along its longest axis (either towards or away from the huddle). Testing sessions lasted 5 min and were video-recorded and later scored by blinded observers. The latency to reach the huddle was measured as an index of social propensity.
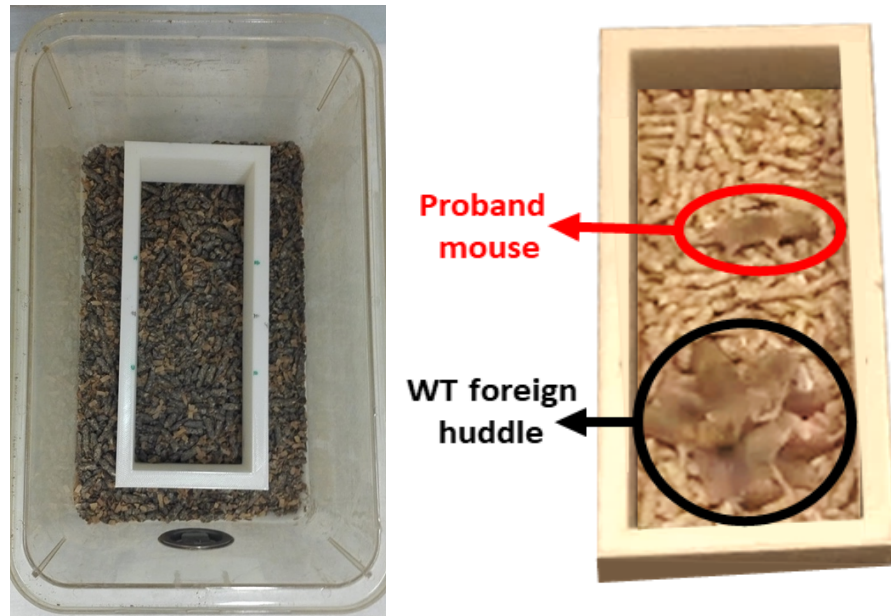
Figure 2. *Social Interaction Arena and paradigm: Social interaction is setup by placing one pup group at an end of the arena. One mouse is picked from the litter and is placed at the other end and the latency is measured between when it is placed and when it reaches the huddle again. This shows a willingness to be integrated into a group as opposed to self-isolation. A normal mouse would reintegrate itself into the group.*

