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Research paper

Preventive effects of resveratrol against early-life impairments in the animal model of autism induced by valproic acid

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ABSTRACT

Background: Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by social interaction deficits and repetitive/stereotyped behaviors. Its prevalence is increasing, affecting one in 36 children in the United States. The valproic acid (VPA) induced animal model of ASD is a reliable method for investigating cellular, molecular, and behavioral aspects related to the disorder. *Trans*-Resveratrol (RSV), a polyphenol with anti-inflammatory and antioxidant effects studied in various diseases, has recently demonstrated the ability to prevent cellular, molecular, sensory, and social deficits in the VPA model. In this study, we examined the effects of prenatal exposure to VPA and the potential preventive effects of RSV on the offspring.
Method: We monitored gestational weight from embryonic day 6.5 until 18.5 and assessed the onset of developmental milestones and morphometric parameters in litters. The generalized estimating equations (GEE) were used to analyze longitudinal data.
Results: Exposure to VPA during rat pregnancy resulted in abnormal weight gain fold-changes on embryonic days 13.5 and 18.5, followed by fewer animals per litter. Additionally, we discovered a positive correlation between weight variation during E15.5-E18.5 and the number of rat pups in the VPA group.
Conclusion: VPA exposure led to slight length deficiencies and delays in the onset of developmental milestones. Interestingly, the prenatal RSV treatment not only prevented most of these delays but also led to the early onset of certain milestones and improved morphometric characteristics in the offspring. In summary, our findings suggest that RSV may have potential as a therapeutic intervention to protect against the negative effects of prenatal VPA exposure, highlighting its importance in future studies of prenatal neurodevelopmental disorders.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by impairments in two primary domains: deficits in social communication and interaction, as well as the presence of repetitive and stereotyped behaviors (American Psychiatry Association (APA), 2013). Over recent years, the prevalence of ASD among children in the general population has risen, with one in 36 children being diagnosed with ASD in the United States (Maenner et al., 2023). Diagnosis is primary clinical and typically occurs when children exhibit traits associated with these two domains, meeting diagnostic criteria. However, tools facilitating the early identification of developmental milestones have gained increasing importance.

Unusual sensory responses have been reported in up to 69–90% of children with ASD (Baranek et al., 2006), indicating atypical sensory development in this disorder. These sensory impairments have been described as early as 6 months of age in children later diagnosed with autism at 24 months, persist to postnatal life, and can predict social communication impairments and repetitive behaviors (American Psychiatry Association (APA), 2013), potentially aiding ASD diagnosis

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(Robertson and Baron-Cohen, 2017). Before 24 months of age, children with ASD may exhibit behavioral alterations such as hypo-responsiveness to novel stimuli, atypical motor behaviors, and impairments in social, communication, and play behaviors. While a definitive diagnosis from 18 to 24 months is rare, these early observations contribute to a better understanding of sensory development and its relevance for early ASD detection and interventions (Siemann et al., 2020). Motor disturbances are a common feature in these patients, with prevalence rates exceeding 50% depending on the parameter assessed. Deficits in fine motor skills and altered movement planning are also frequently noted and these impairments may emerge as early as six months of age, predicting language outcomes manifesting around 3 years of age (Bradley et al., 2016). Investigating motor impairments in ASD is pivotal for early intervention and improved outcomes.

The gestational period and disruptions during this phase are linked to an elevated risk of neurodevelopmental disorders in offspring. Prenatal risk factors such as maternal infections (Jiang et al., 2016), advanced parental age (Wu et al., 2017), and maternal use of the anticonvulsant and mood stabilizer valproate (VPA) during the first trimester of gestation (Modabbernia et al., 2017) have been associated with ASD outcomes in offspring (Christensen et al., 2013; Modabbernia et al., 2017).

VPA has been shown to induce ASD-like impairments in rodents, establishing a reliable animal model of ASD (Rodier et al., 1997; Schneider and Przewłocki, 2005). This model replicates social and repetitive/stereotypical behaviors observed in ASD individuals, demonstrating face, construct, and predictive validity (Mabunga et al., 2015). These animals also exhibit developmental delays in the onset of behavioral milestones, highlighting global dysfunctions and supporting the hypothesis of general sensorimotor impairments early in life (Ruhela et al., 2019; Schneider and Przewłocki, 2005), along with immune system impact, increased oxidative stress and pro-inflammatory responses (Deckmann et al., 2019; Gottfried et al., 2015).

Resveratrol (RSV), a polyphenol found in grapes, is known for its classical anti-inflammatory and antioxidant properties, and has demonstrated potential benefits in treating cardiovascular diseases, tumors, and brain disorders (Vang et al., 2011). Our research group has previously shown that RSV prevents social, sensory, cellular, and molecular alterations in the VPA model (Bambini-Junior et al., 2014; Deckmann et al., 2019; Fontes-Dutra et al., 2018; M. M. Hirsch et al., 2020; Santos-Terra et al., 2021). However, the preventive role of RSV in early postnatal periods and its impact on developmental milestones remains insufficiently understood.

Investigating early development in postnatal life can offer insights into the effects of prenatal risk factor exposure during early gestation on postnatal outcomes. Developmental milestones can be valuable indicators of growth and maturation in individuals, reflecting the intricate progression of physiological, cognitive, and behavioral elements. Their importance lies in the ability to identify potential delays in neurological development at an early stage, enabling early interventions.

Therefore, our study aims to analyze the effects of prenatal exposure to VPA on pregnant female rats, as well as their litters' early postnatal life, evaluating behavioral hallmarks commonly performed in the literature. We also aim to observe if prenatal treatment with RSV can have a preventive effect on VPA-induced abnormalities in both pregnant female rats and their VPA-exposed litters.

2. Experimental procedures

2.1. Animals

Sixty-day-old primiparous female Wistar rats were obtained from the Center of Reproduction and Experimentation of Laboratory Animals (CREAL) at the Federal University of Rio Grande do Sul (UFRGS). The rats were housed in plastic cages (410 mm/340 mm/160 mm – length/width/height) without environmental enrichment, accommodating four to five rats per cage. Rats were grouped based on similar weight and individually marked on the tail. Cage dimensions provided a total inside area of 1110 cm^2 with an area of above 277.5 cm^2 per animal. For reproduction animals, cages were arranged with alternating female and male cages to expose females to the male scent. The animals were maintained under a standard 12/12-h light/dark cycle (light cycle 7 am to 7 pm), and at a constant temperature of 22 ± 1∘C in the Unity of Animal Experimentation (UEA) at the Clinical *Hospital of Porto Alegre* (HCPA). The handling of animals followed the National Council for the Control of Animal Experimentation (CONCEA) of Brazil, with ad libitum access to food and water. The ethics committee at HCPA approved this project (HCPA-FIPE #170280 and #160477).

