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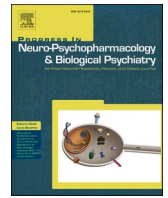
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Female mice prenatally exposed to valproic acid exhibit complex and prolonged social behavior deficits

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized mainly by deficits in social communication and stereotyped and restricted behavior and interests with a male to female bias of 4.2/1. Social behavior in ASD animal models is commonly analyzed in males, and seldomly in females, using the widely implemented three-chambers test procedure. Here, we implemented a novel procedure, the Live Mouse Tracker (LMT), combining artificial intelligence, machine learning procedures and behavioral measures. We used it on mice that were prenatally exposed to valproic acid (VPA) (450 mg/kg) at embryonic day 12.5, a widely recognized and potent ASD model that we had previously extensively characterized. We focused on female mice offspring, in which social deficits have been rarely documented when using the 3-CT procedure. We recorded several parameters related to social behavior in these mice, continuously for three days in groups of four female mice. Comparisons were made on groups of 4 female mice with the same treatment (4 saline or 4 VPA) or with different treatments (3 saline and 1 VPA). We report that VPA females show several types of social deficits, which are different in nature and magnitude in relation with time. When VPA mice were placed in the LMT alongside saline mice, their social deficits showed significant improvement as early as 1 h from the start of the experiment, lasting up to 3 days throughout the duration of the experiment. Our findings suggest that ASD may be underdiagnosed in females. They also imply that ASD-related social deficits can be ameliorated by the presence of typical individuals.

1. Introduction

Autism spectrum disorder (ASD) is a pervasive developmental disorder that is currently diagnosed based on two main clusters of behaviors as per the current version of the Manual of the American Psychiatric Association: (i) deficits in social communication and interactions and (ii) repetitive and restrictive behavior and thoughts patterns. Symptoms need to have been present since childhood and negatively affect the person's life (American Psychiatric Association, 2013). ASD prevalence is constantly increasing due, in all likelihood, to a combination of factors (Järbrink and Knapp, 2001). These include (i) heightened awareness of the disease by family and practitioners leading to a better diagnosis and recognition and (ii) a potential increase in environmental insults from

our modern way of living (Maenner et al., 2020; Hertz-Picciotto and Delwiche, 2009). In 2017, Loomes et al. reported a 3:1 male to female bias; however, in the most recent report from the Autism and Developmental Disabilities Monitoring (ADDM) network, ASD was reported with a bias of 4.2:1 male to female (Maenner et al., 2023; Loomes et al., 2017). Several hypotheses have been associated with this sexual dimorphism, including the possibility that females are underdiagnosed as ASD symptoms may be expressed differently with regard to gender (Loomes et al., 2017).

While the specific causes of ASD are still not clearly identified, there is a general consensus that there is no single etiology, but rather a combination of genetic and environmental factors (Chaste and Leboyer, 2012). Over 1000 genes have been implicated in ASD, each being

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responsible for only a minor proportion (Vorstman et al., 2017). A common ground for most of these genes is that they seem to be implicated in neuronal transmission, with many of them being at the interface of glutamatergic and GABAergic synapses (De Rubeis et al., 2014; Wang et al., 2017; Guang et al., 2018). Taken together, however, gene mutations are thought to account for only 25% of ASD cases, so that environmental factors may account for the rest. The effects of these environmental factors are most inflicting during prenatal development and include toxins, pharmacological agents and severe viral and bacterial infections (Bölte et al., 2019).

Knowledge of the genetic and environmental determinants of ASD has led to the development of various genetic and environmental mouse models mimicking ASD symptomatology and in most cases showing good construct and face validity, or at least striving towards that direction (Bey and Jiang, 2014; Thabault et al., 2022). Among these animal models, valproic acid (VPA) administration in a single dose (250–500 mg/kg) to pregnant females at a critical age of embryonic development (E 12.5) has been constantly shown to be a reliable ASD model recapitulating several behavioral, cellular and molecular phenotypes of the disorder (Thabault et al., 2022; Rodier et al., 1996; Schneider and Przewlocki, 2005; Al Sagheer et al., 2018; Nicolini and Fahnestock, 2018; Chaliha et al., 2020). Social deficits in these ASD models are often explored using the 3-Chambers test (3-CT) apparatus that was first described by Crawley's team in 1999 (Crawley, 2004); this is an easy to set up test that yields straightforward scores to be analyzed statistically (Silverman et al., 2010). As such, the 3-CT is very useful for initial screenings but does not permit the analyses of complex social behavior, over an extended period of time, within a group of mice.

Here, we have implemented the recently described Live Mouse Tracker (LMT) procedure, which enables automatic live tracking and subsequent analysis of complex social behavior in a group of mice with virtually no time limit other than the one set by the experimenter (de Chaumont et al., 2019). This is achieved by a depth-sensing infrared camera combined with machine learning and radio frequency chips for animal identification. The social profile of each animal can thereby be obtained, and its individual and group behavior analyzed in detail. Using this procedure, we measured here a wide range of collective and individual social behaviors continuously for 3 days in groups of female mice that were prenatally exposed to either a saline (SAL) solution (0.9% NaCl) or VPA (450 mg/kg) at E12.5. We performed our studies in female mice given the possible underdiagnosis of ASD in females in clinical and pre-clinical settings. Analysis was performed on several sets of groups of 4 mice of the same (saline or VPA) or mixed (saline and VPA) treatment from different litters and that were raised after weaning in different cages so that tested mice did not have any previous contact with each other. Our study provides large-scale behavioral analysis of several parameters related to female social behavior at different periods of time, ranging from 1 h to 3 days.

2. Methods and materials

2.1. Hardware- and software-related information

The LMT equipment needs to be built from scratch by the user according to the previously described procedure as no commercial version is yet available (de Chaumont et al., 2019). This system enables individual identification of groups of mice over several days, followed by automatic live tracking using a random-forest machine learning process for animal and posture identification, radio-frequency identification (RFID) chips detected by floor antennas for mouse identification, and an infrared-depth RGB-D camera. A Kinect camera is set 63 cm above a 50 × 50 cm² cage, perpendicular to the floor. RFID antennas (134–125 kHz) are placed under the cage and induce an electric current in the RFID chips carried by a mouse, which is thereby identified. Behavioral labelling is performed in real time with a detection of the shape and posture of each individual, as well as relative positions between

individuals. A filter is set up to determine that the moving objects are indeed mice, by means of a machine learning algorithm which is involved in preparing the data, searching for the identity and orientation of each mouse. All software can be downloaded directly from the following site: <http://www.bioimageanalysis.org/lmt/>. Full source code is available at <http://icy.bioimageanalysis.org/plugins/livemousetracker>. This includes Java code and CAD hardware resource files. Python analysis scripts are available at <https://github.com/fdechautmont/lmt-analysis>.