Light-dark box. Anxiety-related behaviors were measured using a novel version of the light-dark box test [14] adapted for pups. Briefly, the apparatus was developed through a 3D-printed model, consisting of two compartments: a larger (13.5 x 9.5 x 10 cm) white compartment (light box), surmounted by a 200-lux bright light; and a smaller (8.5 x 9.5 x 10 cm) black enclosed chamber (dark box) (see Fig. 3). The light box features a sliding wall to adapt the size of this compartment to different developmental stages of pups. The floor of the arena was based on white paper (which was changed at the end of each test to avoid odor retention) with an uneven surface, specifically designed to reflect light and amplify the anxiogenic properties of this compartment. The test was conducted placing male and female pups in the middle of the light box, at 3-5 cm from the entrance of the dark box (depending on the age of the pups). Behavioral measures included latency to enter the dark compartment to enter the dark compartment with all four paws.
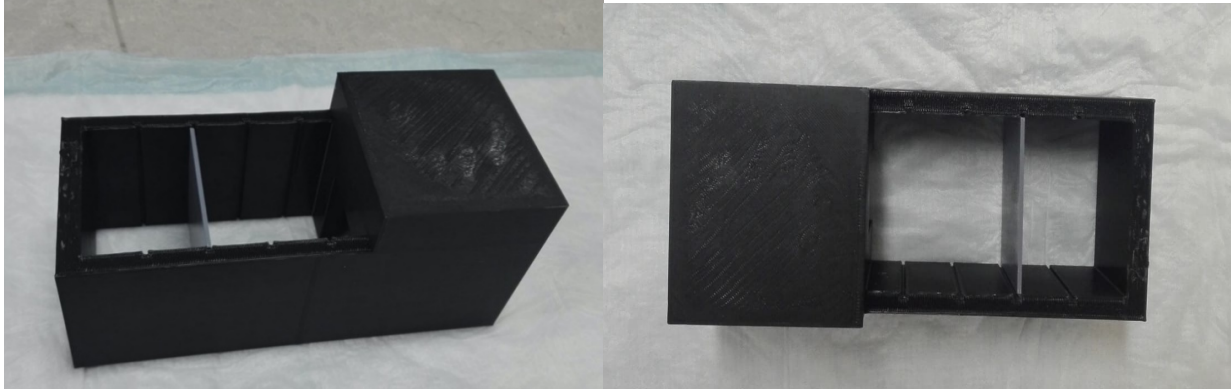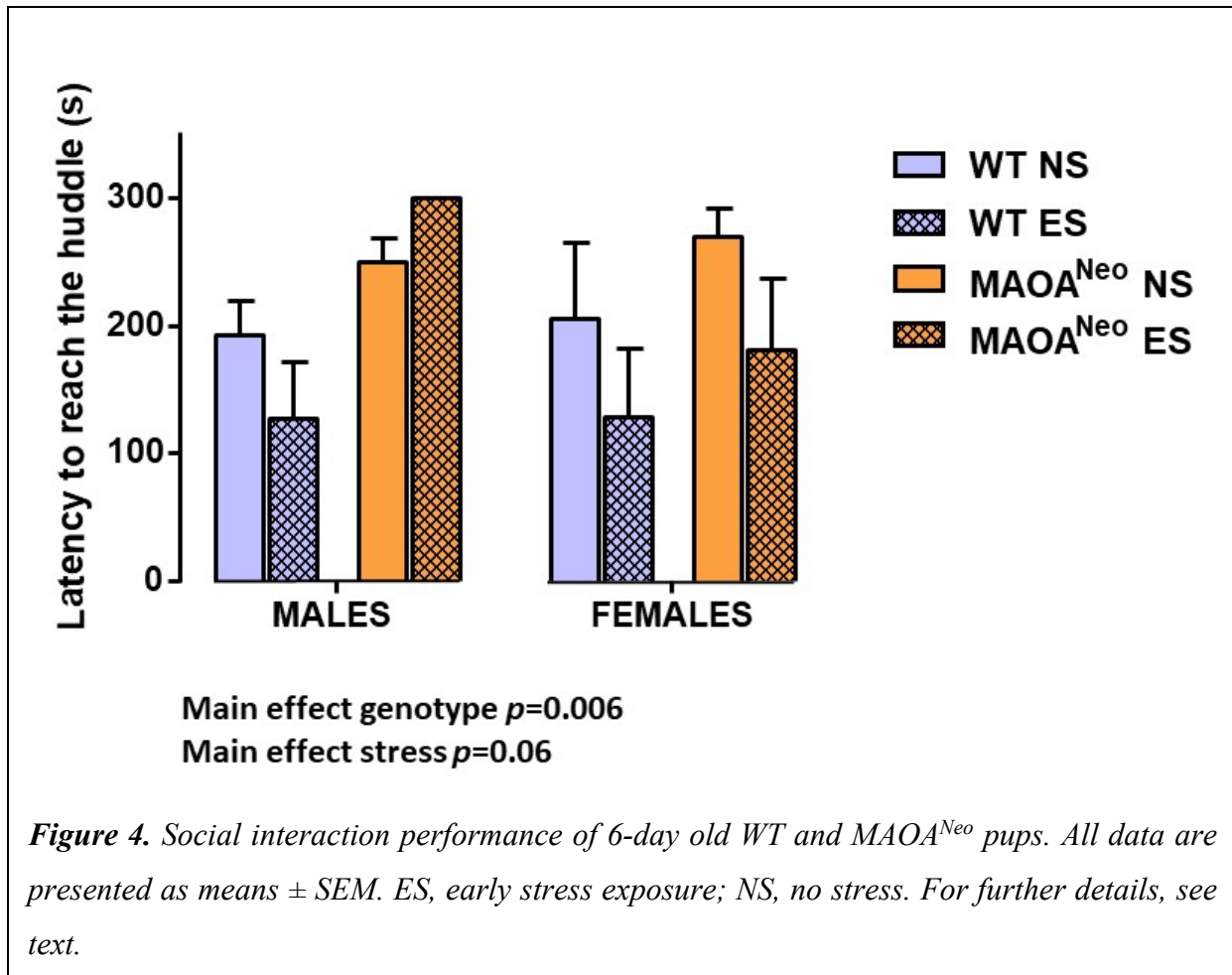
Figure 3. *Dark Light Box Arena: The Dark light box is set up by placing one pup from the huddle X distance away from the dark entrance and the latency is measured between the drop-off time and the entrance time. This shows the risk-taking nature of the animal to be without cover and in a bright space as opposed to a covered dark area which is what normal mice prefer.*