Estrous cycles of female rats were assessed by fresh vaginal lavage under a tenfold optical microscope magnification. Primiparous females in pro-estrous and early estrous phases were monogamously mated overnight with slightly larger males from neighboring cages during the dark cycle. Pregnancy was confirmed by the presence of spermatozoa in the vaginal smear the following morning. The confirmation day was designated Embryonic day 0.5 (E0.5). A total of 38 pregnant rats were randomly assigned to one of four groups: Control, RSV, VPA, or RSV+VPA.

2.2. Prenatal weighing and administration of RSV and VPA

Pregnant females were weighed daily on a rodent weighing balance at E0.5 and from E6.5 to E18.5. RSV administration: female received a daily subcutaneous injection of 3.6 mg/kg RSV (Fluxome, Stenløse, Denmark), freshly dissolved at 36 mg/mL in dimethyl sulfoxide (DMSO) or received an equivalent volume of the vehicle DMSO, as previously described (Bambini-Junior et al., 2014; Fontes-Dutra et al., 2018). VPA administration: On Embryonic day 12.5 (E12.5), pregnant rats received a single intraperitoneal injection of 600 mg/kg VPA (Acros Organics, NJ, USA), dissolved in 0.9% saline to attain a concentration of 250 mg/mL or received an equivalent volume of 0.9% saline solution. Experimental groups: Control (subcutaneous DMSO from E6.5 to E18.5 and intraperitoneal saline solution at E12.5), RSV (subcutaneous RSV from E6.5 to E18.5 and intraperitoneal saline solution at E12.5), VPA (subcutaneous DMSO from E6.5 to E18.5 and intraperitoneal VPA at E12.5), and RSV+VPA (subcutaneous RSV from E6.5 to E18.5 and intraperitoneal VPA at E12.5).

Pregnant rats were isolated on E19.5 to give birth and all dams gave birth on E22.5 and the litters enter at postnatal development at the same stage of maturity. The birth day was considered postnatal day 0 (P0). The number of pups per litter was counted immediately after birth, with no reports of miscarriages or stillbirths. To maintain gender balance, an equal number of males and females per litter were used for evaluations.

2.3. Developmental milestones evaluations

Behavioral milestones were assessed at P6, P8, P10, P12, P14, P16, P18, and P20. After these evaluations, animals were weaned at P21. All assessments were conducted in a specialized room, with animals weighed using a rodent weighing balance and their length measured with a sanitized ruler (excluding the tail length). We carefully observed the emergence of expected developmental milestones, which encompassed criteria such as *Eyes Opening*, *Incisor Teeth Appearance*, and *Ear Reflex*. Instances where the defined milestones did not manifest adequately or where there was an absence of expected progression were not taken into consideration. All apparatuses were sanitized after each test, and observations were carried out on individual animals. To minimize maternal stress, evaluations were conducted on one litter at a time, with gentle handling and minimal manipulation. As the development of each pup was evaluated individually, comparisons were performed individually to better represent performance within groups.

To ensure objectivity, two trained observers conducted all evaluations individually. Observers were unaware of the litter and

experimental group. Measurements were registered only when both observers reached a consensus. *Ear reflex* and *Eyelid reflex* were elicited using a cotton swab stimulus near the ear and eye. The day of appearance of the ear unfolding twitch and eye muscular contraction (blink) was recorded. Incisor teeth appearance was noted when the teeth crown emerged in the hard palate (Mammel et al., 2020).

Cliff Avoidance was evaluated by placing animals on an 80 cm high platform and recording the day they exhibited the ability to turn 180 degrees (Fox, 1965; Hou et al., 2018; Ruhela et al., 2019; Schuch et al., 2016; Wolf et al., 1996).

Negative Geotaxis was assessed by placing pups on a 45-degree inclined platform with a slightly roughened surface, with their heads pointing downwards. The first day on which the animal successfully completed a full rotation, with its head facing upward, was recorded. If the animal fell during the task, it was not considered (Hou et al., 2018; Ruhela et al., 2019; Schuch et al., 2016).

Righting Reflex was evaluated by placing the animal on its back on a paper towel, with the first appearance of a coordinated torsional move using the limbs and/or trunk considered the appearance day. This test is dependent on the intact vestibular and somatosensory system (Wolf et al., 1996). A typical response occurs almost immediately, in one second, with the behavior considered absent if the reflex was absent for more than 30 s (Hayashiuchi et al., 2017; Ogawa et al., 2020; Oka et al., 1992; Wolf et al., 1996).

Startle Reflex was evaluated by generating a loud handclap (approximately 80 dB) (Papadakis and Stavroulakis, 2020) about 30–45 cm from the animal. The rapid contraction of skeletal muscles was considered a startle reflex response (Hou et al., 2018; Koch, 1999).

Limb Positioning and Grip were assessed using a 2.5 mm covered wire installed in a plate; Gently stimulating the back of fore and hind limbs, extension and hyperextension of the limbs were considered positive for positioning reflex. The first appearance of grasping behavior upon placing the limbs on the wire was recorded (Schönfeld et al., 2017).

The *March Reflex* was assessed by gently placing each pup in the center of a plastic circle with a radius of 6.5 cm. The day of appearance of the reflex was recorded when the animal exited the circle within 30 s (Lubics et al., 2005).

2.4. Statistical analysis

The longitudinal data (maternal weight, pups' weight, and length) were analyzed using generalized estimating equations (GEE) after confirming normality through a Shapiro-Wilk test. GEE extends generalized linear models for analyses of repeated measurements, accommodating time-varying covariates. The working correlation matrix was set to the exchangeable matrix for the compound symmetry structure of the data. Inference tests were conducted using Wald Chi-Square Test, followed by post-hoc Bonferroni correction for determining statistical significance. The correlation between relative weight variation (adjusted by initial pregnancy weight) and the number of pups per litter was analyzed using Spearman Rank Correlation Coefficient. Additionally, the number of pups and the developmental milestone onset were analyzed using two-way ANOVA, followed by a post-hoc Tukey test ($p < 0.05$). All statistical analyses were conducted by the Biostatistics Unity of the Research and Post-Graduation Group of Clinical Hospital of Porto Alegre (GPPG/HCPA) using IBM SPSS software (version 28.0.0). Graphs and figures were created using GraphPad Prism software (version 8.0.1 (244)). The data were reported as mean ± SD.

