Central nervous system infection following vertical transmission of Coxsackievirus B4 in mice

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<u>***Running title***</u>*: Coxsackievirus B4 infection of the foetal CNS*

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Abstract:

Coxsackie B viruses (CV-B) are important pathogens associated with several central nervous system (CNS) disorders. CV-B are mainly transmitted by the faecal-oral route, but there is also evidence for vertical transmission. The outcome of *in utero* CV-B infections on offspring's CNS is poorly explored. The aim of this study was to investigate vertical transmission of CV-B to the CNS. For this purpose, pregnant *Swiss albino* mice were intraperitoneally inoculated with CV-B4 E2 at gestational days 10G or 17G. Different CNS compartments were collected and analyzed for virus infection and histopathological changes. Using plaque assays, we demonstrated CV-B4 E2 vertical transmission to offspring's CNS. Viral RNA persisted in the CNS up to 60 days after birth, as evidenced by a sensitive semi-nested(sn) reverse transcripton(RT)-PCR method. This was despite infectious particles becoming undetectable at later time points. Persistence was associated with inflammatory lesions, lymphocyte infiltration and viral dsRNA detected by immunohistochemistry. Offspring born to dams mock- or virus-infected at day 17G were challenged by the same virus at day 21 after birth (-+ and ++ groups, respectively). Sn-RT-PCR and histology results compared between both ++ and -+ groups, show that *in utero* infection did not enhance CNS infection during challenge of the offspring with the same virus.

Key words: Type B Coxsackieviruses (CV-B); Vertical transmission; Central nervous system (CNS); Persistence;

Introduction

Type B Coxsackieviruses (CV-B) are small non-enveloped positive-sense single stranded RNA viruses, belonging to the species *Enterovirus B*, from the *Enterovirus* genus of the *Picornaviridae* family (Adams et al. 2013; Knowles et al. 2012). They encompass 6 serotypes (CV-B1 to 6) that have been distinguished from CV-A on the basis of the pathology induced when inoculated to newborn mice (Crowell & Landau, 1997).

Coxsackie B viruses can cause a wide range of human diseases such as myocarditis (Klingel et al. 1992), pancreatitis (Ramsingh, 2008), or chronic inflammatory myopathy (Tam & Messner, 1999). They are also known to be responsible for numerous central nervous system (CNS) clinical manifestations, especially among newborns and children. CV-B infections mainly cause meningitis and encephalitis in infants (Kamei et al. 1990; Kumar et al. 2012; Michos et al. 2007) but have been also linked to other neurological manifestations such as limb paralysis (Yui & Gledhill, 1991), acute transverse myelitis (Minami et al. 2004), and encephalitis lethargica (Cree et al. 2003). Several studies have reported that CV-B infections during childhood increase the risk of the later development of some neuropathologies like schizophrenia (Rantakallio et al. 1997) and amyotrophic lateral sclerosis (Woodall, 2004).

Coxsackie B viruses are generally transmitted by faecal-oral contamination or by respiratory droplets (Muehlenbacks et al. 2015) but they can be also vertically transmitted (Bendig et al. 2003; Evans & Brown, 1963; Kaplan et al. 1983; Modlin & Rotbart 1997; Ouellet et al. 2004). CV-B infections during pregnancy have been well described (Bendig et al. 2003; Euscher et al. 2001; Ornoy & Tenenbaum, 2006). Foetuses and neonates are susceptible to CV-B infections, where their CNS is frequently targeted (Hunt et al. 2012). Such infections can cause foetal damage leading to abortions and stillbirth (Axelsson et al. 1993; Frisk & Diderholm, 1992; Hunt et al. 2012; Wright et al. 1963) or severe neonatal morbidity (Euscher et al. 2001; Ouellet et al. 2004; Satosar et al. 2004).

The association between congenital CV-B infections and CNS complications is well described. A study conducted by Kibrick (1961) showed that 76% of newborns with CV-B infections exhibited CNS lesions. Euscher *et al.* (2001) have demonstrated the placental transmission of CV-B during pregnancy and that such infections can lead to severe CNS sequels, with neurodevelopmental abnormalities and mental retardation. In addition, Konstantinidou et *al.* (2007) have described a case of foetal morbidity following intrauterine transplacental transmission of CV-B3. Histological analysis showed villitis, interstitial pneumonitis and necrotizing meningoencephalitis.

Despite the numerous clinical studies describing CV-B infections in pregnancy where complications lead to foetal or neonatal death or live birth with serious abnormalities, to date, only few experimental studies have been carried out in mouse models to investigate the pathogenic mechanisms of CV-B infection during pregnancy (Bopegamage et al. 2012; Hwang et al. 2014; Marosova et al. 2011; Modlin & Crumpacker, 1982).

The studies mentioned above did not thoroughly explore the effects of CV-B vertical transmission on the CNS. The current study was undertaken to investigate the effect of an *in utero* CV-B infection on offspring's CNS, and the ability of such an infection to enhance viral persistence and infection following subsequent challenge of young offspring with the same virus.

Materials and methods

Virus and cell line. Virus stock: the CV-B4 E2 strain used in these experiments (isolated from the pancreas of a ten years old boy died from diabetic ketoacidiosis (Yoon et al. 1979), and kindly provided by J.W. Yoon, Julia MC Farlane Diabetes research center, Calagary, Alberta, Canada), was propagated in HEp-2 cells (BioWhittaker) in Eagle's Minimal Essential Medium (MEM; Gibco BRL) supplemented with 10% FBS, 1% L-Glutamine, 50 μg streptomycin ml-1, 50 U penicillin ml-1. Supernatants were collected three days post-inoculation (p.i.) and then clarified at 2000 g for 10 min. Virus titre was determined as $TCID_{50}$ on HEp-2 cells by the method of Reed and Muench (1938) and stored in aliquots at -80° C until required.

Mice. Adult *Swiss albino* mice (purchased from Pasteur institute, Tunis, Tunisia) were handled in accordance with the standards of general ethics guidelines and used for all experiments. For planned gestation, three females per male were caged under specific pathogen-free conditions with unlimited access to food and water. Successful fertilization was checked by vaginal plugs formation which corresponds to the first day of pregnancy.

Virus inoculation of timed-pregnant mice and pups. Pregnant mice were inoculated intraperitoneally at two different time points, either at day 10 or 17 of gestation (day 10G or 17G), with 200 µl of CV-B4 E2 at a dose of 1.4×10^5 p.f.u. Control animals were inoculated with 200 µl of sterile MEM.

For each experimental condition, six mice (three offspring born to each of two dams) were sacrificed (using isoflurane) at different p.i. times. Brain, cerebellum, brain stem and spinal cord were removed and rinsed with PBS. One part was snap frozen at -80°C for virus titration and molecular biology assays, and the other one was fixed in 4% formalin for histological analysis.

To study the effect of an infection during pregnancy on a subsequent challenge of the young offspring, pups from dams inoculated at day 17G were separated from their mothers 3 weeks after birth (day 21), and four groups of pups were formed (Fig. 1): A first group of pups born to infected mothers and intraperitoneally challenged with 200 µl of CV-B4 E2 at a dose of 1.4×10^5 p.f.u. three weeks after birth (at day 21) (group ++). A second group of pups born to mock-infected mothers and intraperitoneally challenged with the same virus dose at the age of 21 days (group -+). A third group of pups born to infected dams and that remained unchallenged (group +-). Finally, a fourth group containing pups born to mock-infected mothers and that remained unchallenged (group --). The same procedure of sampling and analysis was followed for those four groups and results were compared, especially between ++ and -+ groups.