Statistical analyses. Data were analyzed with three-way ANOVAs (analysis of variants), with genotype, stress, and sex as factors. As data did not fit a parametric distribution, they were log-transformed before ANOVA testing. Significance threshold was set at 0.05, but alpha levels < 0.10 were discussed as marginally significant.
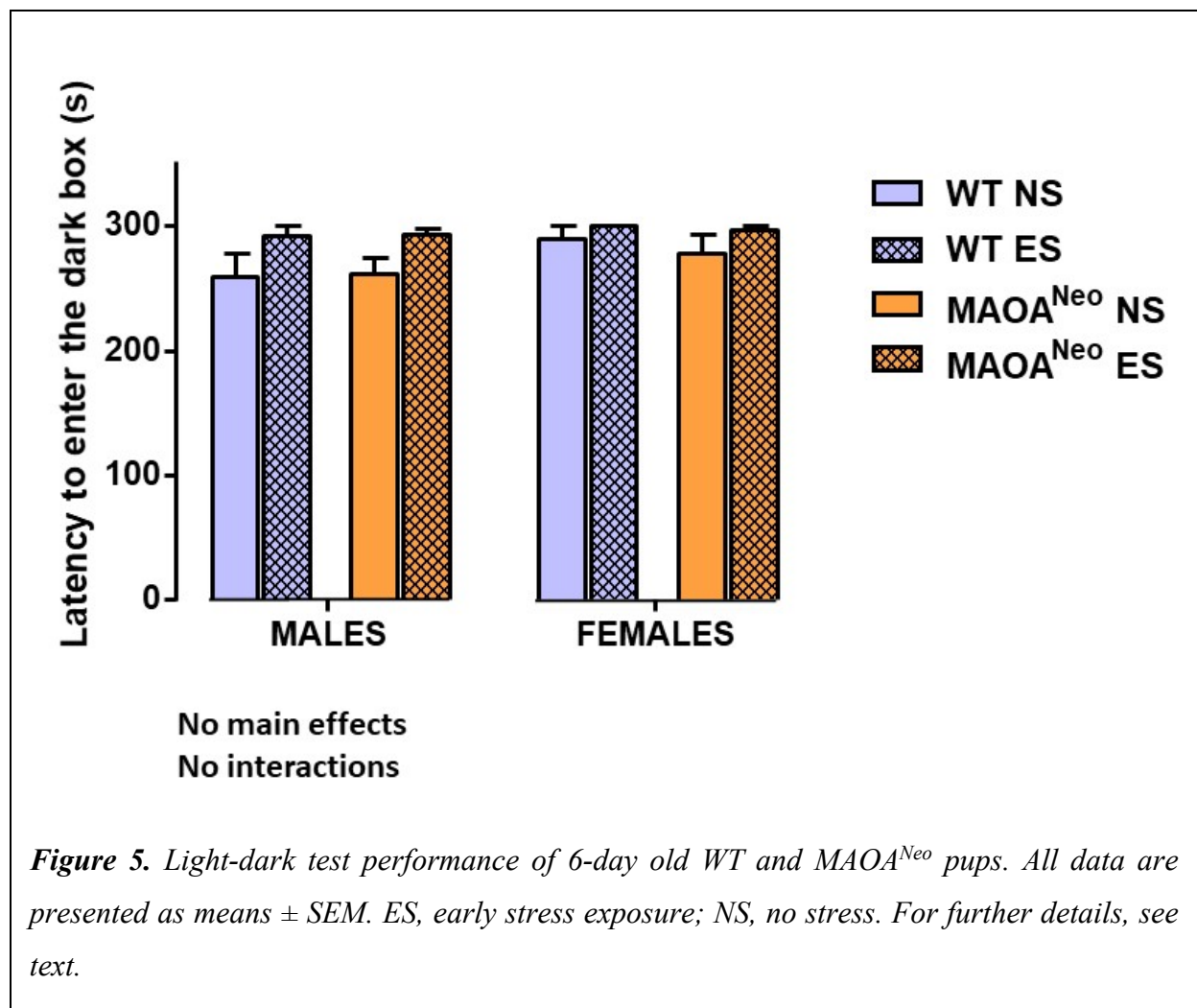
Results:

Behavioral profile on PND 6.

The analysis of social interactions of 6-day pups with a huddle of foreign WT mice (Fig. 4) revealed a main effect of genotype ($F_{1,67}=8.023$, $p=0.006$), indicating that MAOA^{Neo} male and female pups (irrespective of ES exposure) spent significantly more time than their WT counterparts to join the huddle. No main effect of sex was found ($F_{1,67}=0.859$, $p=0.3$), while a trend for main effect of stress was revealed ($F_{1,67}=3.447$, $p=0.06$). No statistically significant interactions were detected.

Figure 4. *Social interaction performance of 6-day old WT and MAOA^{Neo} pups. All data are presented as means ± SEM. ES, early stress exposure; NS, no stress. For further details, see text.*
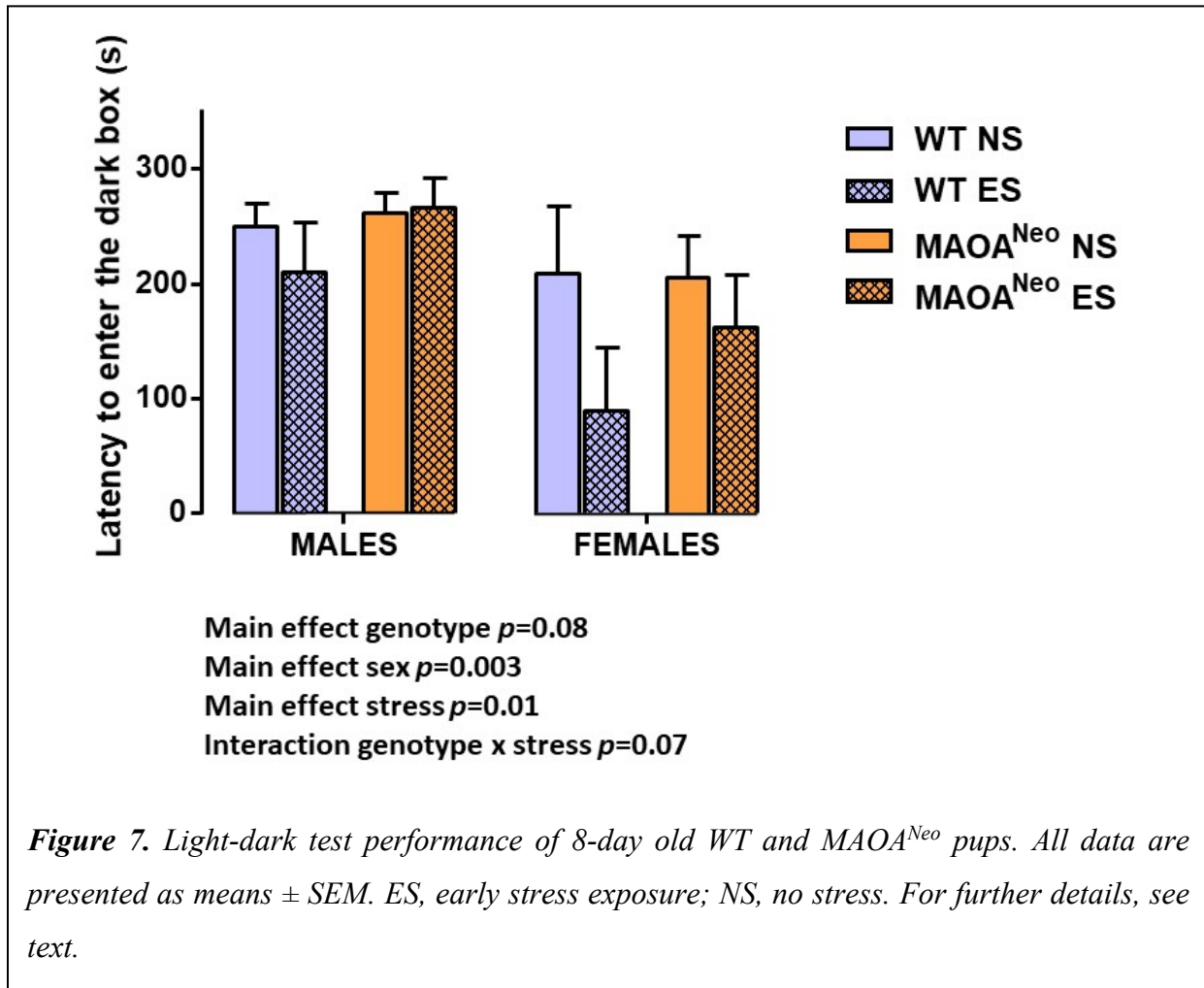
The performance in the light-dark box paradigm, conversely, failed to reveal any effect, as almost all test mice failed to enter the dark compartment within the 5 min of the test (see Fig. 5). We interpreted that this may be due to the fact that the developmental stage was too early for mouse pups to perform in this task, even though they develop light avoidance reflex at P6 [15].