3. Results

3.1. Maternal weight variation and pup numbers

The gross gestational weights are detailed in Table 1A. The weights recorded on pregnancy days 0.5 and 6.5 did not differ among groups ($p = 0.8530$ and $p = 0.6490$, respectively). Delving into the analysis of maternal weight fluctuations during gestation (Fig. 1 and Tables 1A–1D), we observed a significant weight loss in dams that received VPA on both day 13.5 and day 18.5 ($P < 0.05$). Moreover, as depicted in Fig. S1, a tail kink was observed in the VPA-exposed group, a prevalent trait among VPA-exposed animals. Furthermore, as illustrated in Fig. S1, the VPA-exposed group exhibited a tail kink (A), a prominent characteristic present across all VPA-exposed animals, varying in degrees of curvature. Notably, the administration of RSV did not exhibit efficacy in mitigating these morphological changes (B).

Starting from day 6.5, which marked the initiation of RSV treatment, we aligned subsequent data points with the measurements taken on the preceding day. To explore the potential implications of this weight variation on postnatal outcomes, we subsequently investigated the pup count per litter. (Fig. 2 and Table S2). The VPA group exhibited a lower number of pups per litter compared to the Control and RSV groups ($p < 0.05$). Correlation analysis between relative pregnancy fold-change weight variation and total pup count (Fig. 3 and Table S3) revealed a significant correlation in the VPA group from pregnancy days 15.5–18.5 (*$p < 0.05$, **$p < 0.01$), indicated by Spearman Rank Correlation coefficients and corresponding p-values of 0.635 ($p < 0.05$), 0.666 ($p < 0.01$), 0.736 ($p < 0.01$), and 0.780 ($p < 0.01$) on pregnancy days 15.5, 16.5, 17.5, and 18.5, respectively. In contrast, the Control, RSV, and RSV+VPA groups displayed no significant correlation between fold-change weight variation and total pup count during the analyzed pregnancy period.

3.2. Pup weight and length

Evaluating pup weight and length from postnatal day 6–20, we observed significantly increased weight ($p < 0.05$ in the RSV group during all assessed periods for both female (Fig. 4A and Table S4) and male (Fig. 4B and Table S5) cohorts. The statistical analysis of weight variation of pups is demonstrated at Table S6. Likewise, RSV-treated animals exhibited greater length compared to other experimental groups ($p < 0.05$) throughout all observed periods in both females (Fig. 4C and Table S7) and males (Fig. 4D and Table S8). The statistical analysis of length variation of pups is demonstrated at Table S9.

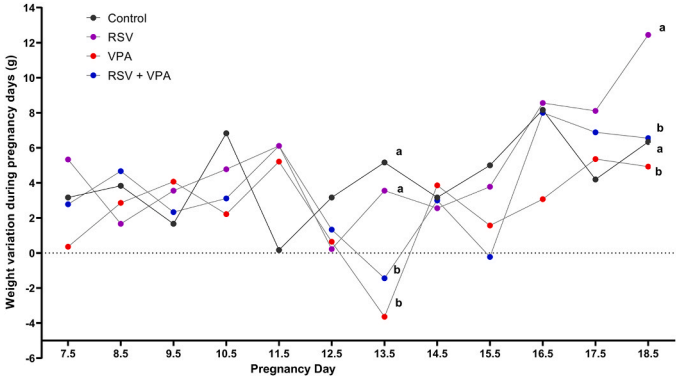

Fig. 1. Weight variation among pregnant rats. Weight variation during pregnancy days 6.5–18.5 at each time point. Control = Gray dot (n = 6), RSV = Purple dot (n = 9), VPA = Red dot (n = 14) and RSV+VPA = Blue dot (n = 9). Different letters indicate significant statistical difference for non-VPA x VPA groups from generalized estimating equations (GEE), followed by Bonferroni test ($p < 0.05$): day 13.5 (one day after VPA injection) - mean ± SD Control: 5.167 ± 3.76, RSV: 3.555 ± 2.01, VPA: − 3.643 ± 7.00, RSV+VPA: − 1.444 ± 5.83; VPA: Wald Chi-Square= 29.080) and day 18.5 (Control: 6.333 ± 5.24, RSV: 12.444 ± 3.81, VPA: 4.929 ± 4.89, RSV+VPA: 6.556 ± 5.72; VPA: Wald Chi-Square= 29.080). For additional information, refer to Tables 1A–1D.

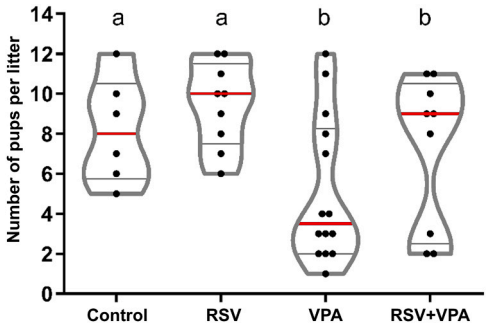

Fig. 2. Number of pups per litter. Control (n = 6 dams, 49 pups), RSV (n = 9 dams, 85 pups), VPA (n = 14 dams, 71 pups) and RSV+VPA (n = 9 dams, 65 pups). Different letters indicate significant statistical differences for non-VPA x VPA groups by two-way ANOVA followed by Tukey test ($p < 0.05$). Values are shown as mean ± standard deviation: Control: 8.167 ± 2.63, RSV: 9.444 ± 2.12, VPA: 5.071 ± 3.62, RSV+VPA: 7.222 ± 3.80; VPA: F= 5.841; p = 0.0212. For additional information, refer to Table S2.