2.2. Animals

Animal housing and experimental procedures were performed in accordance with the European Union directive (2010/63/EU) and validated by the regional ethics committee (Approval # 2020022610505052, 2,021,011,214,447,548 and 2,022,021,015,414,724). C57BL/6Jrj mice (Janvier labs, France) were housed at the local Prebios animal facility, in ventilated cages with access to food and water ad libitum. Room temperature was maintained at 23 °C on a 12 h light/dark cycle.

2.3. Experimental design

Twenty-seven females and 22 males were used for mating. For mating, 2 females were placed with a single male and left overnight, and males were retrieved the next morning. When mating was unsuccessful (absence of plug in the next 12 h and 24 h following mating and weight monitoring every day for the next 7 days), female mice were mated with a different male, 7 days after the first attempt. Pregnant mice received a single i.p. injection of either VPA (450 mg/kg) or NaCl 0.9% at gestational day 12.5 (E12.5), a critical developmental time when the neuronal tube is closing in rodents, followed by neurogenesis and neuronal migration (Nicolini and Fahnestock, 2018; Morriss-Kay et al., 1993). Weaning of offspring was performed on postnatal day 28 (P28). Sex and age-matched pups were separated and raised by groups of 4 in a randomized fashion to avoid littermate effects. All mice were identified by ear tags and with a RFID tag inserted subcutaneously under isoflurane anaesthetic with local analgesia (lidocaine 2% in ointment form). Female mice were then allocated to two experimental groups depending on prenatal treatment: VPA females ($n = 37$), and SAL females ($n = 37$). The experimental timeline is presented in Fig. 1.

Behavioral analysis was performed on young adult mice of an average age of (i) 66 days for the 3-CT, ranging from a minimum of 51 days to a maximum of 89 days and (ii) 70 days for the LMT, with an age range from 53 days to 97 days (see suppl. Fig. 1). All experiments were performed on females, within the same range of age, originating each from a different litter, and that have not been previously housed together prior to the experiments. Each mouse was used only once for behavioral analysis. Experiments were run on several sets of 4 mice/group, during 30 min within the light cycle for the 3-CT procedures and for 3 continuous days (72 h) for LMT.

2.4. Individual social behavior as measured with the three-chamber test

Social interaction was assessed using the 3-CT as previously described (Al Sagheer et al., 2018) on VPA ($n = 20$), and SAL females ($n = 15$). The apparatus consists of a Plexiglas box (60 × 45 × 22 cm) partitioned into three chambers with retractable doorways. Mice were monitored using an unbiased automated video tracking system (VideoTrack, Viewpoint-France) which calculates several parameters for each observed animal such as position, speed, trajectory, and various other behaviors set up by the experimenter including duration of presence in each chamber and within the vicinity of the wire-cup. Data were retrieved using the VideoTrack 3.2 software (Viewpoint-France). The first phase (PHASE-I) comprises two identical non-social stimuli (inverted wire-cups) placed in opposite chambers. The second phase (PHASE-II) comprises a non-social stimulus and a social stimulus (a

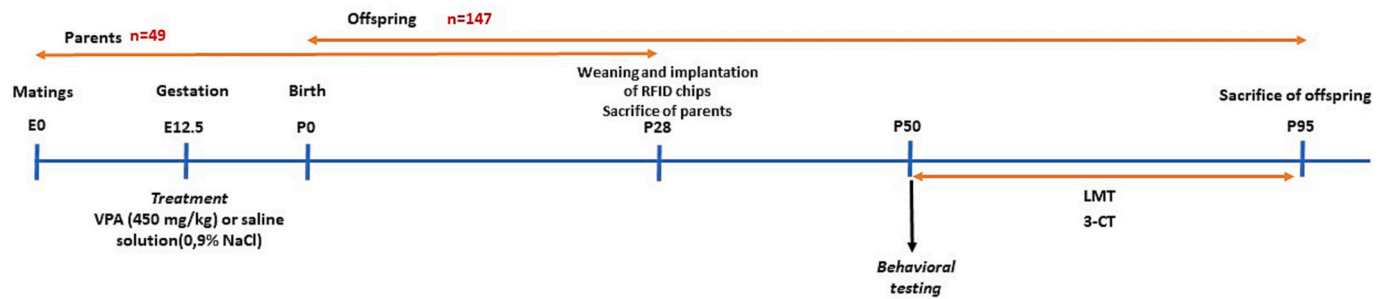


Fig. 1. Experimental design and timeline. Pregnant female mice were i.p. injected at E12.5 with either saline (0.9% NaCl) or a single dose of VPA (450 mg/kg). Offspring were weaned at 4 weeks of age, implanted with a RFID tag and allocated to 2 experimental groups with regard to prenatal exposure. Behavioral analysis was performed for 3 continuous days for the Live Mouse Tracker (LMT) and for 30 min within the light cycle for the 3-Chamber test (3-CT) procedure.

naïve age and sex-matched mouse with no previous contact with the tested mouse). Subsequently, a sociability index (SI) was calculated as follows: (time exploring social chamber – time exploring non-social chamber) / (time exploring social chamber + time exploring non-social chamber). In the third phase (PHASE III), a novel mouse is put within the inverted wire-cup of the opposite chamber than the one already housing the mouse from PHASE II. Each phase was 10 min long, with time spent in each chamber and around the inverted cup recorded using a video tracking system.

2.5. Group behavior as measured with the Live Mouse Tracker (LMT)

Thirty five behavioral events were defined with regard to the intrinsic and relative positions of mice, which were extracted automatically as previously described (de Chaumont et al., 2019). Behavioral features were split into five categories: (i) individual behavior (such as rearing, in green in Figs. 4 and 5), (ii) social dyadic events between two mice (such as breaking and making a group of 3, in red), (iii) dynamic events between two mice (such as social approach and contact, in blue), (iv) subgroup events with several mice (such as making and breaking, in orange), (v) group-making and group-breaking events (such as being in a group of 3, in purple). All behavior from all these five categories were measured and analyzed. For the sake of clarity, only 1 or 2 behaviors from each category is shown here and is represented by a different color in Figs. 4 and 5. These are:

- Oral-oral contact: The focal animal is making a nose contact with another animal (i.e., the nose is at a whisker distance from the nose of the other animal).
- Oral-genital Contact: The focal animal is making a nose contact with the ano-genital region of another animal (i.e., the nose is at a whisker distance from the tail basis of the other animal).
- Social Approach: The focal animal gets closer to another one, with the approaching animal walking at a higher speed than the approached animal.
- Approach contact: The focal animal gets closer to another one, with the approaching animal walking at a higher speed than the approached animal; the approach ends by a contact between the two animals.
- Rearing: The focal animal is straightened on its hindlegs (either unsupported or against the wall).
- Breaking of a group of 3: The focal animal approaches a group of three animals and leave a group of two animals; the focal animal has the highest speed among the three animals in contact.
- Making a group of 3: The focal animal is joining a group of two animals to form a group of three animals in contact.
- Group of 3: The focal animal is with two other individuals.