Figure 5. *Light-dark test performance of 6-day old WT and MAOA^{Neo} pups. All data are presented as means ± SEM. ES, early stress exposure; NS, no stress. For further details, see text.*
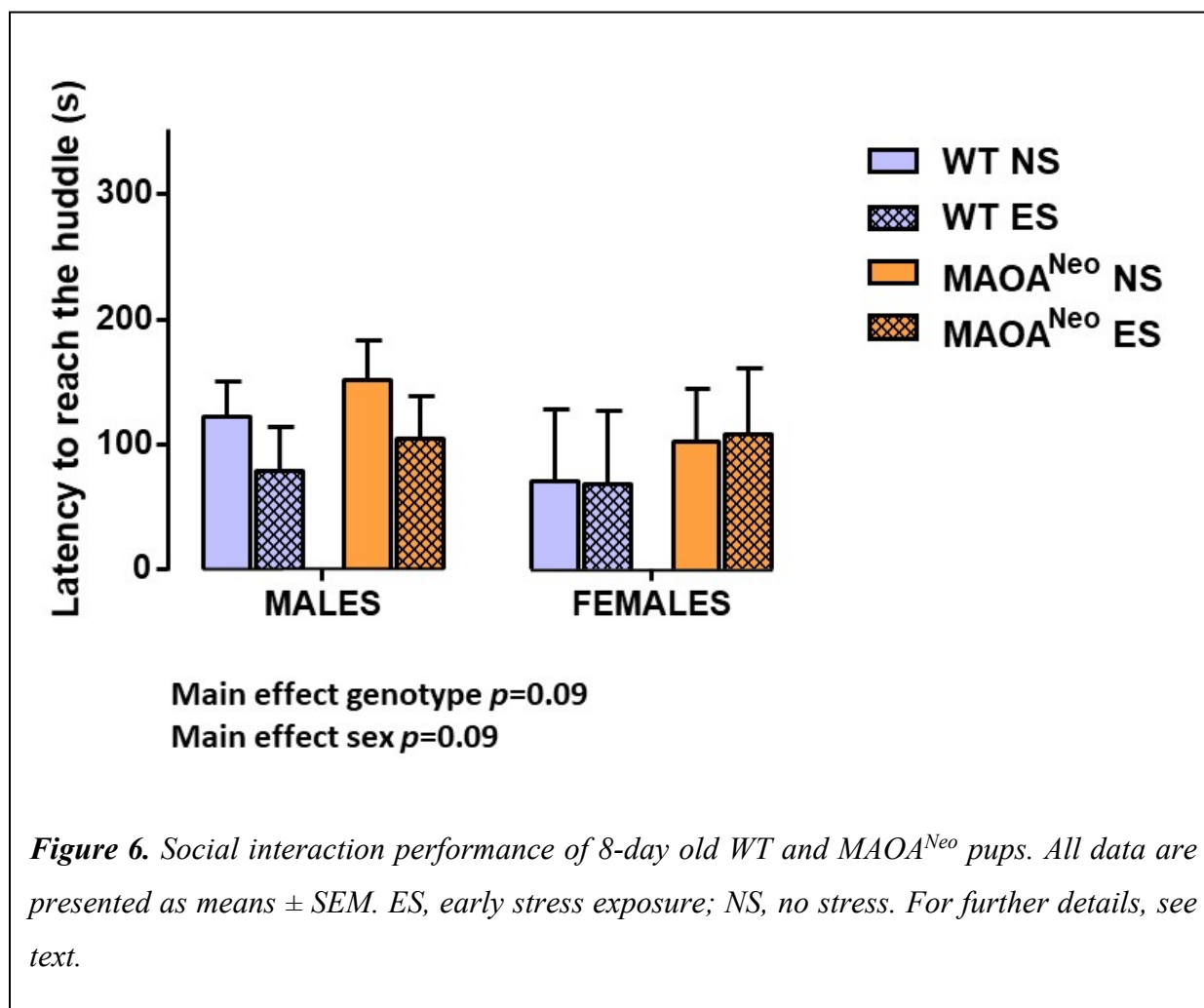
Behavioral profile on PND 8.

