Neuroscience Letters xxx (xxxx) xxx

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Short communication

No effect of prior *Dengue virus* 1 infection in mouse dams on long-term behavioral profiles in offspring infected with *Zika virus* during gestation

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ARTICLE INFO

Keywords:
Zika virus
Dengue virus 1
Development
Behavior
Anxiety
BDNF

ABSTRACT

Zika virus (ZIKV) is a mosquito-borne Flavivirus structurally and antigenically related to *Dengue virus* (DENV). *Zika virus* has been associated with congenital anomalies and most ZIKV outbreaks have occurred in endemic areas of DENV. The present study investigated the effects of prior DENV serotype 1 (DENV1) immunity in immunocompetent female Swiss mice on gestational ZIKV infection in offspring. Physical/reflex development, locomotor activity, anxiety, visual acuity, and brain-derived neurotrophic factor (BDNF) levels were evaluated in offspring during infancy and adolescence. Anti-DENV1 and anti-ZIKV antibodies were detected in sera of the progenitors, whereas no ZIKV genomes were detected in the offspring brain. Pups from dams with only DENV1 immunity presented alterations of physical/reflex development. Pups from all infected dams exhibited time-related impairments in locomotor activity and anxiolytic-like behavior. Offspring from DENV/ZIKV-infected dams exhibited impairments in visual acuity during infancy but not during adolescence, which was consistent with morphometric analysis of the optic nerve. Pups from DENV1-, ZIKV-, and DENV/ZIKV-infected dams exhibited a decrease in BDNF levels during infancy and an increase during adolescence in distinct brain regions. In summary, we found no influence of prior DENV1 immunity on gestational ZIKV infection in offspring, with the exception of alterations of early visual parameters, and an increase in BDNF levels in the hippocampus during adolescence.

1. Introduction

Zika virus (ZIKV) is a mosquito-borne Flavivirus that is closely related to other worldwide arthropod-transmitted human pathogens, such as *Dengue virus* (DENV) [1]. Much of the Brazilian population has already been infected by one or more DENV serotypes. All DENV infections promote long-lasting immunity against the specific serotype and temporary immunity against others [2]. Previous studies have reported immunological cross-reactivity between ZIKV and DENV [3,4].

In 2015, Brazil experienced a ZIKV epidemic in several regions of the country [5]. In adult immunocompetent humans, ZIKV infection usually causes a mild, self-limiting reaction known as Zika fever, and other manifestations, such as Guillain-Barre syndrome [6]. The number of microcephaly cases has increased during ZIKV outbreaks. *Zika virus*--positive particles have been detected in fetal brain tissue [7] and amniotic fluid [8] from ZIKV-infected mothers, providing the first evidence

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https://doi.org/10.1016/j.neulet.2020.135448
Received 19 June 2020; Received in revised form 17 September 2020; Accepted 8 October 2020
Available online 28 October 2020

that this Flavivirus can be vertically transmitted [9]. ZIKV infection during a critical phase of gestational development has been associated with a broad spectrum of congenital anomalies, such as microcephaly [10].

Brain development can be affected by genetic and environmental factors, including viral infections. During this vulnerable period, any disturbances in crucial events can cause irreversible effects on brain structure and function. Growth factors, such as the brain-derived neurotrophic factor (BDNF), play a crucial role during the critical phase of brain development [11]. The BDNF and other neurotrophic factors can be expressed in response to many insults, including viral infection [12].

Despite the structural similarities, the co-circulation of DENV and ZIKV, and the fact that a large portion of the population already has immunity to one or more DENV serotypes, little is known about the consequences of subsequent Flavivirus infections in the offspring. Therefore, the aim of the present study was to evaluate whether prior maternal immunity to DENV1 in mice exacerbates the effects of gestational ZIKV infection in offspring.

2. Material and methods

2.1. Animals

Male and female Swiss mice were obtained from the animal facility of the Federal University of Alfenas. The animals were housed at 20−22 °C under a 12 h/12 h light/dark cycle with water and commercial food pellets *ad libitum*. All of the animal procedures were approved by the Ethics Committee of the Federal University of Alfenas (protocol no. 22/2016) and were in compliance with the Principles of Laboratory Animal Care of the National Institutes of Health.

