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Decreased convulsive threshold and memory loss after anti-NMDAR positive CSF injection in zebrafish

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ABSTRACT

Anti-*N*-methyl-D-aspartate receptor (anti-NMDAR) encephalitis initially promotes memory deficits, behavioral changes, and epileptic seizures. We developed a new animal model of anti-NMDAR encephalitis using a single cerebroventricular injection of CSF from patients in adult zebrafish. We observed a reduction of the seizure threshold and recent memory deficits in those animals injected with CSF from patients with anti-NMDAR encephalitis. The locomotor activity was similar in the CSF and control groups. This zebrafish model consistently recapitulates symptoms seen in patients with anti-NMDAR encephalitis. It may provide a reliable, fast and cost-effective platform to investigate new therapeutic strategies to anti-NMDAR encephalitis.

1. Introduction

The most common initial symptoms of anti-*N*-methyl-D-aspartate receptor (anti-NMDAR) encephalitis are recent memory loss, behavioral changes, and epileptic seizures (Sansing et al., 2007; Iizuka et al., 2008). Patients may evolve with autonomic dysfunction, movement disorders, loss of consciousness and coma. It was first described in 2007 on young women with ovarian teratoma (Dalmau et al., 2007), but the frequency of associated tumors varies with gender and onset age.

The majority of the anti-NMDAR antibodies recognizes the receptor subunit GluN1 (Dalmau et al., 2008). After antibody binding, there is a decrease in the NMDAR density in the neuronal post-synaptic cell membrane due to the internalization and degradation of receptors crosslinked to antibodies. The hypothesis is that the reduction of NMDAR results in excessive release of glutamate in the prefrontal and subcortical structures, which would contribute to the development of the clinical symptoms (Dalmau et al., 2017).

The animal models for anti-NMDAR encephalitis are currently based on rodents using cerebrospinal fluid (CSF) samples of patients. They were critical for the understanding of the pathogenic role of these antibodies, but they require exposure of the brain to a continuous intraventricular infusion of the human anti-NMDAR positive CSF over few days. Therefore, relatively large amounts of patient CSF samples may be required to consistently reproduce the disease in these models (Hughes et al., 2010; Mikasova et al., 2012; Planagumà et al., 2015).

Among other available animals suited for disease models, zebrafish (*Danio rerio*) has gained a prominent place in the study of neuropsychiatric diseases. It is a teleost (Grunwald and Eisen, 2002) with small size, easy handling, breeding and relatively low-cost maintenance (Kalueff et al., 2014; Stewart et al., 2014). The development of zebrafish is well characterized and there are many physiological and genetic similarities to humans (Barbazuk et al., 2000; Dooley and Zon, 2000) (Howe et al., 2013). The nervous system is well characterized, and several types of neurotransmission systems are preserved between zebrafish and humans (Stewart et al., 2015). In addition, the behavioral repertoire is comparable to other vertebrates (Rico et al., 2011; Stewart et al., 2014).

Several studies have already been conducted with zebrafish models to understand the function and involvement of the NMDAR in memory and behaviour in psychiatric and neurodegenerative disorders, as well as in inflammatory demyelinating CNS diseases, such as multiple sclerosis (Roberts et al., 2013; Rosa-Falero et al., 2014; Karttunen et al.,

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2017; Burrows et al., 2019). There is a very high similarity (~90%) between the human and zebrafish GluN1 subunit of the NMDAR (Cox et al., 2005), making zebrafish suitable for the development of a new animal model to evaluate the effects of anti-NMDAR antibodies. We report here a model of anti-NMDAR encephalitis using a single cerebroventricular injection of CSF from patients in adult zebrafish.

2. Material and methods

2.1. Ethics statements

All experiments were conducted in accordance to standard guidelines according to Brazilian Law No. 11794/2008 and the recommendations of the National Council for the Control of Animal Experimentation (CONCEA - Conselho Nacional de Controle de Experimentação Animal). This study has been approved by the Ethic Committee for Use of Animals - CEUA from Pontifical Catholic University of Rio Grande do Sul (PUCRS) (protocol number 8514).