2.6. Data analysis

Data are expressed as mean ± Standard Error of the Mean (SEM) and analyzed using GraphPad Prism-7 software (La Jolla, California, USA). Data that followed a normal distribution were analyzed using one-way or two-way analysis of variance (ANOVAs) whenever appropriate. A Greenhouse-Geisser correction was applied for results presented in Figs. 5 and 6. Upon significant main effects, Tukey's multiple comparisons was performed. When data did not follow a normal distribution, we conducted non-parametric tests (Kruskal-Wallis or Mann-Whitney as indicated) followed by Dunn's multiple comparison tests. For all analyses, a p value of <0.05 was considered significant.

2.7. Data accessibility statement

All raw data and detailed statistical analysis that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1. Social interaction analysis using the three-chamber test

The 3-CT was used to assess individual social behavior of female mice prenatally exposed to VPA (450 mg/kg) or SAL at E12.5 as previously described (Al Sagheer et al., 2018). Using this procedure, we have previously shown that only male mice prenatally exposed to VPA showed a dramatic decrease (down 7-fold) in their social behavior, whereas VPA females had normal sociability (Al Sagheer et al., 2018).

Here, we show that, following habituation in phase I (Fig. 2A), both SAL and VPA females spent more time in the social chamber than in the empty chamber during the phase II of the test (Fig. 2B) (62% versus 38%, and 56% versus 44%, respectively) [$F(3, 64) = 43, p < 0.0001$]. VPA mice seemed to show even more preference for the social chamber than the SAL group ($p < 0.05$) (Fig. 2C). However, when a female mouse was introduced in the opposite chamber during phase III (Fig. 2D), VPA female mice failed to show social novelty preference, contrary to SAL female mice [$F(3, 64) = 6,8, p < 0.001$].

3.2. Social interaction analysis using the Live Mouse Tracker system for 3 continuous days

We analyzed several variables of social behavior as described in the methods section, for 3 days, on homogenous (same treatment within the group) or mixed groups (one VPA and three SAL mice within the group) in several sets of 4 female mice that were prenatally exposed to either SAL or VPA (see Suppl. Fig. 2 for a screen captioning of mice tracking). Mouse behavior was segmented into 5 groups of behavior as described in

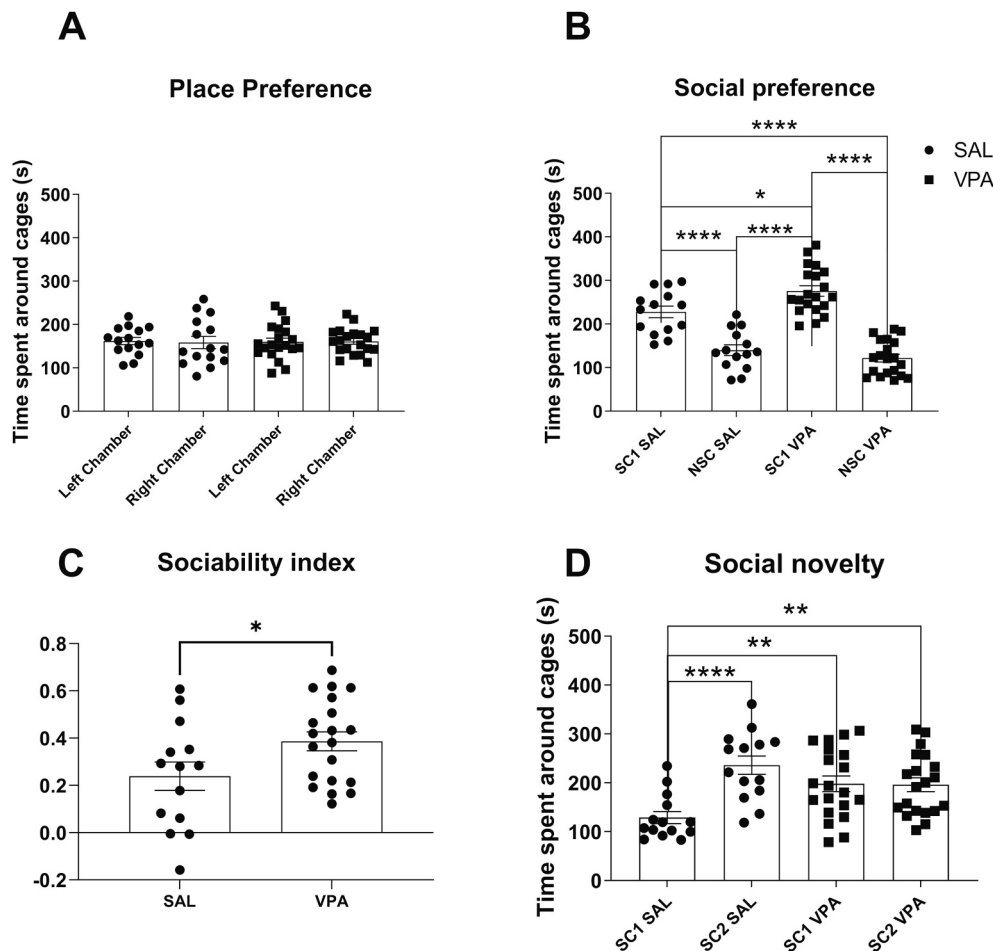


Fig. 2. Social behavior as analyzed in the 3-chamber test (3-CT) procedure. (A) There was no spontaneous preference to either chamber during the 10 min. Habituation period (Phase I-Place Preference) in SAL or VPA mice. (B) In Phase II (Social preference), an engaged mouse was added in one chamber (social chamber 1: SC1) while the other chamber remained empty (non-social chamber: NSC). (C) Results of Phase II are expressed as sociability index as detailed in materials and methods. Note that both Sal and VPA female mice showed social preference. (D) A novel mouse was introduced in the chamber 2 (social chamber 2: SC2) in addition to the engaged mouse in SC1 to evaluate social novelty recognition (Phase III). During Phase III, VPA female mice spent equal time interacting with SC1 and SC2 while saline mice spent more time interacting with SC2. This indicates that VPA SAL mice have no preference for social novelty, contrary to the SAL mice. All data are expressed as means \pm SEM; One-way ANOVA followed by Tukey's multiple analysis was performed ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Saline females ($n = 14$), VPA females ($n = 20$).

the methods section and as initially proposed (de Chaumont et al., 2019). As reported in Fig. 3 (A, B and C), no altered behaviors were identified at the end of the three-day period in VPA groups compared to control groups.

We then performed the same analysis on several sets of heterogeneous groups of 3 SAL and 1 VPA female mice and we monitored their behavior for three continuous days. As shown in Fig. 3 (D, E and F), no differences were found between the VPA and SAL mice when analyzed together within a heterogeneous group.

Thus, analysis of social behavior for a total of three days did not show social deficits in relation to VPA pre-natal exposure whether mice were tested in SAL or VPA homogeneous groups or in SAL and VPA mixed groups.

3.3. Social interaction analysis using the LMT on various time periods

Group social interactions can vary greatly in time. Analyzing results over the total time period of three days may mask major differences in the behavior of different groups. Thus, we further analyzed the data obtained from the LMT system and segmented it to periods of 1 h, 3 h and 1 day (Fig. 4).