2.2. Experimental design

A summary of the experimental procedures is shown in Fig. 1. Female Swiss mice were previously infected with a single intravenous injection of 1×10^5 plaque forming units (pfu) of DENV1 and then were mated with male DENV1-naive Swiss mice. Pregnant female mice were then infected with a single intravenous injection of 1×10^5 pfu of ZIKV on gestational day 7 (GD7) to GD10. Control groups were exposed to the same experimental conditions but received only sterile saline. Thus, four experimental groups were evaluated: SALINE/SALINE, SALINE/ZIKV, DENV/SALINE, and DENV/ZIKV. The day of birth of the offspring was considered postnatal day 0 (PD0). All litters were culled to have an equal number of pups (4 males and 4 females). The culled pups were euthanized by cervical dislocation, and their brains were stored for ZIKV detection by real-time quantitative polymerase chain reaction (RT-qPCR). Physical/reflex measurements were performed between PD5 and PD41 (S1 Table), and the day of detection of each parameter was recorded [13]. For the behavioral tests, the same 88 animals (n = 11/sex/group) were used for the assessments on PD18-20 (infancy) and PD40-42 (adolescence). Different groups of pups (40 animals; n = 5/group/age) were randomly assigned to measurements of BDNF levels in different brain areas and the optic nerve analysis. The assessment of physical and reflex development, locomotor activity and anxiety-like behavior were described in the Supplementary Material (S1 File).

2.3. Viral titration

The ZIKV sample was isolated from a symptomatic patient from Recife/Pernambuco, Brazil, and provided by Dr. Luiz Tadeu Moraes Figueiredo, University of São Paulo. The DENV sample (Mochizuki strain) was obtained from the Vaccines Laboratory bank of microorganisms of the Federal University of Alfenas [14]. Both viruses amplification and titration were performed as previously described [15].

2.4. Anti-DENV1 and anti-ZIKV antibodies in adult female mice and ZIKV detection in the pups brain

Serum samples were collected fifteen days after DENV1 infection, to confirm the presence of anti-DENV1 antibodies (n = 8/group) and after the weaning period to confirm the presence of anti-ZIKV antibodies (n = 4/group) by ELISA, as previously described [16].

Total RNA was extracted from newborn mouse brain tissue using Trizol reagent. The RT-qPCR protocols were performed as previously described [17].

2.5. Visual acuity

The visual acuity were evaluated in the Morris water maze (n = 11/sex/group), as previously described [18]. A visible circular platform (9 cm diameter) was placed 1 cm below the water surface, and an object was positioned on the platform as a proximity cue of its location. The animal's starting position in the maze and visible platform position were randomly changed in each trial (4 trials/animal). A digital camera recorded each trial, and EthoVision 3.1 software (Noldus, The Netherlands) was used to quantify the latency to find the platform.

2.6. Optic nerve analysis

The myelination percentage (number of myelinated fibers in relation to the total number of fibers in the cross section), the absolute cross section area of optic nerves and the ratio between the myelinated fibers and the total nerve cross section area were assessed (n = 5/group/age). The myelinated axons were manually counted, considering Toluidine blue staining around the complete circumference of the fiber. For the cross section area, the nerve circumference was delimited and the total area was calculated (in μm^2), according to the reference scale bar [19]. All the quantifications were performed using ImageJ® by researchers blind to the experimental conditions.