2.2. Animals

In the present study, we used adult (4–6 months), female and male wild-type zebrafish (*Danio rerio*) from the AB background. The animals were from our breeding colony and maintained in automated recirculating systems (Zebtec, Tecniplast, Italy). The aquariums were filled with reverse osmosis-filtered water (enriched with salt - Instant Ocean), free of bacteria and fungi. The temperature (26 °C ± 2 °C) and pH (6.5–7.5) were controlled as well as the concentrations of ammonia, nitrite, nitrate and chlorine. The animals were submitted to a light/dark cycle of 14/10 h and received commercial flakes three times a day supplemented with brine shrimp (de Marchi et al., 2019).

2.3. CSF from patients with anti-NMDAR encephalitis

The CSF injected into the animals was obtained from lumbar puncture of two patients with confirmed diagnosis of anti-NMDAR encephalitis fulfilling the diagnostic criteria proposed by Graus et al. (2016). Both patients presented with typical symptoms like abnormal behaviour, cognitive dysfunction, and seizures over 2–4 weeks. Antibodies against anti-NMDAR subunit GluN1 were confirmed in both serum and CSF, and the antibody titre in the CSF was above 1:100. The CSF samples from each patient were identified as CSF01 and CSF02 for this study.

2.4. Group division

The animals were divided into 4 experimental groups:

- Naïve control group: animals with no intervention.
- Saline control group: animals submitted to the CVMI procedure with injection of saline solution (0.9% NaCl).
- CSF group: animals submitted to the CVMI procedure with injection of undiluted CSF from patients with anti-NMDAR encephalitis (CSF01 and CSF02).

2.5. Cerebroventricular microinjection (CVMI)

For microinjection in the zebrafish telencephalon, the animals were previously anesthetized with 0.1 g/L tricaine, after being placed in a tricaine-moist sponge and the gills were irrigated with the same anaesthetic throughout the procedure. A small incision was made with a 25 × 7 disposable needle (BD, Bioscience Brazil) on the zebrafish skull plaque, to create a small access in the telencephalon. A maximum amount of 8 nL of CSF or saline was injected with a proper microinjector into the zebrafish telencephalon. The procedure was adapted from the previously described by Kizil and Brand (2011). The animals were then transferred to an aquarium with an aerator for recovery after the procedure, where they remained until the experiments (Kizil and Brand, 2011).

2.6. Locomotor activity

The locomotor activity of the animals was performed 24 h after CVMI and was measured between 9:00 am and 1:00 pm. This behavioral task was used to verify the distance travelled, mobility time and velocity, which was calculated with the distance and mobility time. The animals stayed from the CVMI until the time of the experiment in the same behaviour analysis room that the experiments would be performed to reduce stress and interference of environmental changes. The animals were individually placed in an experimental aquarium (30x15x10 cm), adapted to the environment for 60 s, after which a five-minute video was recorded. The videos were recorded with a Logitech camera and analysed using Ethovision XT software, dividing the aquariums into equal virtual sessions with one horizontal line (Gerlai et al., 2000).

2.7. PTZ induced-seizures

For the induction of convulsive state, we exposed the animals to Pentylenetetrazole (PTZ) purchased from Sigma-Aldrich (St. Louis, MO, USA) at the concentration of 10 mM according to the protocol described for Menezes and Da Silva (2017). They were placed in an aquarium (13 × 11.5 × 8 cm) filled with 500 mL of water (Menezes and Da Silva, 2017). The PTZ exposure was performed among naïve, saline controls, and CSF groups. We measured the time (s) to reach the stages of seizure (Mussulini et al., 2013). The last 3 seizure stages were selected because they were easy to identify: circular movements (Stage III); clonic seizure-like behaviour (Stage IV); and fall to the bottom of the aquarium and tonic seizure-like behaviour (Stage V). The animals were observed until they reached Stage V or 10 min of experiment. The latency was measured in seconds (s).

2.8. Inhibitory avoidance task

Aversive memory was evaluated in animals through the inhibitory avoidance test as described by Blank et al. (2009). There were two sessions, training and test, with a 24 h interval between them. The test apparatus consists of an aquarium (18 × 9 × 7 cm) with a guillotine door (9 × 7 cm), which divides the aquarium into two compartments of equal size, one black and one white (Altenhofen et al., 2017). In the dark part two electrodes were placed, which when activated produce an electrical shock of 3 ± 0.2 V. The training was performed after 24 h from CVMI. The animals were individually placed in the white area of the aquarium with closed partition and after 1 min of adaptation, the partition was lifted, allowing them to move to the dark side. When they crossed to the dark side, the partition was closed, and they received a pulsed electric shock administered for 5 s. The animals were then removed and placed in the temporary housing.