Virus titration by plaque assay. Snap frozen tissues were weighed and crushed using a tissue ruptor (Qiagen) in 1% penicillin/streptomycin PBS, and then centrifuged at 2000 g for 10 min at 4°C. Supernatants were diluted 10-fold in MEM with 2% FCS, then inoculated onto confluent HEp-2 cells (3×10^6 cells/well) in 6-well culture plates. After 4 hours of incubation, medium containing virus dilutions was removed, cells were

washed with PBS, 3 ml of medium (9 vol) supplemented with 4% agarose solution (1 vol) were then added to each well. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 and examined daily for CV-B4 cytopathic effect up to 7 days p.i.. Cells were then fixed with 1 ml of 10% formaldehyde and stained with crystal violet for 30 min. Finally, wells were rinsed with water and plaques were counted for the highest dilution showing cytopathic effect. Results were expressed as means of p.f.u. mg^{-1} of tissue +SDs.

RNA extraction and semi-nested-RT-PCR analysis. Total RNA extraction from snap-frozen tissues was performed using Tri-reagent (Sigma) as described previously (Chomczynski & Sacchi, 1987). Extracted RNA was subsequently dissolved in 50 µl of nuclease-free water (Promega), quantified with the Quant-iT Ribogreen RNA assay kit (Molecular probes, Invitrogen) according to manufacturer's instructions, and diluted to be used in reverse transcription (RT)-PCR assays. Genome amplification was performed using a single tube method with Superscript[TM] One Step RT-PCR with Platinium Taq Kit (Invitrogen), as previously described (Jaïdane et al. 2006). Primer sense EV1 (5'-CAAGCACTTCTGTTTCCCCGG-3') and antisense EV2 (5'-ATTGTCACCATAAGCAGCCA-3') were selected within the 5' NTR of the enteroviral genome generating a 435 bp fragment (Leparc et al. 1994). The reaction was performed in a total volume of 50 µl containing 10 µl (100 ng) of RNA, 0.4 µM each EV1 and EV2 primers, 0.2 mM each dNTP, 1.2 mM $MgSO_4$, and 1 µl of reverse transcriptase enzyme/Platinum Taq mix. Samples were subjected to a first step of reverse transcription for 30 min at 50°C, followed by 2 min of denaturation at 94°C; 40 cycles consisting of denaturation for 30 s at 94°C, annealing for 45 s at 55°C, and extension for 45 s at 72°C, and then a final extension step for 10 min at 72°C. Samples revealing negative, we attempted to increase the sensitivity of our approach by submitting RT-PCR products to a second round of amplification through semi-nested(sn)-PCR, in which primer sense EV1 was used with internal primer antisense EV3 (5'-

CTTGCGCGTTACGAC-3$^{'}$) and the Jump Start Accu Taq LA DNA Polymerase Mix (Sigma), to generate a 362 bp fragment within the 5' NTR, as described previously (Jaïdane et al. 2012). The reaction was carried out with 5 µl of amplified DNA samples and 0.4 µM each primers EV1 and EV3 in a total volume of 50 µl containing 1U of JumpStart Accu Taq LA DNA polymerase, 0.2 mM each dNTP, 2.5 mM MgCl$_2$, 5 mM Tris-HCl, 15 mM ammonium sulfate (pH 9.3), and 1% Tween 20. Samples were subjected to 3 min of denaturation at 94°C, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 30 s at 72°C, followed by a final extension step for 7 min at 72°C. RNA extracted from CV-B4 E2-infected HEp-2 cells was reverse transcribed and amplified according to the procedure described above, and served as a positive control. A negative control (no RNA) was also included in each reaction. All reactions were performed by using a preheated thermocycler (Mastercycler Gradient Eppendorf). Amplification results were revealed by using the Gel Doc 2000 system (Bio-Rad) following a "DNA grade" (Type 5, Euromedex) agarose gel electrophoresis. A 100-bp DNA ladder (Invitrogen) was used as a molecular mass marker. For samples that were negative, beta-actin mRNA was amplified as described above and used as an internal control to ensure the integrity of extracted RNA and the absence of RT-PCR inhibitors.

Histological analysis. Formalin-fixed, paraffin-embedded tissues were cut into 4 µm thick sections. The slices were deparaffinised twice in xylene for 10 min, then rehydrated through sequential concentrations of alcohol (100, 95, 70 and 50°) and finally with double distilled water. Sections were then stained with haematoxylin for 15 min, washed in running water, differentiated with 1% HCl/ethanol and incubated in water for 5 min. For cytoplasm staining, sections were then immersed in 0.5 % eosin solution for 3 min and washed in water for 10 min .Sections were

dehydrated again through sequential concentrations of alcohol (50, 70, 95 and 100°). Finally, slides were mounted and observed under a light microscope.

Immunohistochemistry (IHC). The detection of viral dsRNA in formalin-fixed, paraffin-embedded CNS tissue sections was performed using a standard immunoperoxidase approach, as described previously (Richardson et al. 2010). Briefly, following dewaxing and rehydration, samples were incubated with proteinase K (Dako, Ely, Cambs, UK) for 4 minutes. Following a blocks with 0.2M glycine/PBS and then 10% normal goat serum, the sections were incubated with an isotype control or dsRNA-J2 antibody (Scicons, Budapest, Hungary; 1/100, overnight at 4°C) that had been biotinylated by Capra Science (Angelholm, Sweden). This antibody detects dsRNA >50 bp in length and so is unlikely to detect endogenous cellular dsRNA species (Richardson et al. 2010). Following washes and peroxidase blocking, the sections were treated with the Vector Elite ABC Kit (Vector Labs, Peterborough, UK) optimized for biotinylated antibodies, as per the manufacturer's instructions. Staining was visualized with 3,3'-Diaminobenzidine (DAB; Dako). Sections were counterstained with haematoxylin (Dako), dehydrated and mounted in distrene, plasticiser, xylene (DPX). Images were captured under a Nikon Eclipse 80i microscope (Nikon, Guildford, Surrey, UK).

Statistical analysis.

The Student test was performed, using GraphPad Prizm 5 software, to determine if means of viral titres, at days 0 and 5, among offspring of mice which were infected at day 10G or 17G are significantly different. The same test was performed to determine if means of viral titres, at day 26 and 30, between pups challenged at the age of 21 days and born to dams either infected or not during pregnancy, are significantly different. The

Two tailed Fisher's Exact Test was also performed to examine the significance of the difference between mock-infected and virus-infected mice in terms of the presence of staining signal for viral dsRNA. In all cases, differences were considered significant at $P < 0.05$.

Results

Infectious particles were isolated from different compartments of the CNS of offspring born to CV-B4 E2-inoculated dams.

Using the plaque assay method, infectious particles were detected and viral titres were determined at different time points in different zones of the CNS (brain, cerebellum and brainstem) of, respectively, 16/18 (88.88 %) and 11/18 (61.11 %) offspring of dams inoculated with CV-B4 E2 either at day 10G or 17G (Table 1). These data prove that CV-B4 E2 was vertically transmitted in our mouse model and reached the CNS in the course of this *in utero* infection. No infectious particles were however detected in the spinal cord.