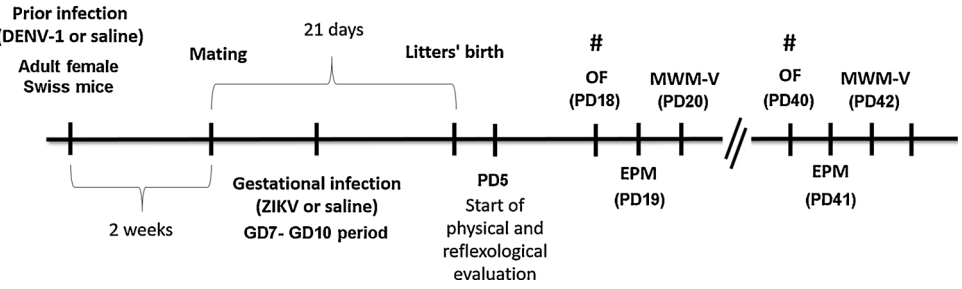

Fig. 1. Experimental design. Adult female Swiss mice were previously injected with DENV1 or saline. An injection of ZIKV or saline was given on GD7-GD10. From PD5 to PD41, physical/reflex parameters were evaluated. Behavioral analyses were performed during infancy (PD18, PD19, PD20) and adolescence (PD40, PD41, PD42). Open field (OF), elevated plus maze (EPM), and Morris water maze (MWM-V). #BDNF and the optic nerve analysis.

2.7. Quantification of BDNF

Tissue levels of BDNF were measured using a commercial kit (Promega, Madison, WI, USA; $n = 5$/group/age), according to the manufacturer's instructions [20].

2.8. Statistical analysis

In the ELISAs, the groups were compared using nonparametric Mann-Whitney *t*-tests and nonparametric Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's multiple-comparison *post hoc* test. The physical/reflex development data were analyzed using one-way ANOVA, followed by Fisher's *post hoc* test. The body weight/length data and behavioral data were compared among groups using repeated-measures ANOVA followed by the Student-Newman-Keuls *post hoc* test. BDNF levels and optic nerve parameters were analyzed using one-way ANOVA followed by the Student-Newman-Keuls *post hoc* test. The data are expressed as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. The S2 Table shows a detailed description of the statistical analysis from all results.

3. Results

3.1. DENV1 and ZIKV immunity in progenitor female mice

Data from the ELISAs showed that DENV1 infection generated anti-DENV1 IgG antibodies in females before ZIKV infection ($p < 0.01$; Fig. 2A). The presence of anti-ZIKV antibodies was also evaluated in the pregnant females (Fig. 2B). All mice in the DENV/ZIKV group were positive for anti-ZIKV antibodies ($p < 0.01$, compared with SALINE/SALINE group), and 50% of mice that were previously injected with saline and then infected with ZIKV during the gestational period were positive for anti-ZIKV antibodies. No anti-ZIKV antibodies were detected in the SALINE/SALINE and DENV/SALINE groups. No significant amounts of circulating anti-DENV1 IgM or anti-ZIKV IgM antibodies were detected in serum in females 14 days after prior DENV1 infection or after the weaning period (data not shown).

3.2. Visual acuity and optic nerve analysis

Fig. 3 shows the results of visual acuity in the Morris water maze and the results of the optic nerve morphometric analysis. We detected a significant effect of age and a significant interaction between treatment and age in visual acuity outcomes (S2 Table). During infancy, pups in the DENV/ZIKV group exhibited an increase in the latency to find the visible platform compared with the SALINE/SALINE group ($p < 0.05$; Fig. 3A). During adolescence, no significant differences in latency were observed (Fig. 3B).

For the optic nerve morphometric analysis, during infancy the DENV/ZIKV group exhibited an increase in the total area of the optic nerve compared with the SALINE/SALINE group ($p < 0.05$; Fig. 3E). During adolescence, the SALINE/ZIKV ($p < 0.05$), DENV/SALINE ($p < 0.001$), and DENV/ZIKV ($p < 0.001$) groups exhibited an increase in the percentage of myelinated fibers compared with the SALINE/SALINE group (Fig. 3D). The DENV/SALINE ($p < 0.05$) and DENV/ZIKV ($p < 0.05$) groups exhibited an increase in the percentage of myelinated fibers compared with the SALINE/ZIKV group (Fig. 3D). The DENV/ZIKV group exhibited an increase in the total area of the optic nerve compared with all of the other groups ($p < 0.05$; Fig. 3F). No significant effect of treatment was observed for the ratio between the myelinated fibers and the total nerve cross section area during infancy (Fig. 3G) and adolescence (Fig. 3H).