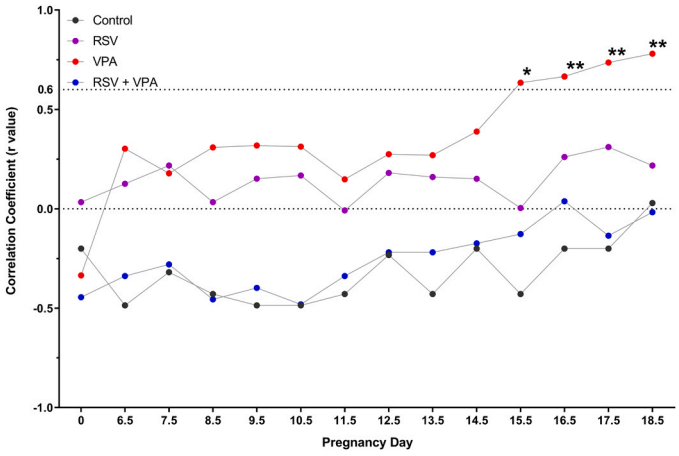

Fig. 3. Correlation between the relative weight variation during pregnancy and number of pups. Control = Gray dot (n = 6 dams n = 49 pups), RSV = Purple dot (n = 9 dams n = 85 pups), VPA = Red dot (n = 14 dams n = 71 pups) and RSV+VPA = Blue dot (n = 9 dams n = 65 pups). Values are shown as Spearman correlation coefficient, r_s. * $p < 0.05$ * *$p < 0.01$. For additional information, refer to Supplementary Table 3.

3.3. Developmental hallmarks evaluations

The results of developmental hallmarks evaluations (Figs. 5–7) are presented alongside raw data and statistical analysis results in Tables S10–S13. The VPA group demonstrated a significant delay in *Eyes Opening* onset (Fig. 5A-B) compared to the control group in both females (5 A, p = 0.0007) and males (5B, p = 0.006). The RSV treatment was able to prevent this delay. We also noted delayed incisor teeth onset in both female (5 C) and male (5D) VPA-exposed groups in comparison to the control group (p = 0008, p = 0.0001) and RSV groups (p = 0.0013, p = 0.0001). Additionally, RSV demonstrated an effect toward earlier *Incisor Teeth* onset in both females (p = 0.045) and males (p = 0.0285). Analyzing *Ear Reflex* in females (5E), we observed that RSV-treated groups exhibited an earlier onset compared to the control and VPA groups ($p < 0.0001$), and the RSV+VPA group displayed an earlier onset compared to the control group ($p < 0.05$). However, no significant difference was found in males (5 F).

Regarding sensorimotor achievements (Fig. 6), the *Cliff Avoidance* onset in females (6 A) occurred earlier in RSV-treated groups than in the control and VPA groups (p = 0.0071). In contrast, males exposed to VPA demonstrated a delayed onset in *Cliff Avoidance* (6B) compared to the control group (p = 0.007), with this delay being mitigated by RSV. In *Negative Geotaxis*, only males from the VPA group exhibited a delayed onset (Fig. 6D) compared to the control group (p = 0.0073). In females, the *Startle Reflex* (6E) displayed an earlier onset in the RSV group compared to the control, VPA, and RSV+VPA groups (p = 0.0012, p = 0.0017, and p = 0.0017, respectively). Conversely, in males (6 F), the VPA-exposed groups exhibited a delayed onset compared to the non-exposed groups (p = 0.0007).

Righting Reflex (Fig. 7A) showed delayed onset in female VPA-exposed groups compared to the control group (p = 0.0394). Similarly, in males (Fig. 7B), both VPA and RSV+VPA groups demonstrated a delayed onset compared to the control (p = 0.0093) and RSV (p = 0.0087) groups.

For *Lower Limb Grip*, the VPA effect was absent in females compared to the control group (Fig. 7C). In males (Fig. 7D), the VPA group exhibited a delayed onset compared to the control (p = 0.0076), RSV (p = 0.0004), and RSV+VPA (p = 0.0054) groups, which RSV effectively prevented.

Lower Limb Positioning (Fig. 7E), showed delayed onset in the female VPA group compared to the Control group (p = 0.0079), a delay mitigated by RSV. For males, (Fig. 7F), RSV-treated groups exhibited an earlier onset compared to non-treated groups (p = 0.0365).

Regarding *Upper Limb Grip*, females in the VPA group displayed a delayed onset (p = 0.0214) compared to the Control group, whereas no significant effect was observed in males (Tables S11 and S13).

No statistically significant differences were noted in the *Eyelid Reflex*, *March Reflex*, and *Upper Limb Positioning* in both female and male animals (Tables S11 and S13).

4. Discussion

In this study, we observed impaired gestational weight variation at E13.5 and E18.5 in dams exposed to VPA. Additionally, a positive correlation emerged between fold-change weight variation at E15.5, E16.5, E17.5, and E18.5, and the number of pups in the VPA group. Interestingly, the VPA group exhibited fewer pups per litter compared to the other groups. Following VPA injection, pregnant females experienced immediate contractions in the lower abdomen, inducing transient alterations in locomotor behavior and breathing patterns. This acute discomfort likely disrupted eating patterns and potentially interfered with embryonic development. Despite its swift absorption profile, the exact mechanisms underlying this phenomenon remain unclear. Previous studies have shown that VPA, when administered intraperitoneally, is swiftly absorbed by rat metabolism, with detectable presence in the brain, liver, and kidney within ten minutes. Within thirty minutes, VPA reaches peak concentrations in these organs, followed by a subsequent decline, with 97% of VPA radioactivity lost within 24 h (Aly and Abdel-Latif, 1980). Although the rapid absorption pattern of VPA is well-documented, the immediate occurrence of abdominal cramps post-injection suggests that VPA might have been present in the intraperitoneal fluid at the time of discomfort. Intraperitoneal saline injection did not elicit any observable reaction.

It is important to note that the gestational period in humans is particularly vulnerable to environmental risk factors, especially during fetal brain development. Insufficient weight gain during gestation increases the risk of developmental delays in communication, motor skills, and social domains in children (Modabbernia et al., 2017).

Animal models exposed to environmental risk factors during prenatal development, such as lipopolysaccharide (LPS), have demonstrated adverse effects on placental weight, embryo count, and subsequent neurodevelopmental abnormalities in postnatal life (Straley et al., 2014). Maternal immune activation, which induces a proinflammatory status during gestation, has been linked to neurodevelopmental abnormalities in offspring (Deckmann et al., 2019; Fontes-Dutra et al., 2020).