The analysis of social interactions with a huddle of foreign WT mice in 8-day old pups (Fig. 6) showed a trend for a significant main effect of genotype ($F_{1,73}$=8.023, p=0.09), again indicating that MAOA^{Neo} male and female mice spent more time to join the conspecifics, irrespective of their

stress exposure. Interestingly, a trend for a main effect was also found in relation to sex ($F_{1,73}$=2.817, p=0.09), pointing to a lower latency of females to reach the huddle. If fully confirmed, this effect may signify a higher level of social propensity in females during this developmental stage. Finally, no main effect of stress was found ($F_{1,73}$=1.353, p=0.2), and the analyses of interactions did not reveal any statistical significance (genotype x sex $F_{1,73}$=0.192, p=0.6; genotype x stress $F_{1,73}$=0.027, p=0.8; sex x stress $F_{1,67}$=0.0007, p=0.9; genotype x sex x stress $F_{1,73}$=0.077, p=0.7).

Figure 7. Light-dark test performance of 8-day old WT and MAOA^{Neo} pups. All data are presented as means ± SEM. ES, early stress exposure; NS, no stress. For further details, see text.

Figure 6. *Social interaction performance of 8-day old WT and MAOA^Neo pups. All data are presented as means ± SEM. ES, early stress exposure; NS, no stress. For further details, see text.*

Our findings on the performance in the light-dark box revealed main effects for both sex ($F_{1,83}$=9.161, *p*=0.003) and stress ($F_{1,83}$=5.701, *p*=0.01) (Fig. 7). Further scrutiny of these results showed that females had a lower latency to enter the dark box, likely indicating their greater anxiety-like behavior. ES-exposed animals showed the same behavioral reaction (irrespective of genotype and sex), indicating that stress exposure increases anxiety responses. A statistical trend was also identified for a main effect of genotype, potentially indicating that MAOA^Neo mice had marginally higher latency to enter the dark box, possibly due to lower anxiety levels. Although no significant interactions were found, a statistical trend was found for the interaction between genotype and stress, which depended on a reduction of the latencies to enter the dark box in ES-exposed WT pups, as compared with the other groups.

Discussion:

The main result of this study is the validation of two novel behavioral paradigms for behavioral testing in mouse pups. The development of tools and procedures for the validation of behavioral abnormalities in early life is critically important to study the neurobiology of neurodevelopmental disorders, such as ASB, autism-spectrum disorder, and Tourette syndrome. From this perspective, our optimization of two protocols may provide an important contribution to future studies in behavioral neuroscience and pharmacology. In this respect, it is worth noting that our laboratory is also developing novel paradigms to further our characterization of mouse pup behavior, such as an elevated plus-maze specifically designed for pups in the first week of life (Fig. 8). These exciting developments may lead to a significant expansion of our understanding of emotional regulation and stress response during early developmental stages. These tools are likely to expand our ability to analyze early predictive signs of behavioral disturbances throughout early life.
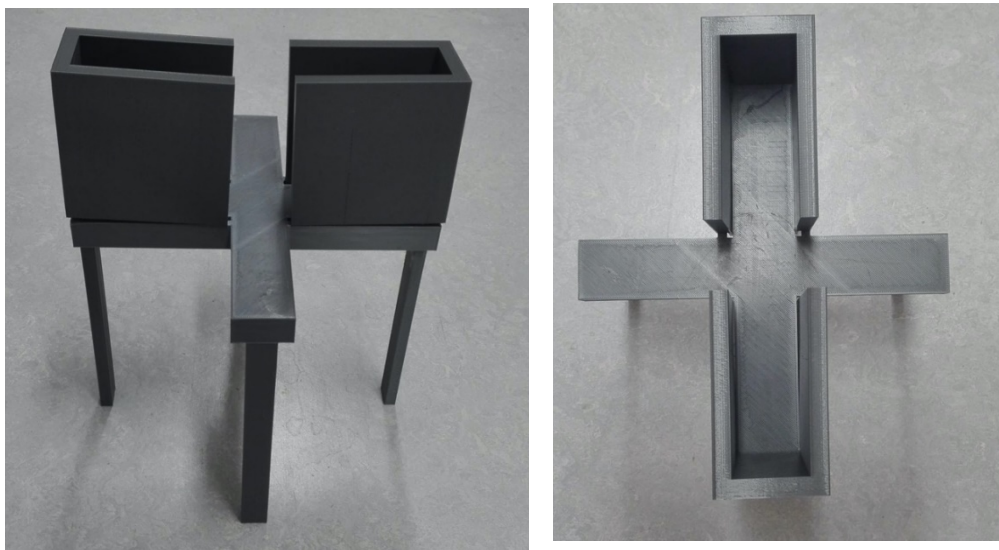

Figure 8. *Elevated Plus-Maze Arena for pups: The elevated plus maze is setup by placing a mouse in the center and measuring their time in the open fields, middle field, and closed fields. This shows how*

daring the animal is to step outside of the safe and enclosed spaces whereas a normal animal would spend a majority of the time in the closed spaces.

Our experiments were negatively impacted by the COVID-19 pandemic, and thus we could not fully explore all the differences between males and females in relation to the effects of stress and *Maoa* genotypes. However, our preliminary results led to several interesting results, which will need to be further defined by future validations and experiments and extended to later developmental stages in pups. First, behavioral analyses on PND6 confirmed previous evidence indicating that MAOA^{Neo} mice exhibit social deficits [16]. The validation of a test that can confirm social deficits at such early stages may be highly promising to study the neurobiological basis of these problems in models of autism spectrum disorder. Furthermore, if our finding of a statistical trend for a negative effect of stress on social propensity is fully validated, the social interaction paradigm in pups may be a valuable instrument to assess the negative effects of early stress on social responsiveness.

The analyses in 8-day old pups (namely, one day after the end of the ES regimen) showed a statistical trend for the negative effect of MAOA^{Neo} genotype. Given that pups exhibit a greater degree of locomotion during that developmental stage, it is possible that further optimizing the arena (potentially by increasing its dimensions) may allow for better capturing of social deficits at this stage. Finally, the results of the light-dark box task in this developmental stage showed that both stress exposure and female sex increase anxiety-like responses already from this developmental stage. This indication may be extremely important to test the mechanisms of sex differences in anxiety-like behaviors already from a developmental stage equivalent to that of childhood. From this perspective, our paradigms may become highly useful in research to dissect the neurobiological mechanisms underlying emotional responses before puberty.

Future Directions and Implications:

Being that the project goals were put on hold, much work still needs to be completed in order to successfully analyze the interaction between MAOA genotypes, ES, and sex. The goal will be to continue further research and extend it to later time points to fully verify how the interactions of these vulnerability factors shape different trajectories of behavioral development. Even with these limitations in mind, my research has shown that the development of tools for behavioral assessment in pups is feasible and holds great promise to advance our understanding of neurodevelopmental disorders, including ASB.

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