The memory test session was tested 24 h after the training session. The session was repeated with the same training parameters except for electric shock, which was not administered during this stage. The latency to enter the dark side (avoidance of previous electrical shock) was measured, and the expected increase in the test session was used as an index of memory retention and learning (Blank et al., 2009).

2.9. Statistics analyses

All data obtained were reported in mean ± S.E.M. and analysed by one-way ANOVA and Tukey's multiple comparisons test as post-hoc test. GraphPad v 6.01 was used for statistical analysis. Training and test latencies for each group were compared by the Wilcoxon matched pairs test. Latencies of multiple groups were compared using Kruskal–Wallis and Mann–Whitney *U* tests. In all analyses, $p < 0.05$ was considered to be significant.

3. Results

In total, 198 zebrafish were included in the studies. Of these, 78 animals were included in the locomotor activity test, 65 animals in the inhibitory avoidance task (memory and learning test), and 44 in the PTZ-induced seizure experiment. Eleven animals died during or after the CVMI procedure (5.5% of the total) and were excluded from the study.

3.1. Locomotor activity is unaffected by CSF with anti-NMDAR

In the locomotor activity task, there were no differences between the control (no CVMI), saline, CSF groups. The mean distance (m) travelled by the animals were 18.10 ± 0.53, 16.24 ± 0.66 and 16.14 ± 0.37 (control, saline, CSF groups, respectively). The mobility time (s) were 284.80 ± 2.79, 282.45 ± 5.70 and 279.73 ± 2.78 (control, saline, CSF groups, respectively). Finally, the mean swimming speed (m/s) were 0.083 ± 0.002, 0.08 ± 0.003 and 0.076 ± 0.002 (control, saline, CSF groups, respectively). There were no differences on the locomotor activity between animals injected with different CSF samples.

3.2. Seizure threshold is lowered by CSF with anti-NMDAR

In the PTZ-induced seizure experiment, the animals injected with CSF from patients with anti-NMDAR encephalitis demonstrated a clear reduction in the seizure threshold compared to controls (Fig. 1). The animals injected with CSF samples had a shorter latency time (106 ± 2.01) to Stage III compared to naïve control (160 ± 5.51) and saline control (165 ± 2.30) groups. Similarly, the latency time to Stage IV were shorter in the CSF group (301.5 ± 6.43) compared to naïve control (356 ± 6.62) and saline control groups (344 ± 5.34). Finally, the latency time to Stage V was also reduced in the CSF groups (362.5 ± 5.44) compared to naïve control (403 ± 6.79) and saline control (389 ± 3.89) groups.

Fig. 1 demonstrates the joint depiction of the results obtained with animals injected with CSF01 and CSF02. Both CSF samples resulted in very similar results reducing the seizure threshold, without any difference on the latency time between the animals injected with CSF01 and CSF02 to achieve Stage III (CSF01 = 103 ± 3.13 and CSF02 = 110.0 ± 2.59), Stage IV (CSF01 = 301 ± 5.10 and CSF02 = 304 ± 4.06), and Stage V (CSF01 = 359.2 ± 8.33 and CSF02 = 366 ± 7.25).

3.3. Memory and learning are affected by CSF with anti-NMDAR

In the inhibitory avoidance task, the latency time to the animals to move to the dark side of the aquarium and receive the electrical shock was not different between the control, saline, and CSF groups during training phase (2.5 ± 0.64, 3 ± 0.74, 3 ± 0.97 s, respectively). As expected for the animals with normal memory and learning capacity, we observed a higher latency time in the test session compared to the training session for the control (24.5 ± 11.41) and saline groups (29.0 ± 8.88), indicating that these animals successfully avoided the dark side where they received the electrical shock during the training phase. In contrast, animals injected with patient's CSF showed no increase of the latency time (4.0 ± 2.05) in the test session (Fig. 2), indicating an important lack of memory and learning to avoid the electrical shock received during the training session.

Fig. 2 shows the joint depiction of the results obtained with animals injected with CSF01 and CSF02. The comparison between the animals injected with different CSF samples has not shown differences in the training phase (CSF01 = 3.0 ± 1.39 and CSF02 = 3.5 ± 2.95) as well as in the test phase (CSF01 = 4.0 ± 1.15 and CSF02 = 4.5 ± 1.91).

4. Discussion

Our results show the zebrafish model is able to recapitulate clinical features seen in patients after a single CVMI of anti-NMDAR positive CSF. We compared the recent memory and convulsive threshold of animals injected with CSF samples from patients with control animals without any procedure and animals with saline injection.