Viral titres peaked at day 0 (day of birth) in both the pups from dams inoculated at day 10G or 17G, with no significant difference detected between these two. These titres rapidly decreased thereafter but remained detectable at day 5 after birth (Fig. 2A and B), with no significant difference between pups born to dams inoculated at either day 10G or 17G. The highest numbers of infectious particles were detected in the brainstem. Viral progeny was not detected in CNS tissues of offspring from negative control dams.

Prolonged detection of viral RNA in different compartments of the CNS of offspring born to CV-B4 E2-inoculated dams.

Infectious virus rapidly became undetectable in the CNS compartments of the offspring. To investigate further the infection and eventual persistence, the presence of viral RNA in the CNS of offspring from dams inoculated at day 17G was studied, up to 90 days after birth, using semi-nested (sn)-RT-PCR. As shown in Table 2 and Fig. 3, CV-B4 RNA was detected until 60 days of age in 8/18 (44.44%) offspring, in the three zones of the CNS that contained infectious particles detected by plaque assay at birth, namely the brainstem, the cerebellum and the brain. Viral RNA was not detected in spinal cord which confirms the results obtained by plaque assay. These results suggest that CV-B4 E2 can establish a persistent infection (until the adult stage) of the CNS following an *in utero* transmission. Viral RNA was not detected in CNS tissues of offspring from control dams.

Histopathological changes in the CNS of offspring born to CV-B4 E2-inoculated dams

In order to explore the eventual abnormalities caused by an *in utero* CV-B4 E2 infection of the CNS, we conducted a histological analysis. CNS (brain, cerebellum, brain stem and spinal cord) tissue sections from offspring born to control and CV-B4 E2-inoculated dams (six offspring from each group, each three born to one different dam) were analysed by haematoxylin/eosin staining at different p.i. times. No histopathological changes were observed in pups from dams inoculated at day 10G. However, two cases of inflammatory lesions with evidence of lymphocyte infiltration were detected in offspring infected with CV-B4 E2 on day 17G (group +-), in the brain (one case) and brainstem (one case), at day 60 of age (Fig. 4). No anomalies were observed in the cerebellum and the spinal cord, or in CNS tissues from the control group.

Effect of a primary infection during foetal life on a subsequent challenge of young offspring

In order to investigate the effect of an *in utero* infection on a subsequent challenge of young offspring, CNS tissues from pups challenged 21 days after birth, and born to either dams infected or not at day 17G (groups ++ and -+, respectively), were subjected to the same analysis by plaque assay, RT-PCR and histological staining, as described above. The results obtained by each procedure were then compared between both groups.

In both the ++ and -+ groups, virus titration revealed the presence of infectious particles in 9/18 (50%) offspring, in the brain, cerebellum and brainstem, until at least day 30 of age (9 days post-challenge) but not at day 60 (Table 3). The spinal cord was negative as throughout the course of the *in utero* infection. No significant differences in the proportion of positive tissues (Table 3) or the mean titres of infectious particles in each tissue at different time points (Fig. 5A and B) was observed between the two groups.

The sn-RT-PCR results revealed that both the ++ and -+ groups showed viral RNA in the brain, cerebellum and brain stem, until day 90 (70 days post-challenge). No significant differences were observed in the proportion of positive tissues at the different time points (10/18 and 8/18 infected mice in the ++ and the -+ groups, respectively). The viral genome remained undetectable in the spinal cord (Table 4).

Histological analysis revealed anomalies of the brain (five cases, inflammatory lesions with lymphocyte infiltration at day 60, and meningitis at day 90) in both ++ (only one of two cases at day 60 is illustrated) and -+ groups (only one of two cases at day 90 is illustrated), and of the brain stem (one case, inflammatory lesions with lymphocyte infiltration at day 90) in the -+ group (Fig. 6). No anomalies were observed in the cerebellum, the spinal cord, and in CNS tissues from the control group.

Viral double-stranded RNA detected in few cells of inflamed and/or persistently-infected CNS tissue sections

In an attempt to identify the cause of tissue damage and to understand the molecular mechanisms of CV-B4 persistence in the CNS, dsRNA staining of paraffin-embedded tissue sections from infected (all inflamed and other selected CV-B4 E2 RNA sn-RT-PCR positive tissues) and negative control offspring was carried out using an antibody specifically recognizing dsRNA (see Material and methods section). Two sections for each sample were stained and analysed in a blinded manner and the isotype control staining on all sections was negative. Following unblinding, 7 of 14 (50%) infected mice were shown to have evidence of dsRNA positive cells (at most, 1 or 2 dsRNA positive cells were present within any of the sections examined). In contrast, no dsRNA positive cells were identified in the 7 mock-infected mice (Two tailed Fisher's Exact Test, p=0.0468). dsRNA immunolabelling was observed in 5/8 (62.5%) inflamed, 7/12 (58.33%) sn-RT-PCR positive, and 5/6 (83.33%) both inflamed and RT-PCR positive tissue sections from infected mice, whereas sn-RT-PCR negative tissues were immunonegative. All control tissue sections from uninfected animals were also negative (Table 5, Fig. 7).

Discussion

Type B Coxsackievirus infections are known to be associated with a range of neurological diseases (Marier et al. 1997; Muir & van Loon, 1997; Rhoades et al. 2011). These infections of the CNS are frequently observed in foetuses and newborns, suggesting a role for vertical transmission of CV-B in pathogenesis (Euscher et al. 2001; Gauntt et al. 1985; Hunt et al. 2012; Konstantinidou et al. 2007; Ornoy &

Tenenbaum, 2006; Romero, 2008; Tebruegge & Curtis, 2009). The current investigation was conducted in an attempt to better understand the effect of an *in utero* infection on offspring's CNS. For this purpose, pregnant outbred *Swiss albino* mice were inoculated with CV-B4 E2 and their offspring's CNS analysed for both virus infection and histopathology at different time points.

Few experimental studies have used animal models to explore CV-B vertical transmission and the issue of CNS infection has not been practically addressed (Bopegamage et al. 2012; Hwang et al. 2014; Larsson et al. 2013; Marosova et al. 2011; Modlin & Crumpacker, 1982).

Vertical transmission of CV-B4 has, however, been reported in some studies (Bopegamage et al. 2012; Hunt et al. 2012; Marosova et al. 2011). In a Slovakian study, CV-B4 was revealed as the most prevalent CV-B serotype encountered in pregnant women (Marosova et al. 2011). In addition, vertical transmission of CV-B4 has been linked to neurological disorders (Hunt et al. 2012). Despite the fact that the CV-B4 E2 strain is more commonly known for its pancreotropism and diabetogenicity in susceptible mouse strains (Yoon et al. 1979), there is evidence of neurotropism as shown by the persistence of that strain in the CNS of *Swiss albino* mice inoculated at one week of age (El Hiar et al. 2012). Indeed, this strain used in these studies (AF311939.1), isolated by Yoon, is closely related to the E2 (S76772.1) strain (99% homology in whole genome sequence) of the three plaque purified variants (E1, E2 and E3) of the CV-B4 Edwards strain isolated from a newborn who died of encephalohepatomyocarditis (Hartig & Webb, 1983; Kibrick & Benirschke, 1958). Histological analysis of the brain of the newborn revealed inflammation and inflammatory cell infiltration in the cortex and meninges.

The inoculation period during pregnancy could be a determinant for the outcome of the infection on offspring (Lansdown, 1975). Our choice for gestational days 10 and 17 arises from recent data reporting that inoculation of CV-B4 at gestational day 10 affects offspring, whereas inoculation at gestational day 17 increases infection of pups challenged with the same virus (Bopegamage et al. 2012).