3.3. BDNF levels

During infancy, the SALINE/ZIKV ($p < 0.01$), DENV/SALINE ($p < 0.01$), and DENV/ZIKV ($p < 0.05$) groups exhibited decreased BDNF levels in the cerebellum compared with the SALINE/SALINE group (Fig. 4A). The DENV/ZIKV group exhibited increased BDNF levels in the cerebellum compared with the DENV/SALINE group ($p < 0.05$). The SALINE/ZIKV and DENV/ZIKV groups exhibited decreased BDNF levels in the striatum compared with the SALINE/SALINE group ($p < 0.05$; Fig. 4C). The DENV/ZIKV group exhibited decreased BDNF levels in the striatum compared with the DENV/SALINE group ($p < 0.05$; Fig. 4C). The DENV/SALINE and DENV/ZIKV groups exhibited decreased BDNF levels in the hippocampus compared with the SALINE/SALINE group ($p < 0.05$; Fig. 4E).

During adolescence, the DENV/SALINE group exhibited increased BDNF levels in the cerebellum compared with the other groups ($p < 0.05$; Fig. 4B). All of the infected groups exhibited increased BDNF levels in the striatum compared with the SALINE/SALINE group ($p < 0.01$; Fig. 4D). The SALINE/ZIKV ($p < 0.05$), DENV/SALINE ($p < 0.05$), and DENV/ZIKV ($p < 0.001$) groups exhibited increased BDNF levels in the hippocampus compared with the SALINE/SALINE group (Fig. 4F). The DENV/ZIKV group exhibited increased BDNF levels in the hippocampus compared with the SALINE/ZIKV and DENV/SALINE ($p < 0.01$).

The data obtained for physical and reflex development, locomotor activity, anxiety-like behavior and the histological sections of the optic nerve were presented in the Supplementary Material (S1 File).

4. Discussion

The co-circulation of DENV and ZIKV has been reported in almost all states in Brazil [3]. The main targets of the humoral response in DENV infections have considerable amino acid sequence identity compared with ZIKV [21]. Several studies have been showed that the main target of antibodies to DENV and ZIKV is the structural envelope (E) protein. The amino acid sequence of E protein had high similarity among the four

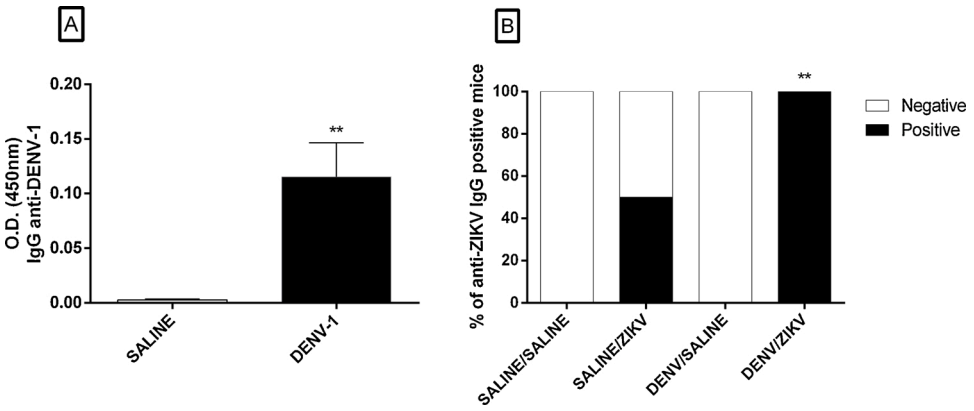

Fig. 2. DENV and ZIKV antibody detection in mice. (A) Anti-DENV1 IgG antibody detection 15 days after DENV1 infection in adult female mice ($n = 8$ females/group). The data are expressed in optical density (mean ± SEM). (B) Anti-ZIKV IgG antibody detection after the weaning period in adult female mice ($n = 4$ females/group). The data are expressed as a percentage of positive and negative results for anti-ZIKV antibody detection. $^{**}p < 0.01$, compared with SALINE or SALINE/SALINE groups.