After one hour in the LMT (Fig. 4A-F), there were several differences

in the number of contacts between the VPA homogeneous groups compared to SAL (Fig. 4A-C). For example, VPA female mice of the homogeneous groups performed significantly fewer social approaches than SAL mice (-12% for social approach versus SAL, $p < 0.01$) (Fig. 4A). No such differences were found in the mixed groups (Fig. 4D). After 3 h of experimentation (Fig. 4G-L), several deficits were identified in the number of social contacts within the VPA mice of the homogeneous groups compared to SAL (-29% for oral-oral contacts $p < 0.0001$; -42% for oral-genital contacts $p < 0.0001$) and some deficits in social approaches were also noticeable (-10% approach to contact, $p < 0.05$) (Fig. 4G). Again, no major differences were found between SAL and VPA mice in the mixed groups (Fig. 4J-L). After 24 h of experimentation (Fig. 4M-R), we observed several deficits in the number of social contacts in the VPA mice in the homogeneous groups compared to SAL (-11% oral-oral contacts $p < 0.0001$; -13% oral-genital contacts, $p < 0.0001$) (Fig. 4M and N), along with a slight reduction in the number of social approaches (-5% approach to contact, $p < 0.05$) (Fig. 4M). No major differences were found in the mixed groups (Fig. 4P-R).

To the exception of rearing behavior, these findings remain consistent when evaluated not as the number of events but as the mean duration of a given event (see supplementary Fig. 3). Please note however that certain behavioral parameters can only be quantified as

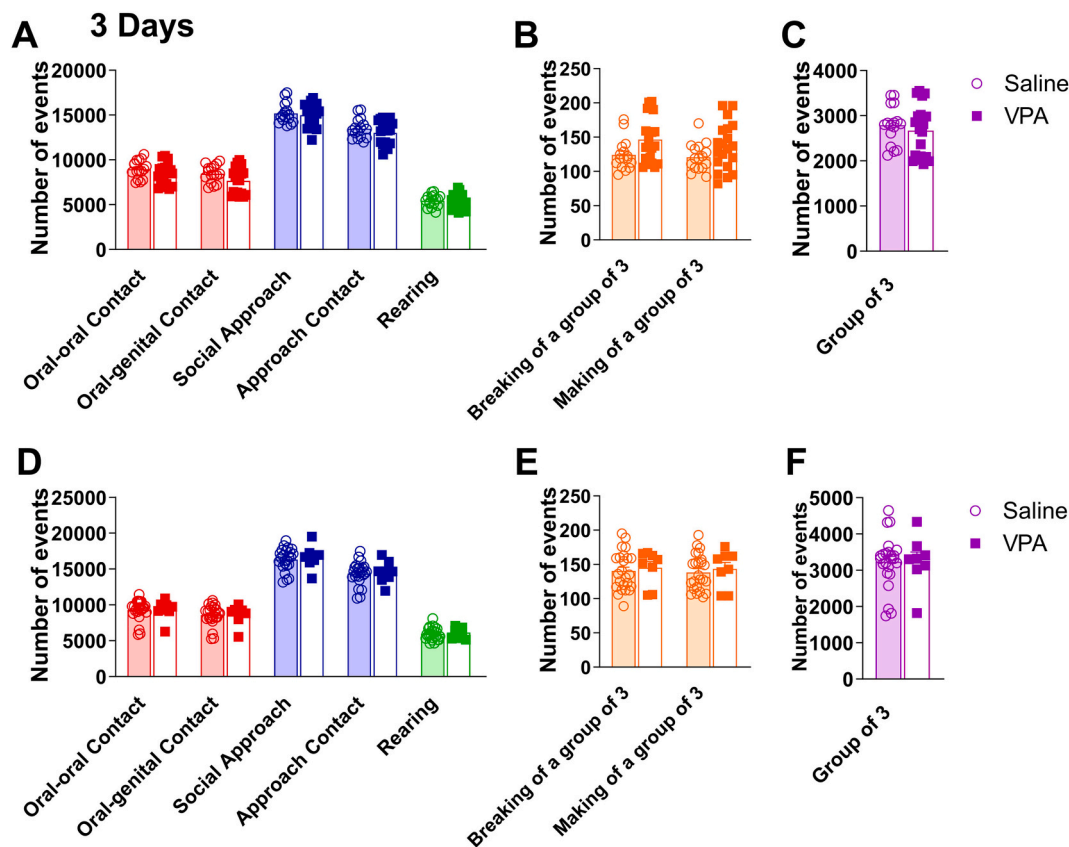


Fig. 3. Social behavior during three continuous days as measured using the Live Mouse Tracker procedure. Behavioral features were split into five categories: (i) individual behavior (green), (ii) social dyadic events between two mice (red), (iii) dynamic events between two mice (approach, escape, following each other...) (blue), (iv) subgroup events with 2, 3 or 4 mice (orange), (v) group-making and group-breaking events (purple). All behavior from all these five categories were measured and analyzed. For the sake of clarity, only 1 or 2 behaviors from each category is shown here and is represented by a different color in figures. In A, B and C, measurements were performed in several sets of homogeneous groups of mice (4 mice receiving the same treatment were analyzed together). In D, E and F, measurements were performed in mixed groups of mice (3 SAL and 1 VPA mice). (A, D) Number of various social contacts and approaches for three days, (B, E) Number of making and breaking contact in a group of 3 mice, (C, F) Number of groups of 3 mice. Note that when measured throughout 3 days, social behavior of saline and VPA mice was similar whether tested in homogeneous groups or within mixed groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

All data are expressed as means \pm SEM; Mann-Whitney test was performed ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Saline female homogeneous group ($n = 16$); VPA female homogeneous group ($n = 20$); saline female mixed group ($n = 24$); VPA female mixed group ($n = 8$).

individual events.

3.4. Evolution in time of social behavior using the LMT

Here, we further analyzed the data by sections of 6 h during the entire length of the experiment and compared homogenous and mixed groups between each other (Fig. 5). We chose to focus on 4 specific behaviors: oral-genital contacts (Fig. 5A), oral-oral contacts (Fig. 5B), social approaches (Fig. 5C) and approaches to contact (Fig. 5D). In all 4 behaviors of the homogenous groups, we observed again a significant decrease in the number of social behaviors in VPA mice compared to SAL mice, mainly at the initial time points of the measurements (Fig. 5A-D). This indicated that SAL and VPA mice of mixed groups expressed similar social behaviors. In addition, we also noted that a few social behavior parameters were significantly higher in SAL mice of the mixed groups compared to SAL mice of homogenous groups (Fig. 5A, C, D) indicating an amelioration of social behavior in the SAL mice in the presence of a VPA mouse.