The detection of viral progeny in foetuses dissected at day 17G is in favour of a transplacental transmission, but a perinatal transmission during delivery cannot be excluded. Contamination of CNS with blood is unlikely since tissues were rinsed three times with PBS. In addition, previous results showed that virus detection in blood is not obvious, since viral RNA is evidenced only transiently during viraemic stages (Jaïdane et al. 2006).

Vertical transmission occurred in dams inoculated at gestational day 10 or 17, with virus replication in offspring's CNS for at least 10 days p.i., but higher virus titres were observed in pups inoculated at foetal day 10, suggesting a greater susceptibility to infection at that developmental stage. This could be explained by the high expression of CAR (Coxsckievirus and Adenovirus receptor) in the foetal CNS, which drops dramatically within days of birth (Honda et al. 2000). It has also been shown that CV-B3 can replicate at high titres, for at least 10 p.i., in the CNS of newborn BALB/c mice, targeting neuronal progenitor cells (which rapidly decreases after birth, as a consequence of differentiation), hence explaining the drastic decrease in susceptibility of newborn pups to CNS infection by 7 days of age in that model (Feuer et al. 2003; Feuer et al. 2009). Likewise, El Hiar *et al.* (2012) demonstrated that the CNS of *Swiss albino* mice inoculated with CV-B4 E2, one week after birth, harboured infectious particles until 15 days p.i. An alternative explanation for the enhanced susceptibility of foetuses, newborn and young mice to CV-B infections could be a lack of immune maturity at that age (Tracy & Gauntt, 2008).

In our current study, the brainstem was the most infected CNS tissue. Intriguingly, no infectious virus could however be isolated from the spinal cord, which differs from the investigation of El Hiar *et al.* (2012) who detected infectious particles in the spinal cord of mice inoculated with the same viral strain one week after birth.

Sn-RT-PCR results demonstrated that CV-B4 E2 infection of offspring's CNS can persist for a relatively long period of time (until day 60 and, in one case, until day 90 from birth) after infectious virus becomes undetectable. Prolonged viral RNA detection despite the lack of infectious virus detection has been described previously. Infectious particles detection is usually restricted to the acute stage of the infection (no longer than two to three weeks, depending on the study) and infectious virus is no longer detectable in the chronic stage. In theory, virus persists in a low rate replicative form (below the detection limit of standard methods) and/or a defective replication form (equivalent amounts of positive- and negative-stranded RNA) (Feuer et al. 2009, Tam and Messner, 1999). The ability of CV‑B RNA to persist for months following an initial infection is well established both in humans and experimental studies (Bopegamage et al. 2005; Bowles et al. 1989; El Hiar et al. 2012; Feuer et al. 2009; Harrath et al. 2004; Klingel et al. 1996; Reetoo et al. 2000; See & Tilles, 1995; Tam et al. 1991; Tam & Messner, 1999). CV-B RNA persistence in the CNS is however only poorly documented. Our results agree with other investigations reporting that CV-B4 E2 and CV-B3 can persist in mouse brain up to 50 and even 90 days p.i. at the RNA level (El Hiar et al. 2012; Feuer et al. 2009; Reetoo et al. 2000). Intriguingly, in the investigation conducted by Bopegamage *et al.* (2012), offspring of dams infected with CV-B4 E2 at day 4, 10, or 17G were negative by sn-RT-PCR performed on brain, heart and pancreas collected at day 30 from birth, despite the use of a tenfold higher viral dose (2×10^6 $TCID_{50}$). This discrepancy may be due to the difference in the route of virus inoculation (oral) and/or in the mouse strain (CD1) used in that study. Indeed,

in the study conducted by Precechtelova et al. (2014), CV-B4 E2 RNA was much longer detected in the pancreas of Swiss albino than in CD1 mice (Precechtelova et al. 2015). To the best of our knowledge, this is the first report describing CV-B persistence in the CNS following vertical transmission. Persistence of viral material is hypothesised to be a potential mechanism in chronic or delayed neuropathologies such as schizophrenia, encephalitis lethargica and amyotrophic lateral sclerosis (Cree et al. 2003; Rantakallio et al. 1997; Woodall et al. 2004). In this context, Feuer *et al.* (2009) previously suggested an association between CV-B3 RNA persistence in the brain and the chronic inflammation and lesions observed in the hippocampus and cortex of surviving mice for up to 9 months p.i.

In the current study, surviving mice were monitored up to 150 days after birth and no death or apparent anomalies, such as paralysis or unusual behaviour, were observed. Despite these findings, histological analysis revealed two cases of inflammatory foci, lymphocyte infiltrations and meningitis in the brain and brain stem of offspring born to infected dams, at a period in which viral material was still detectable (day 60 of age). Relatively few experimental studies have previously described histological abnormalities in the CNS following CV-B infections (Bopegamage et al. 2012; Feuer et al. 2003; Feuer et al. 2009). In the study conducted by Bopegamage et al. (2012), pups born to dams inoculated on the 17th day of gestation and challenged 3 weeks after birth (+/+ group) showed oedema and capillary hyperemia in the brain 5 days post-challenge. Feuer *et al.* (2003) described extensive cellular damage and condensed nuclei within the olfactory bulb, the temporal and entorhinal cortex and the hippocampus in newborn BALB/c mice, 5 days p.i. by CV-B3.

The fact that some studies have associated enterovirus infections during pregnancy with increased risk of the offspring developing chronic diseases, such as type 1 diabetes (Dahlquist et al. 1995; Elfving et al. 2008), allows one to suggest that maternal infections during pregnancy

could play a role in the course of subsequent infections in offspring in agreement with the findings of Bopegamage *et al.* (2012). Our *in utero* infection did not enhance CNS infection during subsequent challenge of the offspring with the same virus, despite the fact that the first infection was still evident at the moment of challenge in our model. However, we cannot exclude that the infection could be increased in tissues other than the CNS. Indeed, in the study of Bopegamage *et al.* (2012), such an effect was evident only in the pancreas which showed more severe histopathological changes than the control groups.

The use of immunohistochemistry (IHC) on CNS tissue sections enabled us to reveal the presence of viral dsRNA at late stages of the infection (day 60) in samples with histopathology and/or positive sn-RT-PCR results for CV-B4 genome. The presence of viral dsRNA could be involved in the delayed histopathological changes observed at that time, and provides a possible mechanism for viral persistence. Indeed, the presence of viral material in the CNS for extended periods of time may contribute to chronic immunopathology (David et al. 1993; Tam & Messner, 1999). dsRNA is thought to be formed during persistent enteroviral infections when positive and negative strands are known to be present in equal amounts (Cunningham et al. 1990; Klingel et al. 1992; Tam & Messner, 1999). The dsRNA structure may give stability and protect viral RNA from degradation, thus promoting long-term persistence. Similar results were proposed from the ratio of positive- to negative-strand RNA, during the chronic stage, in the muscle and the brain of CV-B3-infected mice (Feuer et al. 2009; Tam & Messner, 1999). The low number of cells and the weak intensity of the signal is in agreement with the fact that viral genome is detected only following sn-RT-PCR, but one cannot conclude this is a real form of persistence, or simply a replication intermediate.