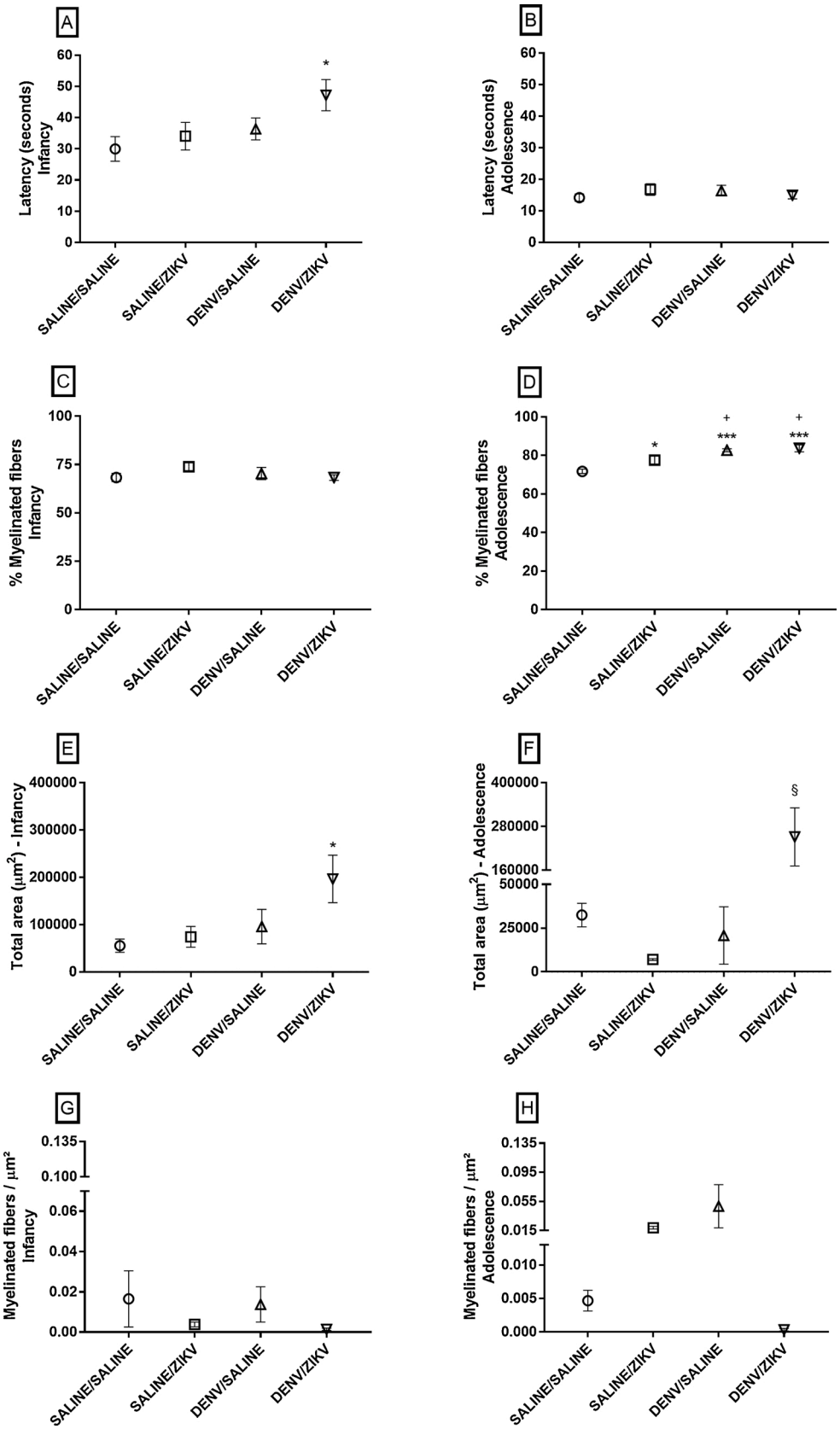

Fig. 3. Latency to find the visible platform in the Morris water maze test during infancy (A) and adolescence (B; $n = 22$/group). Percentage of myelinated fibers during infancy (C) and adolescence (D), total area of the optic nerve during infancy (E) and adolescence (F), and the ratio between the myelinated fibers and the total nerve cross section area during infancy (G) and adolescence (H; $n = 5$/group). The data are expressed as seconds, percentage or area (mean ± SEM). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with SALINE/SALINE group; $^{+}p < 0.05$ compared with SALINE/ZIKV group; $^{\S}p < 0.05$, compared with all other groups.