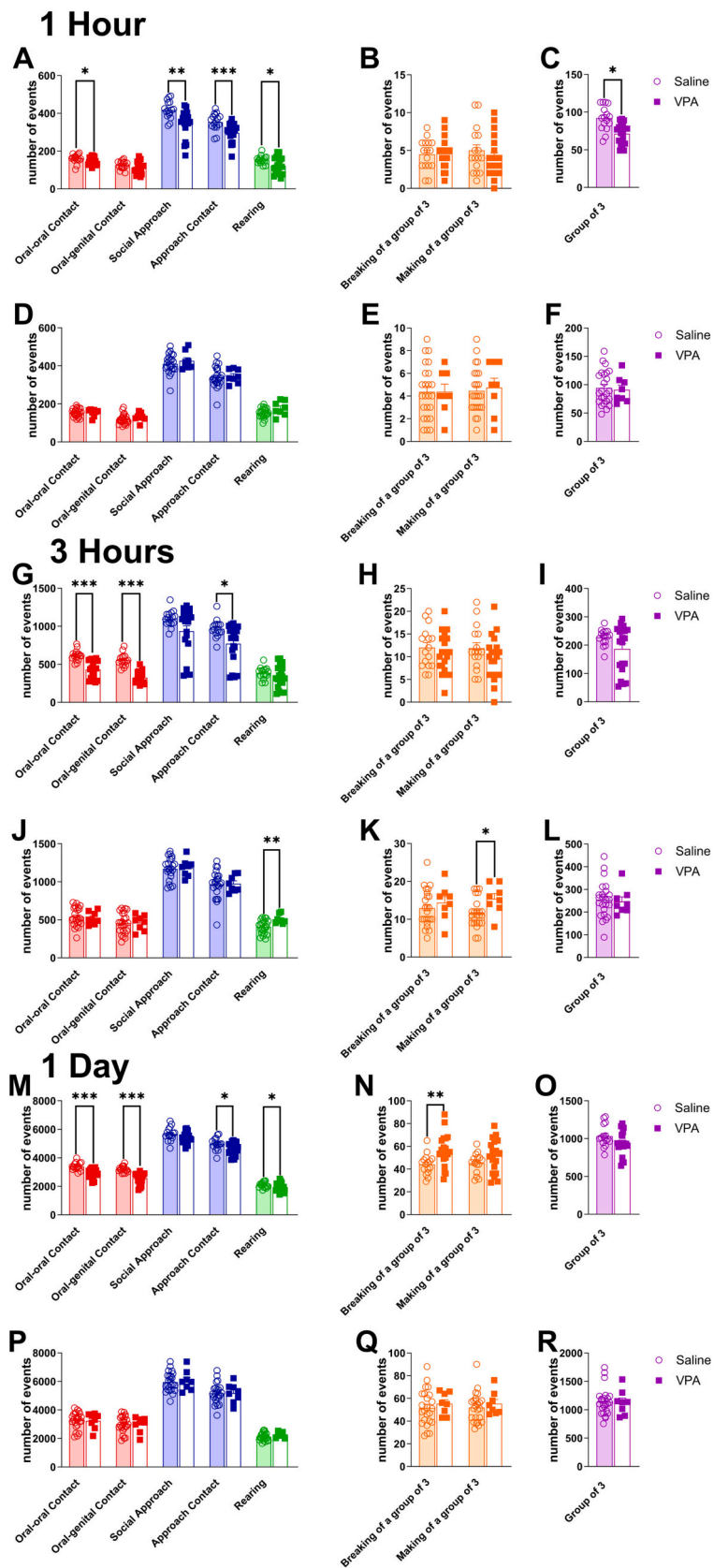
3.5. Comparison of social interaction within mixed groups using the LMT

In order to further explore social behavior of SAL and VPA mice within the mixed groups, and better identify which mouse is actually

interacting with which other mouse, we investigated the number of interactions of each mouse within the mixed groups (3 SAL and 1 VPA) focusing on the same 4 behaviors than above (Fig. 6). Overall, social interactions in mixed groups of mice (3 SAL and 1 VPA), was similar, i.e., mice interacted together regardless of treatment. On one occasion however, after 33 h of measurements, SAL mice even preferred to interact more with the VPA mouse (SAL versus VPA, in green) than with other SAL mice (SAL versus SAL, in blue). This indicates that within a mixed group of SAL and VPA mice, SAL mice sometimes preferred to interact with the VPA mouse rather than with each other. In addition, SAL and VPA mice of the mixed group show the same pattern of social behavior (Fig. 7). These results indicate that, in SAL and VPA mixed groups, mice social behavior becomes similar between SAL and VPA because of an increase in social behavior in both SAL and VPA mice, as observed during the 3 days duration of the experiment.

4. Discussion

Social deficits are one of the two hallmarks of ASD symptoms as defined by the American Manual of Psychiatry (American Psychiatric Association, 2013). As face validity is key in animal modeling of diseases and disorders, it has become mandatory for ASD animal models, whether genetic or environmental, to express some form of social



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Fig. 4. Social behavior at various time periods (1 h, 3 h and 24 h) as measured using the Live Mouse Tracker procedure. Behavioral features were split into five categories: (i) individual behavior (green), (ii) social dyadic events between two mice (red), (iii) dynamic events between two mice (approach, escape, following each other...) (blue), (iv) subgroup events with 2, 3 or 4 mice (orange), (v) group-making and group-braking events (purple). All behavior from all these five categories were measured and analyzed. For the sake of clarity, only 1 or 2 behaviors from each category is shown here and is represented by a different color in figures. Number of various social contacts, approaches and rearing (A, D, G, J, M, P), making and breaking a group of 3 mice (B, E, H, K, N, Q) and formation of groups of 3 mice (C, F, I, L, O, R) within the homogeneous groups (A, B, C, G, H, I, M, N, O) versus mixed groups (D, E, F, J, K, L, P, Q, R) following 1 h, 3 h or 1 Day in the LMT. Note that VPA mice showed several social deficits at different time points when tested together (homogeneous groups) while no such differences were present when mice were tested in SAL and VPA mixed groups. All data are expressed as means \pm SEM; Mann-Whitney test was performed ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