In conclusion, using our experimental murine model, we have demonstrated that CV-B4 E2 can be transmitted from dams to their foetuses' CNS with productive replication in the brain, cerebellum and brainstem until 10 days p.i whether the virus was inoculated at days 10G or 17G. Viral RNA persists in the above mentioned tissues until 60 days after birth with evidence of double-stranded complexes. Histopathological changes were observed in only a few cases, and CNS infection during subsequent challenge of pups was not augmented. Further investigations, using this murine model of CV-B4 vertical transmission to the CNS, are needed to determine the long lasting consequences of CV-B infection during foetal life upon surviving mice.

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Table 1: Viral load for individual samples taken from different compartments of the CNS of offspring born to dams inoculated either at day 10 or 17 of gestation.

		Day 17G			Day 0			Day 5			Day 21			
Inoculation at day 10G	**Brain**	5	0	21.73	166.6	174.9	83.33	22.35	11.39	0				Litter 1
		45.23	23.9	11.48	11.22	0	0	4.23	0	0				Litter 2
	Cerebellum	20.2	31.25	50	337.3	166.6	0	0.99	4.77	1.95				Litter 1
		54.23	25.32	0	12.34	0	0	0.28	52.63	0				Litter 2
	Brainstem	0	142.8	0	56.81	514.7	0	21.51	21.92	100.2				Litter 1
		73.65	67.34	0	0	23.33	0	35.71	14.28	0				Litter 2
	Spinal cord	0	0	0	0	0	0	0	0	0				Litter 1
		0	0	0	0	0	0	0	0	0				Litter 2
Inoculation at day 17G	**Brain**				0	0	16.33	26.3	6.0	0	0	0	0	Litter 1
					6.9	0	0	6.72	0	0	0	1.76	0	Litter 2
	Cerebellum				0	57.14	0	13.88	0	17.24	0	0	0	Litter 1
					0	0	0	0	0	2.94	0	0	0	Litter 2
	Brainstem				250	0	229.1	0	0	0	0	0	0	Litter 1
					8.33	0	0	20.83	178.5	0	0	0	0	Litter 2
	Spinal cord				0	0	0	0	0	0	0	0	0	Litter 1
					0	0	0	0	0	0	0	0	0	Litter 2
		pup 1	pup 2	pup3	pup1	pup 2	pup 3	pup 1	pup 2	pup 3	pup 1	pup2	pup3	

CNS tissues (brain, cerebellum, brainstem and spinal cord) of each of six offspring (each three born to one different dam) were tested by plaque assay, for viral progeny, *each time p.i. (day 17G, and days 0 and 5 after birth for mice inoculated at day 10G, and days 0, 5 and 21 after birth for those inoculated at day 17G). Results are expressed in p.f.u. mg^{-1} of tissue for each sample*

Table 2: Kinetics of viral RNA detection in different compartments of the CNS of offspring born to CV-B4 E2-inoculated dams at day 17 of gestation.

	Day 30			Day 60			Day 90			
Brain	+	+	-	-	-	-	+	-	-	Litter 1
	+	-	-	+	+	-	-	-	-	Litter 2
Cerebellum	+	+	-	+	-	-	-	-	-	Litter 1
	-	-	-	-	+	+	-	-	-	Litter 2
Brainstem	+	+	-	-	-	-	-	-	-	Litter 1
	-	-	-	-	+	+	-	-	-	Litter 2
Spinal cord	-	-	-	-	-	-	-	-	-	Litter 1
	-	-	-	-	-	-	-	-	-	Litter 2
	pup 1	pup 2	pup 3	pup 1	pup 2	pup 3	pup 1	pup 2	pup3	

CNS tissues (brain, cerebellum, brainstem and spinal cord) of offspring born to dams inoculated with CV-B4 E2 at day 17G, were analyzed for viral RNA detection by sn-RT-PCR. Six offspring (each three born to one different dam) were tested each time p.i. (days 30, 60 and 90 after birth). Results are summarized as positive (+) or negative (-) for each sample.

Table 3: **Viral load for individual samples taken from different compartments of the CNS of offspring challenged 21 days after birth and born to dams either infected or not at gestational day 17 (groups ++ and -+, respectively).**

		Day 26			Day 30			Day 60			
Group ++	Brain	10.41	57.87	50	0	0	28.08	0	0	0	Litter 1
		21.3	0	0	0	37.03	0	0	0	0	Litter 2
	Cerebellum	406.5	0	0	0	27.77	0	0	0	0	Litter 1
		0	2	0	0	0	0	0	0	0	Litter 2
	Brainstem	303.3	0	375	22.60	27.32	6.75	0	0	0	Litter 1
		0	0	0	0	0	0	0	0	0	Litter 2
	Spinal cord	0	0	0	0	0	0	0	0	0	Litter 1
		0	0	0	0	0	0	0	0	0	Litter 2
Group -+	Brain	0	1.06	0	0	0	0	0	0	0	Litter 1
		0	0	1.75	0	2.32	0	0	0	0	Litter 2
	Cerebellum	25.56	0	0	0	0	0	0	0	0	Litter 1
		15.34	3	0	0	2	27.3	0	0	0	Litter 2
	Brainstem	100.4	0	6.94	0	42.6	0	0	0	0	Litter 1
		0	0	0	0	6.25	0	0	0	0	Litter 2
	Spinal cord	0	0	0	0	0	0	0	0	0	Litter 1
		0	0	0	0	0	0	0	0	0	Litter 2
		pup 1	pup 2	pup 3	pup 1	pup 2	pup 3	pup 1	pup 2	pup 3	

CNS tissues (brain, cerebellum, brain stem and spinal cord) from six offspring (each three born to one different dam) of each of ++ and -+ groups were analysed by plaque assay, for viral progeny, each time p.i. (days 30, 60 and 90 after birth). *Results are expressed in p.f.u. mg^{-1} of tissue for each sample.*

Table 4: Kinetics of viral RNA detection in different compartments of the CNS of offspring challenged 21 days after birth and born to dams either infected or not at gestational day 17 (groups ++ and -+, respectively).

		Day 30			Day 60			Day 90			
Group ++	**Brain**	-	-	+	-	-	-	+	-	+	Litter 1
		-	+	-	-	+	+	-	-	-	Litter 2
	Cerebellum	-	+	-	-	+	-	-	-	+	Litter 1
		-	-	-	-	+	-	-	-	-	Litter 2
	Brainstem	+	+	+	-	+	-	-	+	-	Litter 1
		-	-	-	-	+	-	-	-	-	Litter 2
	Spinal cord	-	-	-	-	-	-	-	-	-	Litter 1
		-	-	-	-	-	-	-	-	-	Litter 2
Group -+	**Brain**	-	-	-	-	-	-	-	+	+	Litter 1
		-	+	-	+	+	-	-	-	-	Litter 2
	Cerebellum	-	-	-	-	-	-	-	-	-	Litter 1
		-	+	+	+	-	-	-	+	-	Litter 2
	Brainstem	-	+	-	-	-	-	-	-	-	Litter 1
		-	+	-	-	-	-	+	-	--	Litter 2
	Spinal cord	-	-	-	-	-	-	-	-	-	Litter 1
		-	-	-	-	-	-	-	-	-	Litter 2
		pup 1	pup2	pup3	pup1	pup2	pup3	pup1	pup2	pup3	

CNS tissues (brain, cerebellum, brain stem and spinal cord) from six offspring (each three born to one different dam) of each of ++ and -+ groups were analysed by sn-RT-PCR each time p.i. (days 30, 60 and 90 after birth). *Results are summarized as positive (+) or negative (-) for each sample.*

Table 5: IHC for dsRNA results depending on histopathology and sn-RT-PCR findings in CNS tissue sections of offspring challenged or not at day 21 of age, and born to dams inoculated or not with CV-B4 E2 at gestational day 17.