An acute high dose of VPA can hyperactivate biological pathways associated with inflammation, potentially contributing to abnormalities

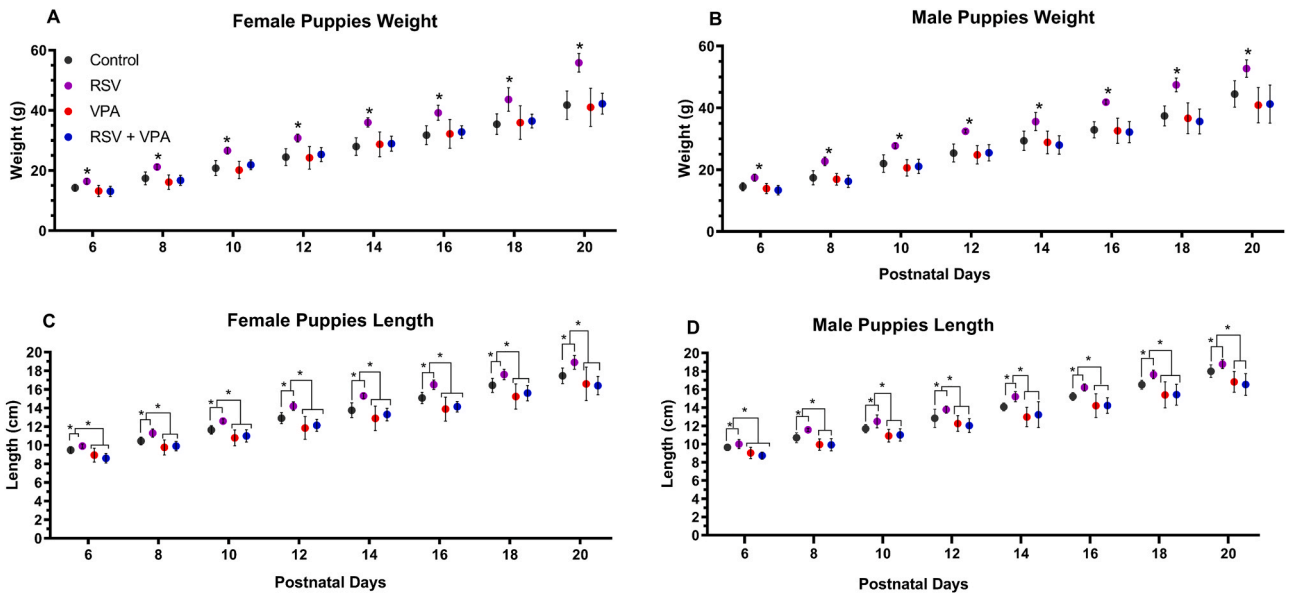

Fig. 4. Weight and length variation of pups during the evaluated period of experimental groups. Weight variation of female (A) and male (B) pups and length measures of females(C) and male (D) pups between postnatal days 6–20. Control = Gray dot (n = 39), RSV = Purple dot (n = 12), VPA = Red dot (n = 40) and RSV+VPA = Blue dot (n = 28). Values are shown as mean ± standard deviation. Statistical analysis: Generalized Estimating Equations (GEE), followed by Bonferroni test. * $p < 0.05$. For additional information, refer to Tables S4–S6 for weight variation and Tables 7, 8 and 9 for length variations.

observed in postnatal life (J.-Y. Huang et al., 2016). Despite the anti-inflammatory effects of VPA at low doses in non-pregnant animals (Amirzargar et al., 2017; Tian et al., 2019), elevated doses like 600 mg/kg, have been reported to induce inflammation in pregnant mice (Lucchina and Depino, 2014). Furthermore, high doses of prenatal VPA exposure in humans are linked to congenital malformations and language impairments, including worse verbal comprehension in children with ASD (Nadebaum et al., 2011; Roullet et al., 2013). Prenatal VPA exposure in humans is also associated with a heightened risk of ASD development in children (Christensen et al., 2013). Moreover, it is noteworthy that children who were exposed to high doses of valproate (>800 mg daily) during pregnancy exhibited a substantial reduction of 9.7 points in their IQ scores compared to those who were not exposed (Baker et al., 2015).

Thus, the correlation between fold-change weight variation during the E15.5-E18.5 period and the reduced number of pups in the VPA group could be related to the high VPA concentration (600 mg/kg) administered. Elevated VPA concentrations can lead to fetal reabsorption and abnormal development, potentially resulting in fewer pups. In an ASD animal model involving pregnant rats exposed to 600 mg/kg VPA, higher than the 500 mg/kg exposure in other models, over 50% of gestations showed complete reabsorption (Favre et al., 2013). Notably, we observed a tail kink, a recurring feature in VPA-exposed offspring, which prenatal RSV treatment was unable to mitigate.

The RSV molecule modulates metabolic mechanisms like mitochondrial biogenesis and activates sirtuin (SIRT) 1, a deacetylase enzyme (Zhao et al., 2020). Previous rodent studies have demonstrated that gestational RSV administration can prevent developmental anomalies in neurodevelopmental disorders models (Chen et al., 2020; Ferreira et al., 2020; Xie et al., 2018; Zhao et al., 2020). Moreover, subcutaneous RSV injection in pregnant ewes increased fetal weight, promoting better fetal oxygenation and growth (Darby et al., 2019). Building on these findings, we postulate that RSV fosters a more favorable gestational environment that aids in recovery from potential insults. The observed increase in fetal weight and length in the RSV group may be attributed to improved uterine conditions supporting enhanced prenatal and postnatal development. Consequently, the weight variation impairments observed in VPA-exposed pregnant rats could be a consequence of an aberrant uterine environment impacting early-life offspring development, akin to the outcomes seen in humans (Mamun et al., 2014; Mayer and Joseph, 2013). The effects of VPA exposure span both early and late postnatal life, with various studies demonstrating social impairments in adulthood (Bambini-Junior et al., 2011; Deckmann et al., 2019; Favre et al., 2013; Schneider and Przewłocki, 2005). Although RSV could not entirely mitigate VPA-induced weight variation and reductions in pup count, it effectively countered developmental hallmark impairments induced by VPA. Notably, RSV facilitated an earlier appearance of certain developmental milestones, while positively influencing weight and length outcomes. This suggests that the RSV effects extend beyond mere prevention, potentially involving enhanced cellular metabolism and tissue development (Vang et al., 2011), as evidenced by increased weight and length compared to the other groups. Prenatal RSV treatment prevented delayed onset of *Eyes Opening*, *Cliff Avoidance*, and *Lower Limb Grip* induced by VPA in males. Although the limbs' development was affected, there was no alteration in the March reflex, indicating that while the motor functional-mechanic works typically, the limbs alterations may be related to sensorimotor integration.