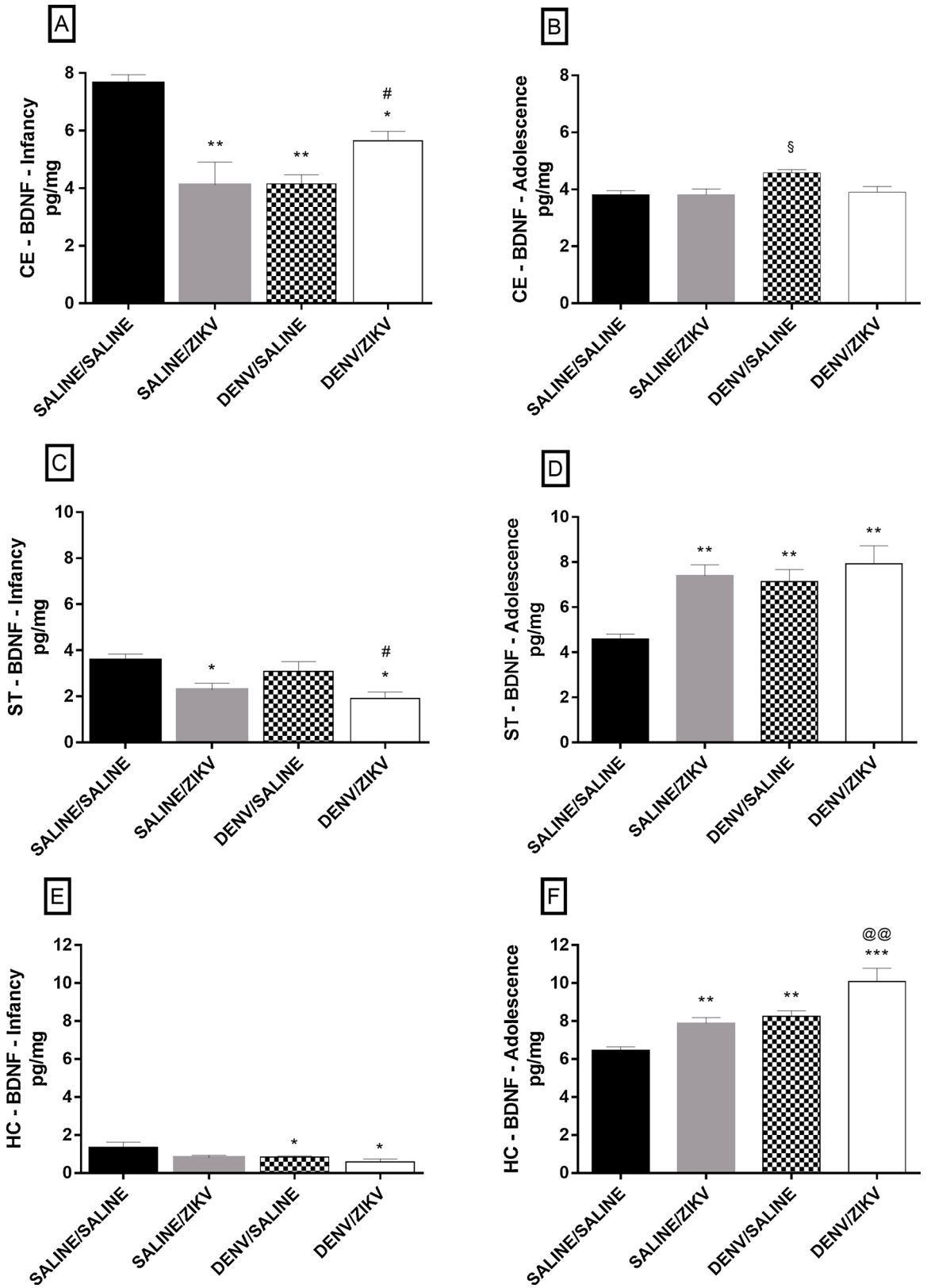

Fig. 4. BDNF levels in the cerebellum (CE), striatum (ST), and hippocampus (HC) during infancy (A, C, E) and adolescence (B, D, F) ($n = 5$/group). The data are expressed as pg/mg tissue (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with SALINE/SALINE group; $^{\#}p < 0.05$, compared with DENV/SALINE group; $^{@@}p < 0.01$, compared with SALINE/ZIKV and DENV/SALINE groups; $^{\S}p < 0.05$, compared with all other groups.

DENV serotypes (60–75 %) and among DENV serotypes and ZIKV (54–59 %) [4,22]. In addition, the T cell response after ZIKV and DENV infection is characterized by T cells able to recognize nonstructural proteins, being these proteins more conserved between among flaviviruses [23]. This structural and genetic relationship between ZIKV and all DENV serotypes supports the hypothesis of antibody cross-reactivity between these two viruses, a characteristic that can complicate the accurate diagnosis of patients and influence the protective and pathological immune responses to these infections.

Although devastating effects of ZIKV infection have rapidly emerged [15,24], few studies have evaluated the effects of prior DENV1 infection on gestational ZIKV infection. The present study used an immunocompetent animal model and found that prior DENV1 infection did not influence the effects of gestational ZIKV infection, with the exception of visual acuity during infancy, and BDNF levels in the hippocampus during adolescence. Pups from ZIKV-infected dams, independent of prior DENV1 immunity, exhibited alterations of physical and reflex parameters, impairments in locomotor activity, and anxiolytic-like behavior. Most of these effects were observed mainly during infancy, suggesting that BDNF may play a preventive role against these alterations during mouse development.

The ELISA results indicated that progenitor females that were infected with ZIKV during the gestational period, in the context of prior DENV1 immunity, had high levels of anti-ZIKV and anti-DENV1 IgG antibodies, suggesting cross-reactive immunity between these two viruses. This increase could be due to the activation of cross-reactive memory B cells produced after DENV1 infection during the ZIKV gestational infection [25]. DENV1 serum antibodies from female progenitors that were previously infected were able to recognize and bind ZIKV epitopes, resulting in the higher detection of anti-ZIKV antibodies.