Sample	Histopathology	Sn-RT-PCR for CV-B4 E2 RNA	IHC for dsRNA
Brain 1, day 60, +- group	+	-	-
Brainstem 1, day 60, +- group	+	+	+
Brain 1, day 60, ++ group	+	+	+
Brain 2, day 60, ++ group	+	-	-
Brain 1, day 90, ++ group	+	+	+
Brain 1, day 90, -+group	+	+	-
Brain 2, day 90, -+ group	+	+	+
Brainstem 1, day 90, -+ group	+	+	+
Brain 2, day 60, +- group	-	+	-
Brain 3, day 60, +- group	-	+	+
Cerebellum 1, day 60, +- group	-	+	-
Cerebellum 2, day 60, +- group	-	+	+
Brainstem 2, day 60, +- group	-	+	-
Cerebellum, day 60, ++ group	-	+	-
Brain, day 60, -- group	-	-	-
Brainstem, day 60, -- group	-	-	-
Cerebellum, day 60, -- group	-	-	-
Brain1, day 90, -- group	-	-	-
Brain 2, day 90, -- group	-	-	-
Brainstem, day 90, -- group	-	-	-
Cerebellum, day 90, -- group	-	-	-

Results of IHC for dsRNA detection in selected brain, cerebellum and brainstem samples from infected and control animals, depending on histopathology and sn-RT-PCR findings, are summarised as positive (+) or negative (-).
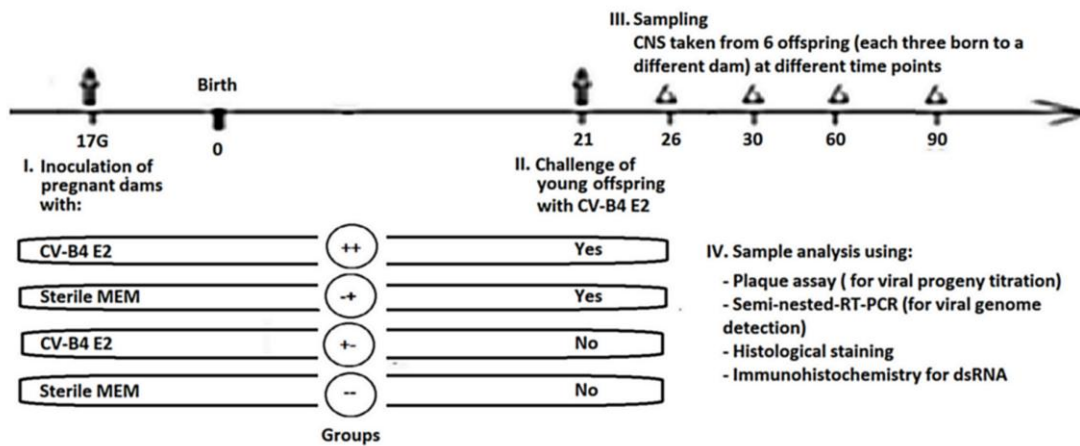
Fig 1. Schematic representation of the experimental design adopted to explore the effect of *in utero* CV-B4 infection on a subsequent challenge of young offspring by the same virus.
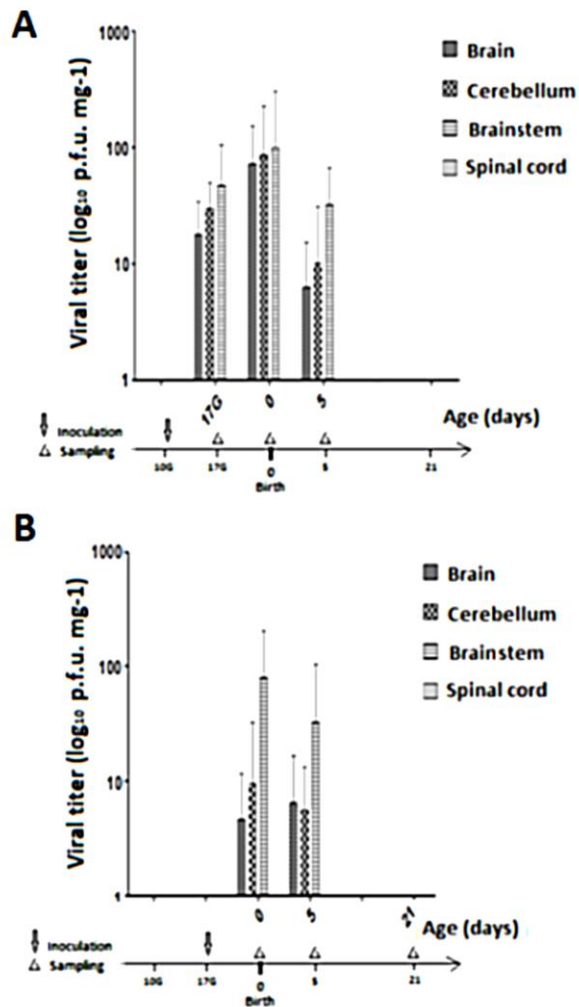
Fig 2. Kinetics of viral progeny in different compartments of the CNS of offspring born to CV-B4 E2-inoculated dams. Viral titres were determined by plaque assay in CNS tissues (brain, cerebellum, brainstem and spinal cord) of offspring from dams inoculated with CV-B4 E2 at day 10G (A) or day 17G (B). Tissues of each of six offspring (each three born to one different dam) were tested each time p.i. (day 17G, and days 0 and 5 after birth for mice inoculated at day 10G, and days 0, 5 and 21 after birth for those inoculated at day 17G). *Results are plotted as mean log10 p.f.u. mg-1 of tissue + SDs, n=6)*
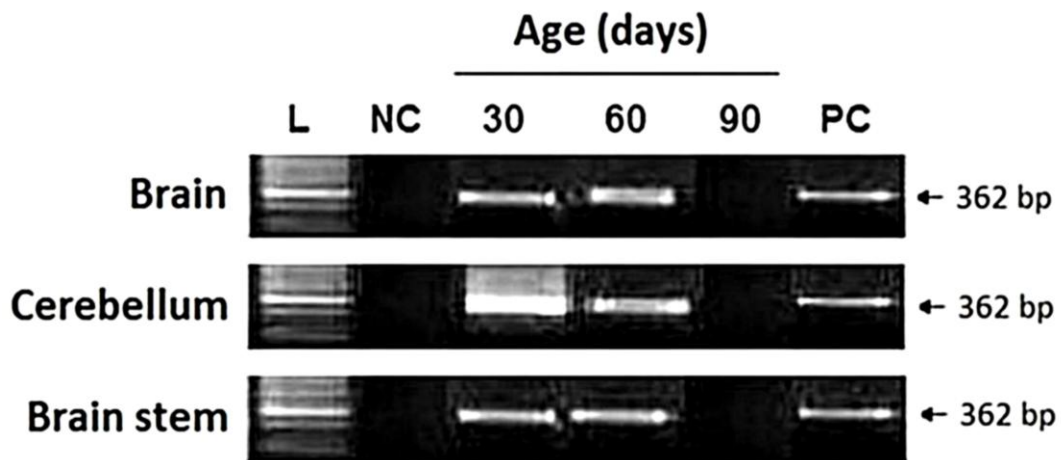