Sensory integration refers to the process of consolidating information from one or multiple sensory domains, channeling these inputs to the brain (Camarata et al., 2020). Eye-tracking assessments in individuals with ASD, including children and infants, have unveiled challenges in maintaining typical eye contact, suggesting that neural networks involved in sensory integration might contribute to atypical behaviors (Black et al., 2017). Reduced responses to vestibulo-ocular stimuli integration are observed in children with ASD and attention-deficit/hyperactive disorder (ADHD) (Carson et al., 2017). This deficit could potentially arrive from delayed visual information processing, rather than an isolated impairment in the vestibular system. A potential repercussion of disturbances in vestibular-visual integration could manifest in the delayed *Eye-Opening* milestone among prenatally VPA-exposed animals. This milestone is pivotal for processing and integrating visual stimuli, potentially affected by disrupted visual wiring patterns that emerge prior to eye opening (Huberman et al., 2008). Therefore, we hypothesize that delays in eye-opening could arise as a consequence of disturbances in vestibular-visual integration, possibly influenced by the early development of visual wiring patterns that occur before eye opening. This perspective suggests a potential interplay

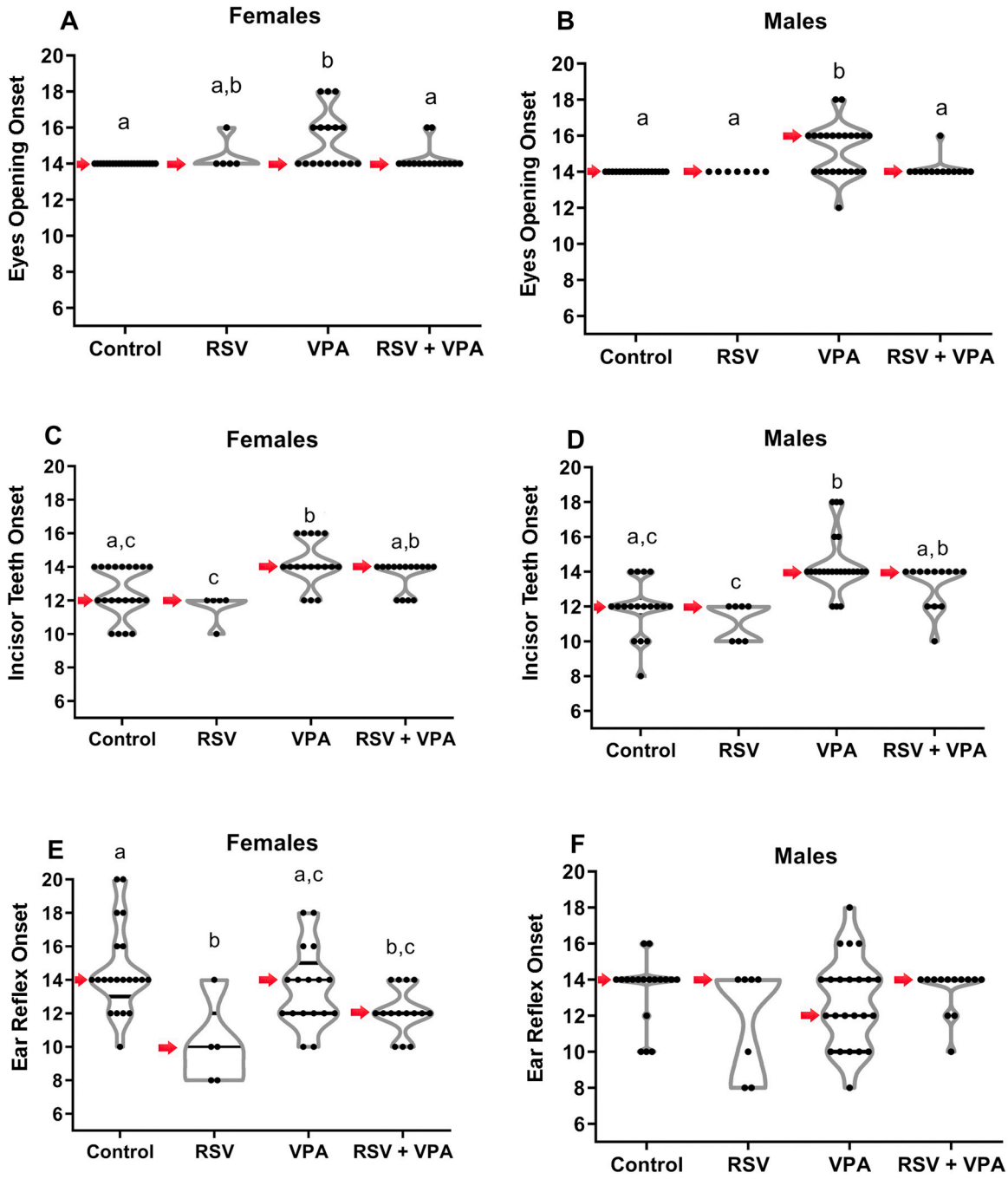

Fig. 5. Onset of hallmarks Eye-Opening, Incisor Teeth Appearance and Ear Reflex. Onset of Eye-Opening in females (A) and in males (B), Incisor Teeth in females (C) and in males (D) and Ear Reflex in females (E) and in males (F). Control (n = 39), RSV (n = 12), VPA (n = 40) and RSV+VPA (n = 28). Horizontal lines represent values, with the median indicated by the thin line and quartiles by the thick lines. The red arrow points to the median (middle quartile). Statistical analysis: two-way ANOVA followed by Tukey test ($p < 0.05$). Different letters indicate significant statistical differences. For additional information, refer to Tables S10 and S11 for females and Tables 12 and 13 for males.

between disruptions in sensory integration and the timing of eye-opening. While our hypothesis leans towards the notion that delays in eye opening are influenced by disturbances in sensory integration, we also recognize the alternate interpretation that disruptions in vestibular-visual integration might contribute to delayed eye opening. This complex relationship underscores the need for further investigation to elucidate the intricate mechanisms that underlie these developmental changes.

Although our study did not uncover alterations in the *Eyelid Reflex* or its neural underpinnings, abnormalities in the integration of sensory-motor domains, reminiscent of ASD patterns, could be present (Wang et al., 2021). Distinct connectivity patterns, particularly in the integration of frontal-posterior inputs have been documented in individuals with ASD (Just et al., 2004, 2012). Studies suggest a spectrum of connectivity alterations in ASD, encompassing under-connectivity, over--connectivity, or both, portraying a diffuse pattern of disrupted connectivity (Cheng et al., 2015; Di Martino et al., 2014).