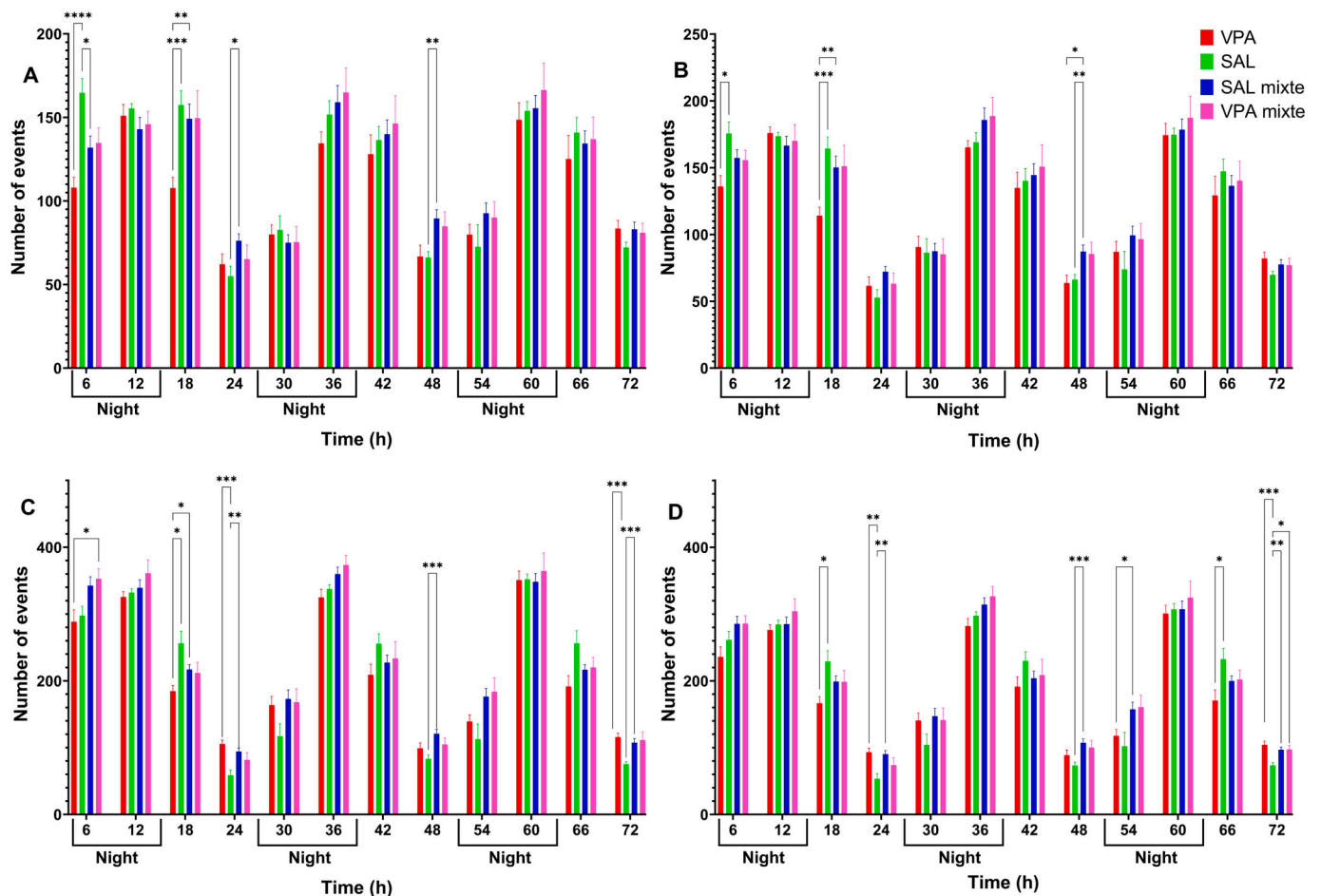


Fig. 5. Evolution in time of social behavior by segments of 6 h using the Live Mouse Tracker procedure. Number of (A) oral-geital contacts, (B) oral-oral contacts, (C) social approaches, (D) social contacts over the three days of the experiment. Note that, at early time points, VPA mice from the homogeneous groups showed several deficits in social behavior while VPA and SAL mice from the mixed group showed similar social behavior. All data expressed as means \pm SEM; two-Way ANOVA with post-hoc Tukey was performed. A Greenhouse-Geisser correction was applied. Only selected differences were highlighted for the sake of clarity ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

deficits before being considered for further analysis and characterization (Crawley, 2004; Silverman et al., 2010; Haratizadeh et al., 2021; Takumi et al., 2020; Ellegood and Crawley, 2015).

Social deficits in ASD children are expressed in the form of social play behavioral deficits and lack of non-verbal social gestures such as pointing, showing, and giving (Tartaglione et al., 2019; Hobson et al., 2013; Jordan, 2003; Rutherford et al., 2007). Mice are very social mammals and exhibit a wide range of social behaviors such as direct cross interactions, sniffing, following, regrouping, collective nesting, maternal care, general play behavior and sexual approaches (Panksepp et al., 1984; Ricceri et al., 2007; Vanderschuren et al., 2016). A significant number of reports have described social impairments in a large majority of ASD animal models, including the VPA models, although most of these experiments were performed on males only and when performed on females no social interaction deficits were observed

(Thabault et al., 2022; Nicolini and Fahnstock, 2018; Tartaglione et al., 2019; Jaber, 2022).

Since its initial implementation, the 3-CT has rapidly become the gold standard to measure social approach, social preference and, more generally, exploratory locomotion in ASD animal models (Ricceri et al., 2007). The procedure is now automated and is widely used, in its original form or with slight variations, among teams working on various animal models of ASD (Yang et al., 2011a). Despite the many advantages of this simple and efficient procedure, the 3-CT suffers from several drawbacks as it cannot evaluate complex social behaviors, in a group of mice, and for a long period of time.

Recently, we published a series of studies on 3 different ASD animal models, genetic (Shank3) and environmental (VPA and poly I:C) all showing major deficits in social behavior that were almost exclusively present in male ASD mice but not in females (Al Sagheer et al., 2018;