A clinical study have reported persistent ZIKV infection in the brain [24]. In the present study, RT-qPCR did not detect ZIKV particles in pups' brain. This finding may be related to the small viral titers in tissue and the age of the pups during the analysis. Other researchers also reported difficulties in detecting ZIKV particles in fetuses using various methods, including RT-qPCR [26,27].

Despite the absence of ZIKV particles in the brain, female pups in the DENV/ZIKV group exhibited alterations of physical and reflex parameters. However, the most pronounced effects were observed in the DENV/SALINE group, suggesting that these effects may occur by maternal passive immunization or through inflammatory responses that are triggered by the infection. In fact, both anti-DENV and anti-ZIKV antibodies are able to cross the placental barrier [28,29]. Barth et al. [30] showed that DENV infection led to transient inflammatory responses that were detected from day 2 to 49 post-infection. Thus, the previous immune response to DENV1 in female progenitors may have been related to inflammatory mediators that affected normal development of the pups and contributed to post-ZIKV effects.

In this study, all of the infected groups exhibited motor impairment during infancy but not during adolescence, with the exception of female SALINE/ZIKV pups. Consistent with our results, Cui et al. reported that congenitally ZIKV-infected mice displayed motor incoordination [31]. In fact, a clinical case report has described motor dysfunction in neonates with congenital ZIKV infection, including arthrogryposis, a fetal malformation that reduces joint movement [32]. Our results also showed a decrease in anxiety-like behavior in all of the infected groups during infancy. Interestingly, during adolescence, only the isolated infections led to anxiolytic behavior, whereas the DENV/ZIKV group exhibited anxiety-like behavior that was similar to the SALINE/SALINE control group. The test of grooming behavior confirmed these data. A decrease in anxiety-like behavior is associated with higher risk exposure. In fact, studies have shown that parasites, such as *Toxoplasma gondii*, cause rodents to increase risk-taking behavior, thereby facilitating transmission to their primary hosts [33].