Fig. 3. **Viral RNA detection in different compartments of the CNS of offspring born to CV-B4 E2-inoculated dams.** CNS tissues (brain, cerebellum, brainstem and spinal cord) of offspring from dams inoculated with CV-B4 E2 at day 17G, were analyzed for viral RNA detection by sn-RT-PCR. Six offspring (each three born to one different dam) were tested each time p.i. (days 30, 60 and 90 after birth). Results are illustrated by a representative agarose gel electrophoresis. *L: 100 bp DNA ladder; NC: negative control; PC: positive control;*
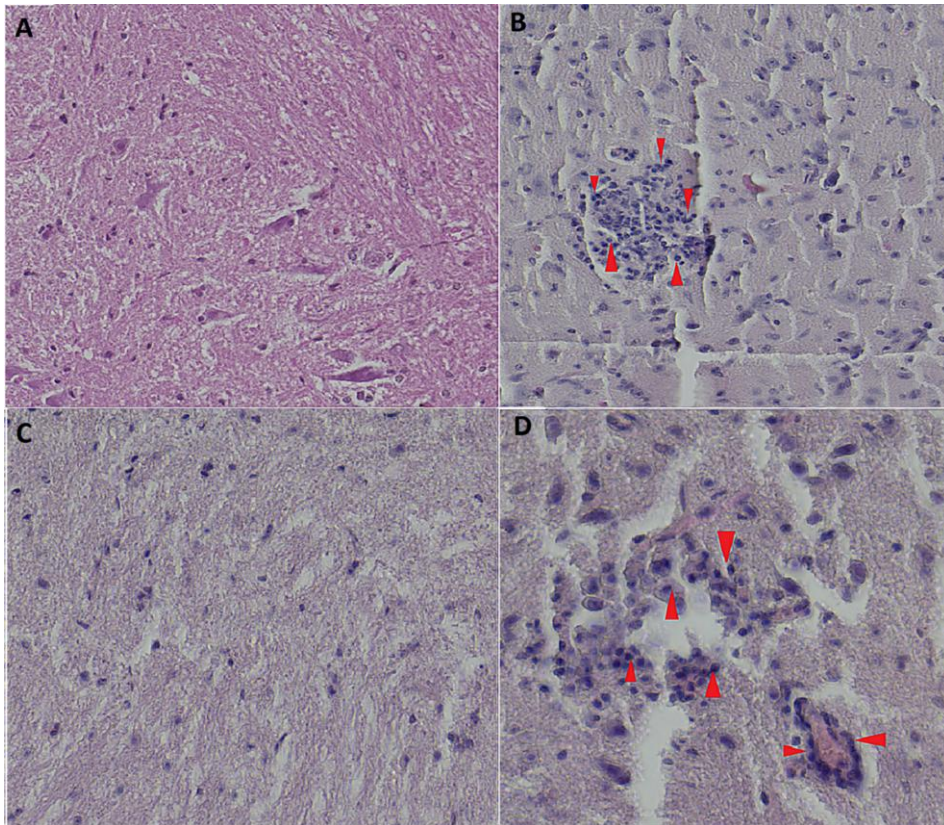
Fig 4. Histopathological changes in CNS tissues of offspring born to dams inoculated with CV-B4 E2. CNS tissue (brain, cerebellum, brainstem and spinal cord) sections of offspring from control and CV-B4 E2-inoculated dams (six offspring from each group, each three born to one different dam) were analysed by haematoxylin/eosin staining at different p.i. times. Histopathological changes were found only at day 60 after birth in 1 out of 6 analysed brains (B) and 1 out of 6 analysed brainstem (D) from mice inoculated at day 17G (group +-). Inflammatory foci with lymphocyte infiltration are indicated by arrows (B, D). No anomalies were observed in tissues from mice inoculated at day 10G and from control mice. Representative sections of brain (A) and brainstem (C), taken at day 60 of age, from control mice. Gr x200.
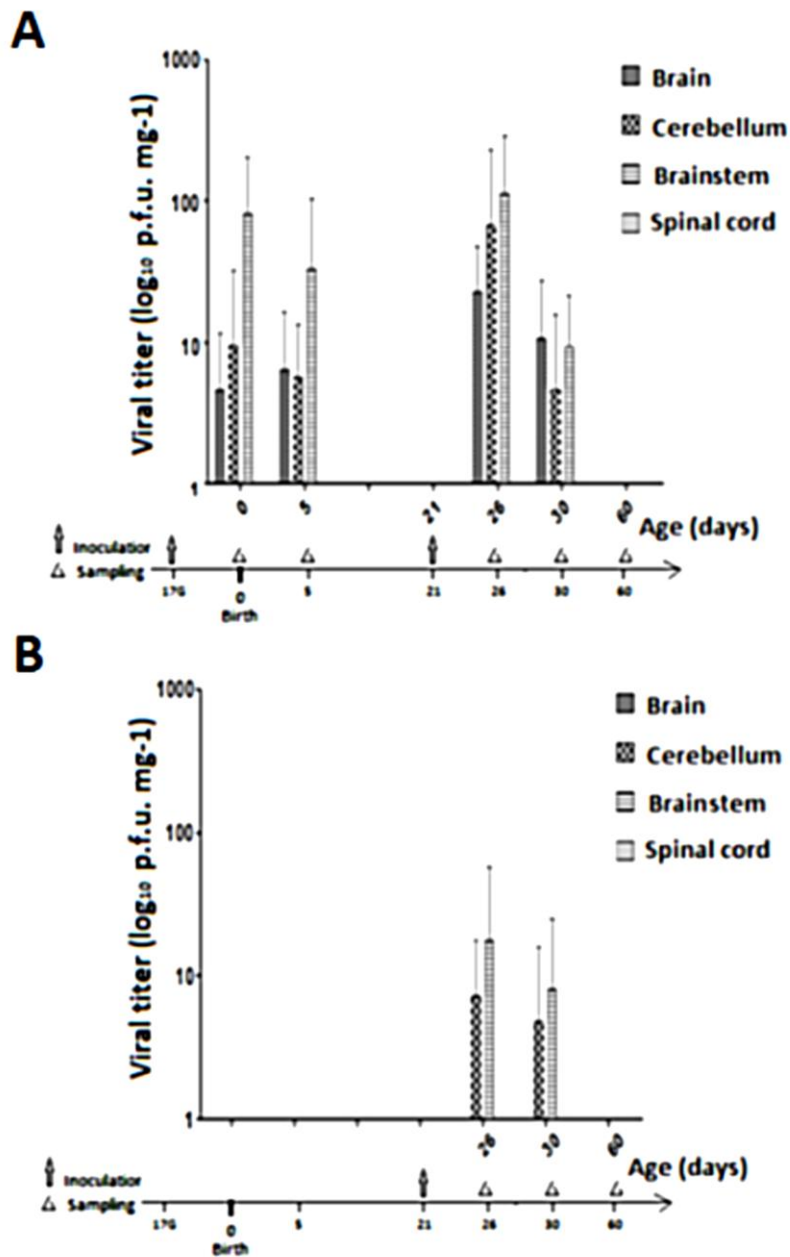
Fig 5. Kinetics of viral progeny in different CNS tissues from pups challenged 21 days after birth and born to either dams infected or not at gestational day 17 (groups ++ and -+, respectively). CNS tissues (brain, cerebellum, brain stem and spinal cord) from six offspring (each three born to one different dam) of each of ++ and -+ groups were analysed each time p.i. (days 26, 30 and 60 after birth) for viral progeny. Viral titres in CNS tissues