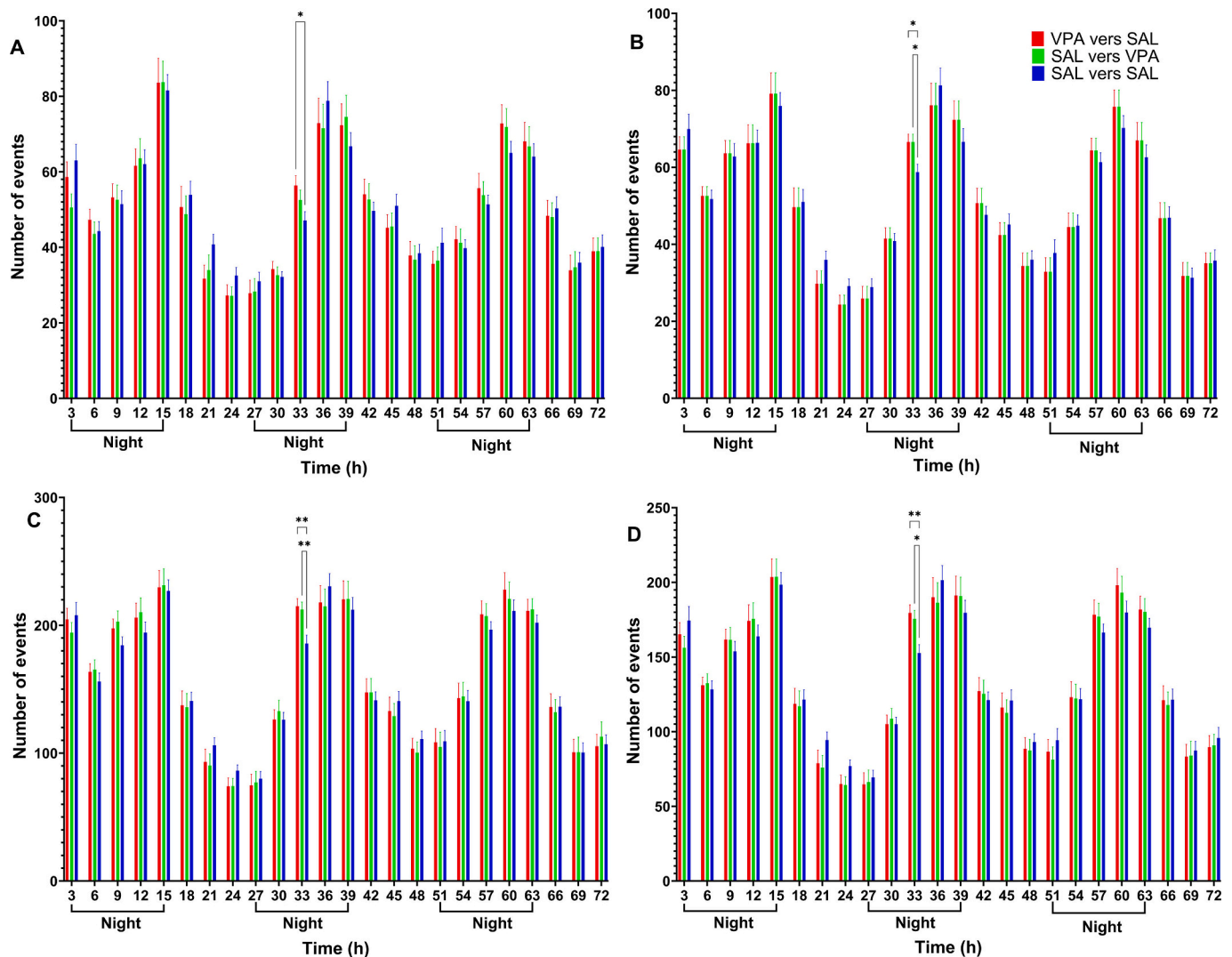


Fig. 6. Comparison of the number of interactions within mixed groups using the Live Mouse Tracker) procedure. Number of (A) oral-genital contacts, (B) oral-oral contacts, (C) social approaches and (D) social contacts during the three days of the experiment. Note that there was generally no preference in the social contacts in the mixed groups in relation with treatment except on few occasions (time point 33 h) where SAL mice seemed even to prefer interacting with the VPA mouse than with another SAL mouse. All data is expressed as means \pm SEM; two-Way ANOVA with post-hoc Tukey was performed. A Greenhouse-Geisser correction was applied. Only selected differences were highlighted for the sake of clarity (* $p < 0.05$).

Jaber, 2022; Haida et al., 2019; Matas et al., 2021; Maisterrena et al., 2022). Of interest is the fact that, both males and females showed abnormal gait and motor coordination behaviors and correlative loss of cerebellar Purkinje cells. This raised the question of whether ASD females were somehow protected from contracting social deficits, whatever the disorder's etiology, or whether the procedure that is used to evaluate social behavior is not sensitive enough to detect mild or specific social deficits.

This is of relevance given the male bias in ASD that is estimated at 4.2:1 according to the ADDM network most recent estimates (Maenner et al., 2023). ASD Females are generally diagnosed later in life (Giarelli et al., 2010) and have a higher risk than males of remaining undiagnosed (Bargiela et al., 2016). There are many possible explanations to this gender bias. In our hands, using both environmental and genetic animal models of ASD, we have systemically reported that female ASD mice showed less social deficits and motor disorders than males (Jaber, 2022). These behavioral differences between males and females were also accompanied by cellular differences as females showed less loss in cerebellar Purkinje cells than males. So it is possible that the male bias in ASD, and in several other psychiatric illness, is in fact due to females

being more resistant to internal (genetic) and external (pharmacological and environmental) stressors than males (Becker, 2012) as we have also previously proposed (Haida et al., 2019). However, one cannot exclude that there may be also a systematic gender discrimination against ASD females as ASD is more often perceived as a male condition (Brickhill et al., 2023). Females may also be underdiagnosed given that they are more emotionally expressive than males and show a better use of non-verbal communication and thus seem to be less perceived as being atypical than males by their peers (Geelhand et al., 2019).

Here, we aimed to explore whether we can identify complex social deficits in female VPA mice and determine their nature and extent. We used the widely accepted VPA ASD animal model, which we have also previously characterized extensively in males and females separately, where VPA (450 mg/kg) is i.p. injected prenatally into the pregnant female at E12.5 (Thabault et al., 2022; Al Sagheer et al., 2018; Jaber, 2022; Maisterrena et al., 2022). Male mice prenatally exposed to VPA show major social deficits and loss of cerebellar Purkinje cells as others and we have previously shown (Al Sagheer et al., 2018; Jaber, 2022; Main and Kulesza, 2017; Mansour et al., 2022).