ASD subjects exhibit difficulty in filtering auditory information from noisy stimuli, rather than a direct auditory impairment (Rotschafer, 2021), similar to the processing of visual stimuli. The delay in *Startle Reflex* response, as well as *Negative Geotaxis*, *Cliff Avoidance* and *Righting*, induced by prenatal exposure to VPA, might partly result from abnormal

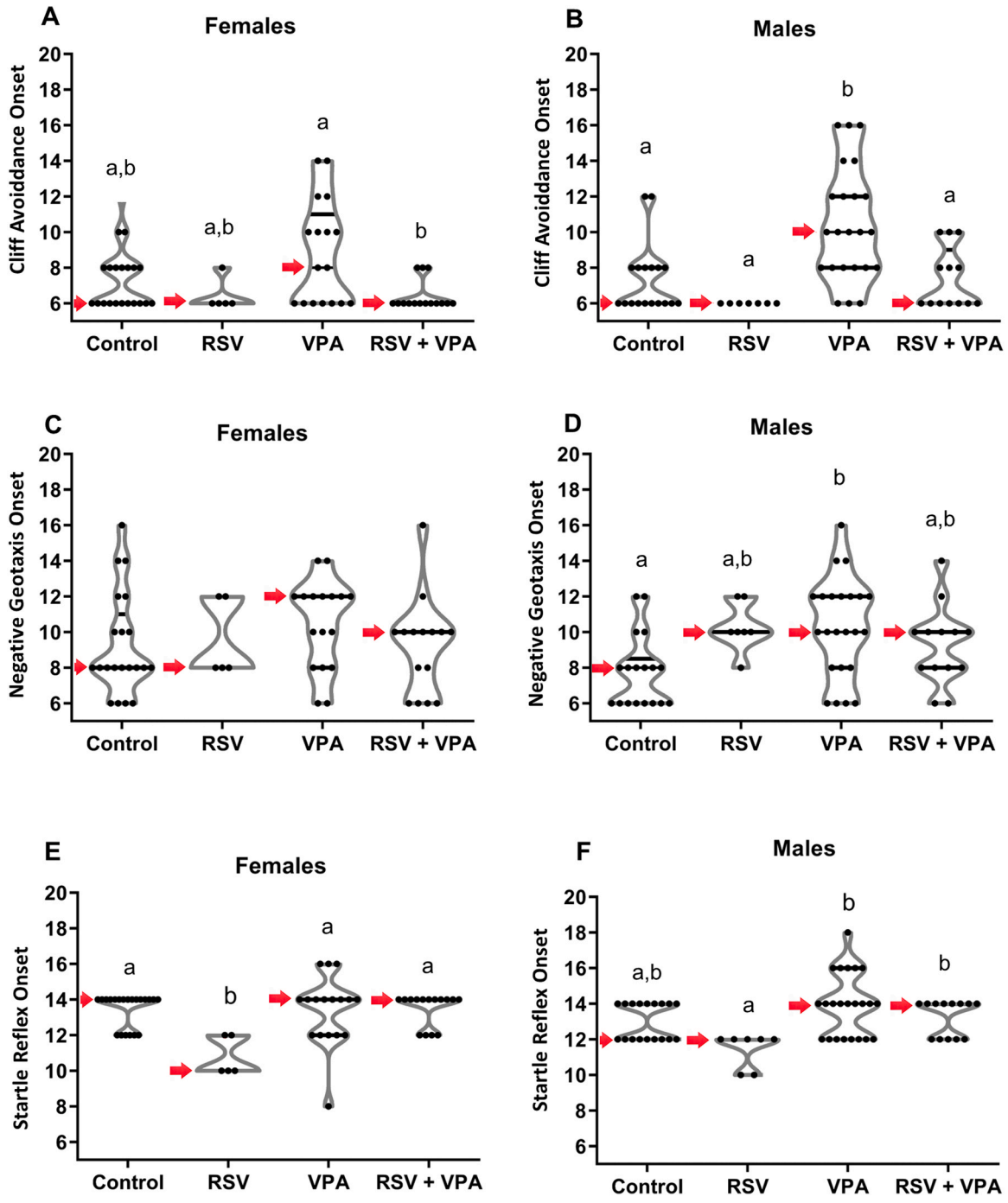

Fig. 6. Onset of hallmarks Cliff Avoidance, Negative Geotaxis and Startle Reflex. Onset of Cliff Avoidance in females (A) and in males (B), Negative Geotaxis in females (C) and in males (D) and Startle Reflex in females (E) and in males (F). Control (n = 39), RSV (n = 12), VPA (n = 40) and RSV+VPA (n = 28). Horizontal lines represent values, with the median indicated by the thin line and quartiles by the thick lines. The red arrow points to the median (middle quartile). Statistical analysis: two-way ANOVA followed by Tukey test ($p < 0.05$). Different letters indicate significant statistical differences. For additional information, refer to Tables S10 and S11 for females and Tables 12 and 13 for males.

processing within the auditory-vestibular axis.

Impairments in the somatosensory system, responsible for processing tactile, proprioceptive, and nociceptive stimuli, are commonly observed in individuals with ASD offering potential diagnostic value (Thye et al., 2018). In the VPA animal model of ASD, somatosensory stimulus processing impairments have been observed, including the whisker nuisance task, and irregular distribution of GABAergic parvalbumin-containing neurons in the primary somatosensory area (Fontes-Dutra et al., 2018). The inhibitory system plays a pivotal role in mitigating sensory information noise, contributing to sensory integration in both primary and secondary somatosensory areas. The maturation of these neurons is also regarded as a marker of early sensory development timing (Reha et al., 2020).