were determined by plaque assay for both ++ (A) and -+ (B) groups. *Results are plotted as mean log10 p.f.u. mg^{-1} of tissue + SDs, n=6).*
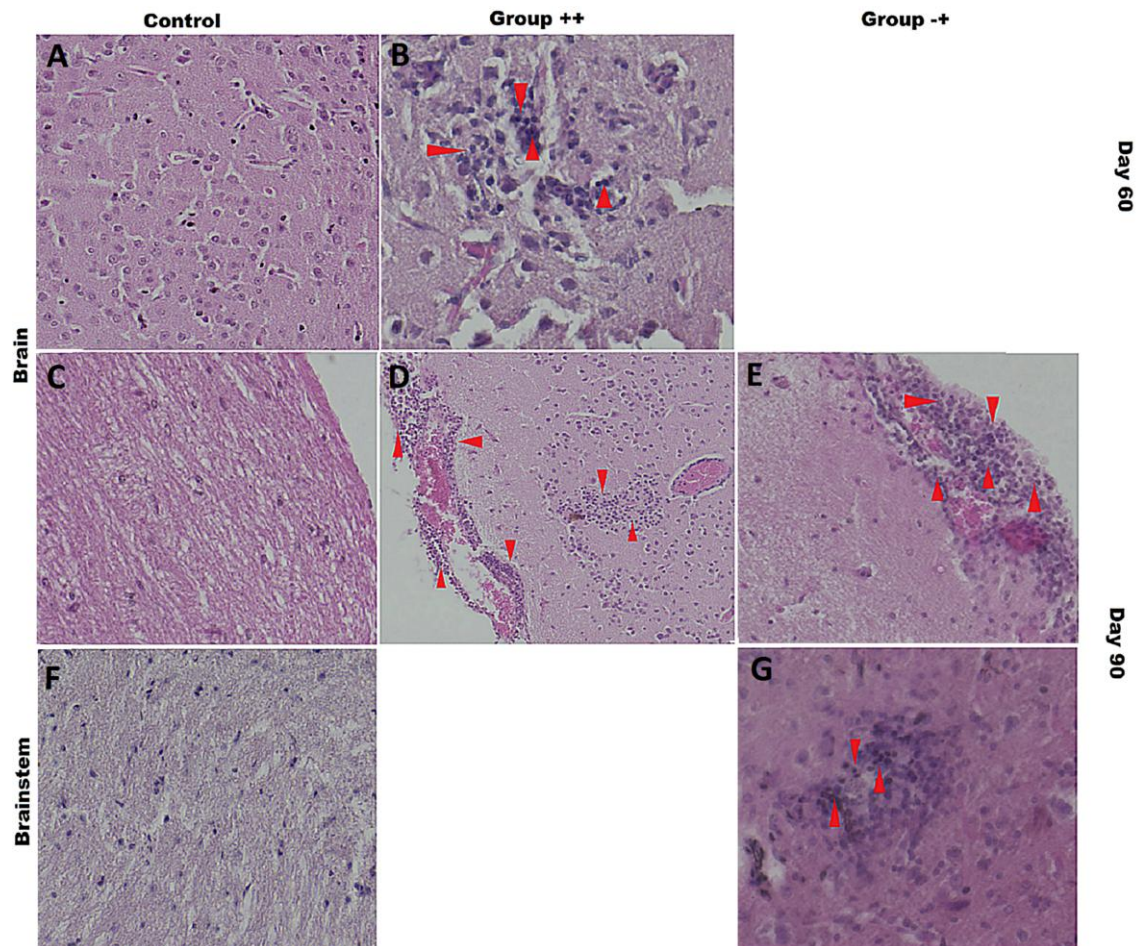

Fig 6. **Histopathological changes in different CNS tissues from pups challenged 21 days after birth and born to either dams infected or not at gestational day 17 (groups ++ and -+, respectively).** CNS tissues (brain, cerebellum, brain stem and spinal cord) from six offspring (each three born to one different dam) of each of ++ and -+ groups were analysed each time p.i. (days 30, 60 and 90 after birth) by haematoxylin/eosin staining. Histopathological changes (indicated by arrows) were found only in the brain at days 60 and 90 in the group ++, and in the brain and the brainstem at day 90 in the group -+. All illustrated sections from infected groups show inflammatory foci with lymphocyte infiltration. Both

illustrated sections of brain taken at day 90 show signs of meningitis (D and E). Representative sections of the brain and the brainstem taken, at the same corresponding days, from negative control mice, are given in the first column (A, C and F). Gr x200
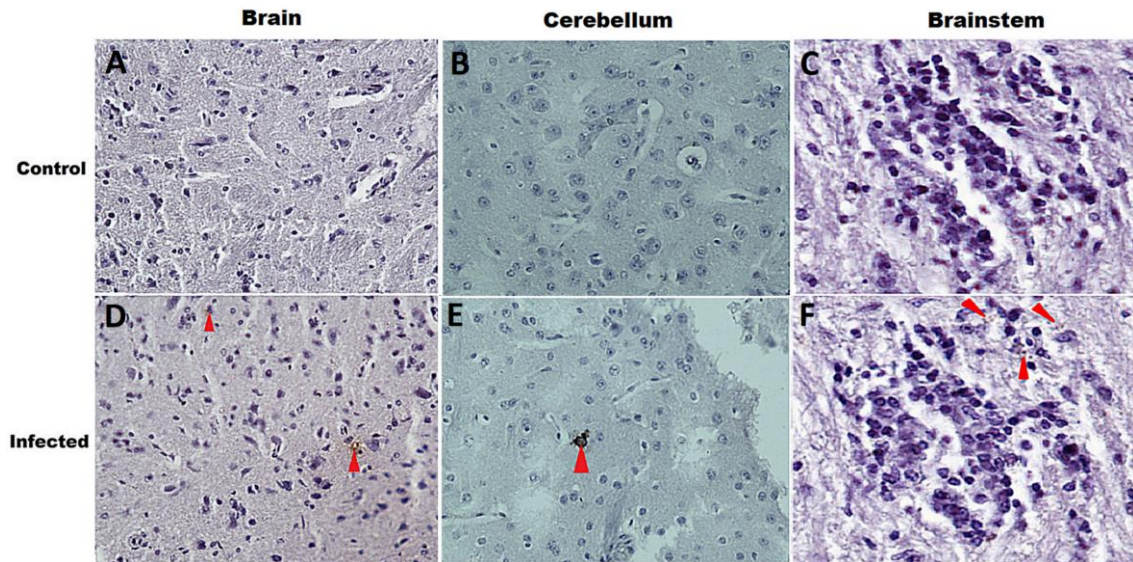

Fig 7. Staining for dsRNA in CNS tissue sections of offspring from dams inoculated with CV-B4 E2 at gestational day 17. Representative illustrations of immunostaining for dsRNA in CNS tissue sections counterstained with hematoxylin: Brain from negative control (A) and from infected offspring (D). Cerebellum from negative control (B) and from infected offspring (E). Brainstem from negative control (C) and from infected offspring (F). All samples were taken at 60 days after birth. A faint brown positive signal for viral dsRNA is indicated by arrows. Gr x400.