We implemented the recently developed LMT apparatus and

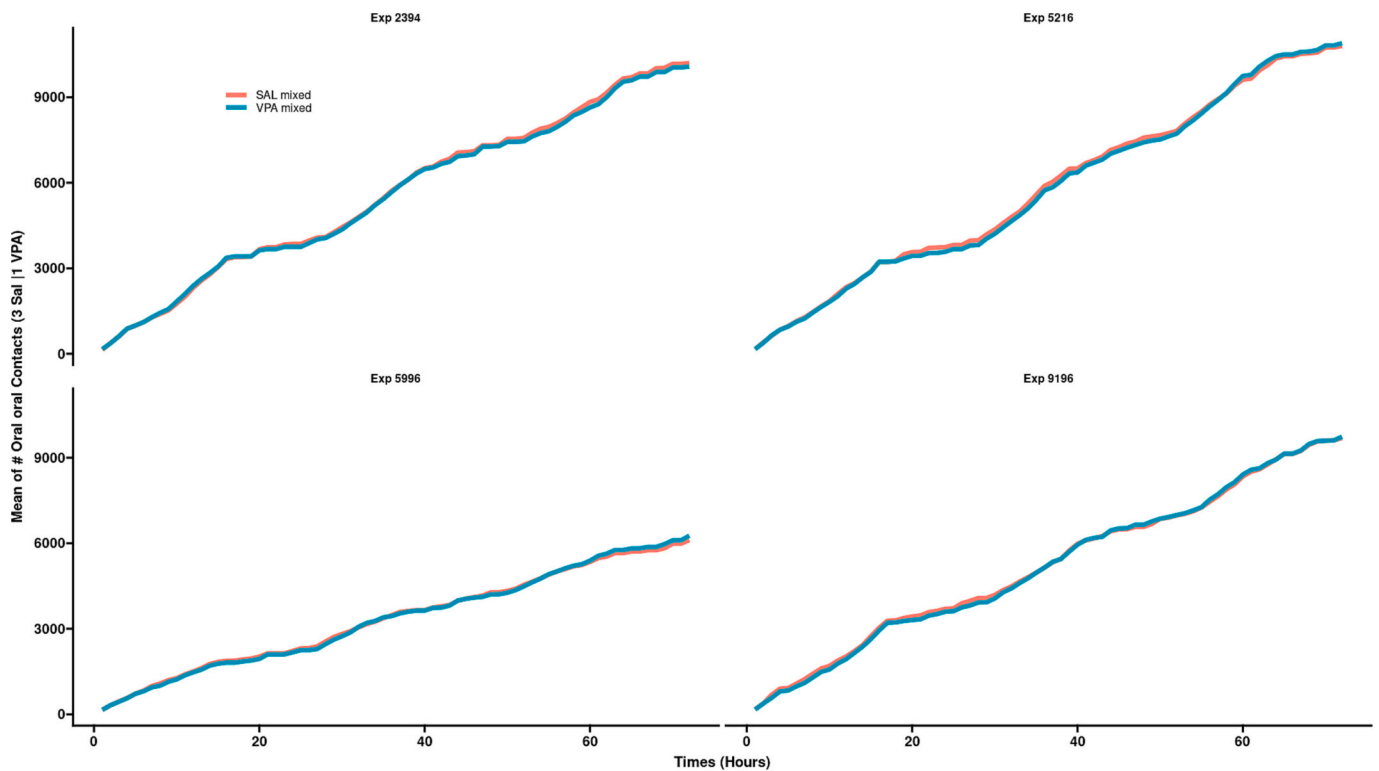


Fig. 7. Evolution of number of oral-oral contacts in 4 different experiments with mixed groups of SAL and VPA mice. Note that saline and VPA mice of different experimental settings had corresponding scores throughout the 3 days of measurements. This indicates similar social behavior of mice whatever the prenatal exposure when tested in mixed groups of 3 SAL to one VPA.

measured social behavior on groups of 4 mice randomized from different litters of the same sex and receiving the same treatment (homogeneous groups) or in groups of 3 SAL and 1 VPA mice. Mice within the same experimental group had no previous contact with each other. Experiments were performed on several sets of groups of 4 mice, for three consecutive days and data were analyzed following 1 h, 3 h, 24 h and three days. We report that: (i) VPA females show several forms of social deficits as compared to SAL females; (ii) social deficits were complex in their nature as they implicated several behavioral traits and variables such as approaches, following, direct interactions, breaking/making a group of mice and nesting (see materials and methods for a complete description of these behaviors); (iii) these social deficits appear early on during the experiments and tend to disappear within three days, possibly suggesting a stress reaction to novelty. (iv) One of the most intriguing findings is that VPA mice, when placed within a group of SAL mice, exhibit significant improvement in several social variables as early as 1 h and lasting up to 3 days of the experiment duration; (v) similarly, SAL mice in mixed groups showed an increase of several variables linked to social behavior in the presence of a VPA mouse. In other words, placing an ASD mouse within a group of normal mice improves the social behavior of both the VPA mouse and that of normal mice. The assessment of whether this beneficial effect endures beyond the 3-day time period of the experiment still requires evaluation. Our study aligns with the findings of Crawley's research team, which previously demonstrated that when BTBR mice—an inbred strain manifesting multiple social deficits—are raised with C57BL/6 J mice from their juvenile age until adulthood, their social deficits exhibit significant improvement (Yang et al., 2011b). Similarly, housing BTBR mice with C57BL/6 J mice from weaning to adulthood, significantly increased the myelin thickness in the prefrontal cortex of BTBR mice (Makinodan et al., 2017).

During the first hour of experimentation, VPA homogeneous group had fewer rearing behavior and social approaches than the SAL homogeneous group indicating less exploration of the cage and social

environment, in line with the 3-CT results. This may be related to higher anxiety levels in VPA mice as shown previously (Gao et al., 2019). Indeed, stress is a frequent comorbidity of TSA and have been shown to negatively impact social interactions (Fuld, 2018; Bishop-Fitzpatrick et al., 2015). These results may also indicate a lack of social interest of VPA mice to interact with other VPA mice. In line, an ongoing study using the LMT apparatus on a genetic animal model of ASD is showing that control and ASD male mice prefer to interact with a control mouse rather than with an ASD mouse (Rusu et al., 2022).

After three hours, there was no longer a decrease in the rearing behavior of VPA mice in the homogenous groups while deficits in social approaches persisted accompanied by a significant decrease in social contacts in the VPA homogeneous group compared to SAL. In the mixed groups, we also noted an increase of grouping behaviors by the VPA mouse indicative of improvements in social interactions in the presence of SAL mice.

Similar alterations in social contacts and approaches were also observed after 24 h in the VPA homogeneous group compared to SAL. In addition, at this time point, there was a significant decrease in the distance traveled by the VPA mice which is indicative of a hypoactivity that started after 24 h of housing in the LMT. Of interest is the observation that these differences were not observed in the heterogenous groups indicating a form of “normalization” of behavior between the VPA and the SAL mice housed together in the LMT. Following these observations, we aimed next at exploring the evolution in time of different social behaviors by comparing the behavior of mice in homogeneous groups versus mixed groups. Three main observations can be drawn from this analysis: (i) mice in mixed groups performed more social contacts than the SAL mice in the homogenous groups, (ii) VPA mice in mixed groups performed more social contacts than the VPA mice in the homogenous groups and, (iii) VPA mice in mixed groups show no differences in many parameters of social interactions and behavior compared to SAL mice in homogenous groups, indicative of an amelioration in their general social

behavior. Taken together, these results indicate that the presence of a VPA mouse in a group of SAL mice has a positive impact on the sociability of both SAL and VPA mice. We then analyzed the interaction between individual mice in the mixed groups where we observed that SAL mice preferred to interact with the VPA mice rather than with other SAL mice. Furthermore, mice of the mixed groups tend to rapidly show homogeneous social behavior scores, regardless of the treatment, over the period of 3 days (Figs. 7) due to a general increase in social behavior in VPA and SAL mice.

These findings showing the cross-influence of ASD mice and control mice on their corresponding social behavior need to be confirmed with further studies in ASD animal models and, more importantly, in humans, as they may have repercussions on currently explored policies of integrating individuals with ASD within groups of typical individuals, including in the school systems. Although research reports on the effects of integrating ASD individuals in regular schools are very scarce, it has been shown that when intervention therapies are implemented in schools, ASD children have significantly higher social network inclusion and received more friendship nominations than ASD children without such an intervention (Locke et al., 2019; Gilmore et al., 2019).

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Credit authorship contribution statement

Alexandre Maisterrena: Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Fabrice de Chaumont:** Software, Resources, Methodology, Conceptualization. **Jean-Emmanuel Longueville:** Methodology, Formal analysis. **Eric Balado:** Methodology, Investigation, Formal analysis. **Elodie Ey:** Software, Resources, Methodology, Investigation, Formal analysis. **Mohamed Jaber:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Authors declare no financial interests that are directly or indirectly related to this work. Authors did not use AI assistance in writing of the manuscript.

Data availability

Data will be made available on request.

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