Considering the disrupted interneuron distribution in somatosensory layers and the importance of the secondary somatosensory cortex in functions like tactile discrimination, object manipulation, grasp, and release (Wolff and Shepard, 2013), we posit that compromised somatosensory input integration contributes to the delays observed in accurate *Limb Grip* and *Positioning* in VPA-exposed rats. The literature extensively documents somatosensory-vestibular-visual integration

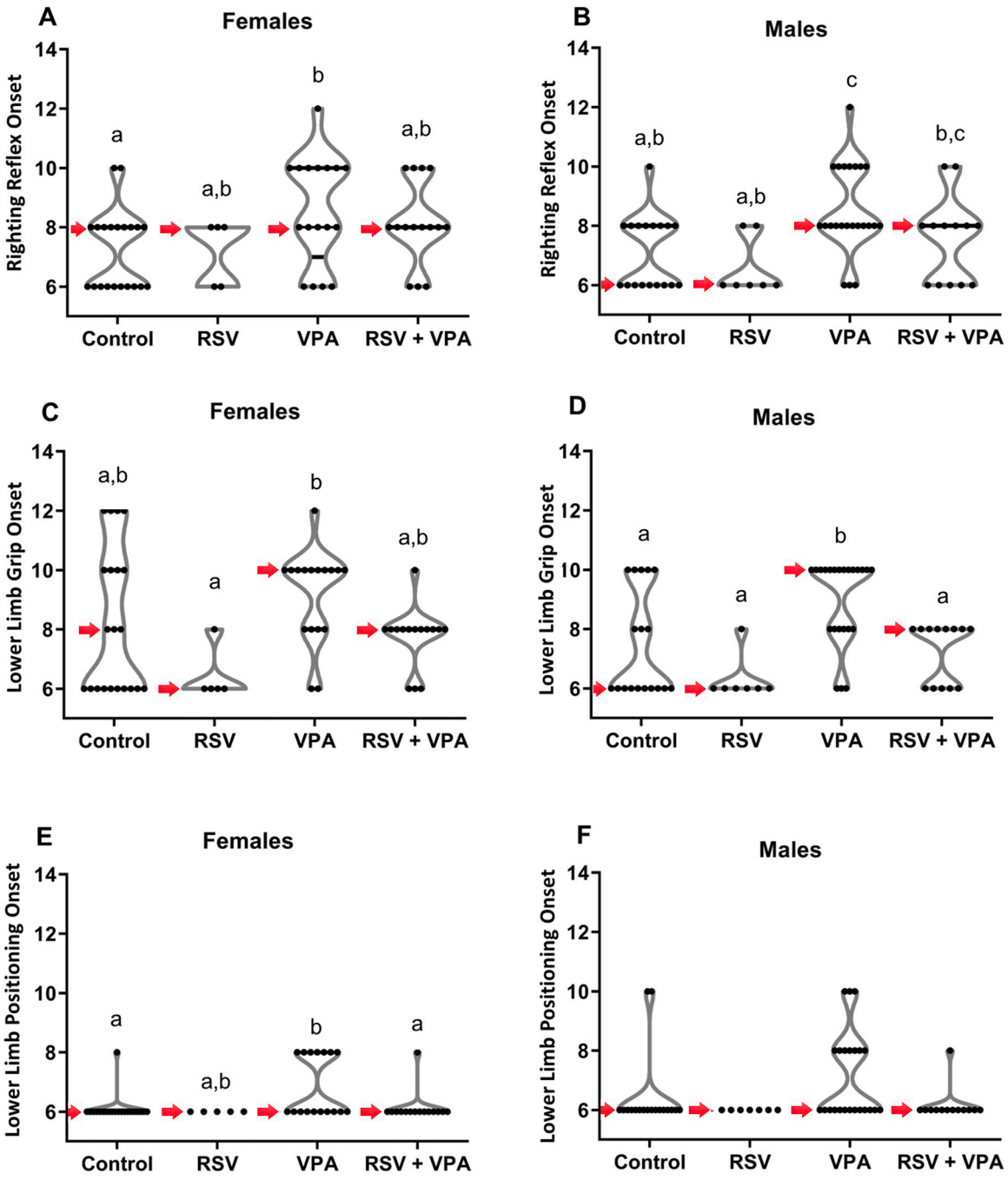

Fig. 7. Onset of hallmarks Righting Reflex, Lower Limb Grip and Lower Limb Positioning. Onset of Righting Reflex in females (A) and in males (B), Lower Limb Grip in females (C) and males (D) and Lower Limb Positioning in females (E) and in males (F). Control (n = 39), RSV (n = 12), VPA (n = 40) and RSV+VPA (n = 28). Horizontal lines represent values, with the median indicated by the thin line and quartiles by the thick lines. The red arrow points to the median (middle quartile). Statistical analysis: two-way ANOVA followed by Tukey test ($p < 0.05$). Different letters indicate significant statistical differences. For additional information, refer to Tables S10 and S11 for females and Tables 12 and 13 for males.

(Cheung and Lau, 2020), and impairment in this complex interplay could directly affect development of behaviors like *Cliff Avoidance*, *Negative Geotaxis*, and *Righting* in VPA-exposed animals.

While our study provides valuable insights into the developmental consequences of prenatal VPA exposure and the potential benefits of RSV intervention, we recognize the critical importance of further literature and clinical evidence to bridge the gap between rat model findings and their potential implications for individuals with ASD. The translation of findings from animal models to clinical contexts requires careful consideration of species differences, as well as the multifaceted nature of ASD.

In summary, our study underscores the harmful consequences of prenatal exposure to VPA on gestational weight variation and pup count in an animal model. The immediate discomfort and altered behavior exhibited in pregnant rats followed by VPA injection further emphasize the potential impact of this teratogen on embryonic development. The association of VPA with inflammatory and immune responses suggests plausible mechanisms behind these outcomes. Additionally, we identified significant deficits in behaviors encompassing both sensory and motor aspects, hinting a potential early impairment in their integration. These findings suggest that early indicators of impaired sensory-motor integration may be useful as predictive markers for future behavioral

impairments in ASD. Our results align with prior research indicating the substantial impact of VPA exposure during early developmental stages in animal models of ASD.

In conclusion, our study provides a stepping stone for understanding the potential impact of prenatal VPA exposure on developmental milestones as well as provides support for the use of RSV as a reliable tool for investigating both the pathophysiology and etiology of ASD.

CRediT authorship contribution statement

Carmem Gottfried: Supervision, Funding acquisition, Project administration, Conceptualization, Data analysis and discussion, Writing – review & editing, Visualization. **Gustavo Brum Schwingel, Mellanie Fontes-Dutra:** Conceptualization, Investigation, Methodology, Data analysis and discussion, Writing – review & editing, Visualization. **Rudimar Riesgo, Victorio Bambini-Junior:** Funding acquisition, Data analysis and discussion, Writing – review & editing, Visualization. **Bárbara Ramos:** Investigation, Methodology, Data analysis and discussion.

Declaration of Competing Interest

The authors report no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2023.09.008.

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