We also found that only DENV/ZIKV offspring exhibited visual impairments during infancy. These data were confirmed by the delay in visual orientation that was previously observed in these same pups. Consistent with our data, a preclinical study also reported visual acuity impairment, reflected by the absence of lamination and a decrease in retina thickness in adolescent mice that were infected with ZIKV during the gestational period [31]. Case reports have also described vision problems in neonates, even in the absence of microcephaly, in regions with a high incidence of ZIKV infection [34].

Our behavioral results were consistent with optic nerve myelination, which is related to visual acuity [35]. All of the infected pups exhibited an increase in myelination during adolescence, and the DENV/ZIKV group had the largest total area of the optic nerve, which may explain the absence of previously observed visual impairments in DENV/ZIKV animals during infancy, suggesting that early myelination was not sufficient to involve the entire optic nerve area in DENV/ZIKV animals. Age-dependent improvements in visual acuity in the DENV/ZIKV group may be related to higher BDNF levels during adolescence. Lower BDNF levels have been reported to induce optic nerve hypomyelination [36], whereas BDNF overexpression accelerates development of the visual system [37].

High BDNF levels in the cerebellum, striatum, and hippocampus are related to improvements in motor coordination [38] and an anxiolytic effect [39]. Moreover, mice that were infected with the Flavivirus *Japanese encephalitis virus*, exhibited a significant increase in BDNF levels in the brain, which appears to reflect a neuroprotective response [12]. In the present study, Flavivirus-exposed pups exhibited a decrease in locomotor activity during infancy and anxiolytic behavior during both infancy and adolescence. These results were consistent with BDNF levels.

In summary, the present study found that prior DENV1 infection did not influence the effects of subsequent gestational ZIKV infection, with the exception of visual acuity during infancy, and BDNF levels in the hippocampus during adolescence. Pups from DENV1-, ZIKV-, and DENV/ZIKV-infected dams exhibited alterations of physical and reflex parameters, lower locomotor activity, anxiolytic behavior, and visual impairments, mainly during infancy. Our results suggest that BDNF levels might be involved in recovery from the effects of both infections during adolescence. Additionally, due to the high similarity between the DENV serotypes and ZIKV, we cannot rule out that the same results could be obtained using mice previously infected with DENV2, 3 and 4. However, further studies are needed to better characterize the effect of a previous DENV immunity induced by other serotypes on gestational ZIKV infection in the offspring.

CRediT authorship contribution statement

Karla Cristinne Mancini Costa: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Investigation, Visualization. **Gustavo Andrade Brancaglion:** Investigation. **Carolina Aparecida de Faria Almeida:** Investigation. **Gabriel Estevam Santos de Amorim:** Investigation. **Luciana Lopes Veloso:** Investigation. **Lucas da Silva Lião:** Investigation. **Gabriel Augusto Pires de Souza:** Investigation. **Bruna Pereira Pinheiro:** Investigation. **Marilene Lopes Ângelo:** Investigation. **Silvia Graciela Ruginsk:** Writing - original draft, Writing - review & editing, Supervision. **Wesley Nogueira Brandão:** Investigation. **Tania Marcourakis:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing. **Carla Speroni Ceron:** Writing - original draft, Writing - review & editing. **Luiz Felipe Leomil Coelho:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Larissa Helena Torres:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the support of Luiz Tadeu Moraes Figueiredo, Rosana Camarini, Ana Carolina Cardoso dos Santos Durão, Lidia Emmanuela Wiazowski Spelta and Pedro Henrique Flamini.

Funding

This work was supported by the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES – Finance Code 001), National Counsel of Technological and Scientific Development (National Council for Scientific and Technological Development (CNPq) - grant 405353/2016-2) and Minas Gerais Research Funding Foundation (FAPEMIG) (grants APQ-01165–16 and PPM-00399–18). TM and LFLC are research fellows of CNPq.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2020.135448.

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