For the first time, we demonstrated a reduction in the seizure threshold with both CSF from patients with anti-NMDAR, using a very well-established protocol in zebrafish to assess the seizure threshold with PTZ (Mussulini et al., 2013). We did not find previous studies with

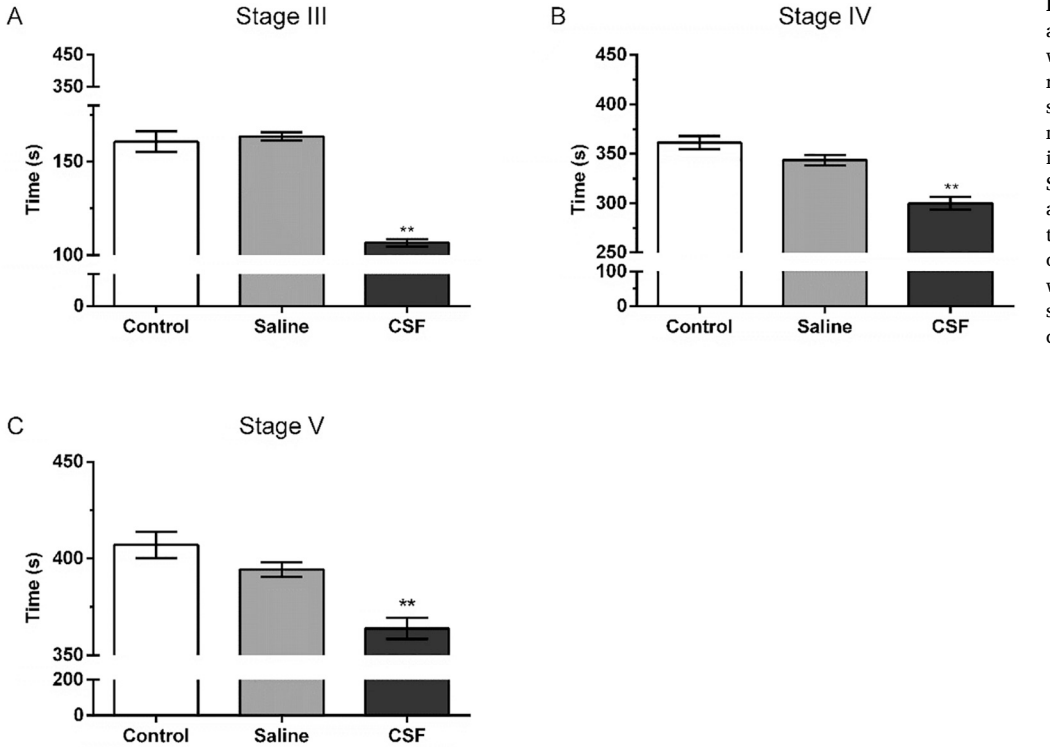

Fig. 1. Reduced seizure threshold in animals injected with CSF from patients with anti-NMDAR encephalitis. The bars represent the time (s) to reach PTZ-seizure stages. (A) Stage III: a circular motion; (B) Stage IV: convulsive behaviour convulsive clonic type; and (C) Stage V: fall to the bottom of the aquarium and convulsive behaviour of tonic type. CSF group consists to the set of results obtained with animals injected with CSF01 and CSF02. Data are presented as mean ± S.E.M. ** $p < 0.001$ compared to control.

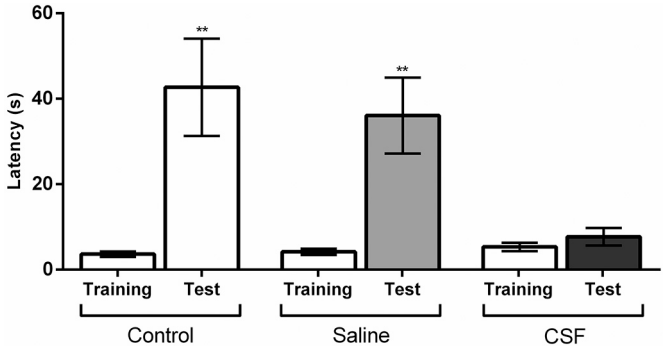

Fig. 2. Recent memory dysfunction in animals injected with CSF from patients with anti-NMDAR encephalitis. Inhibitory avoidance task test in zebrafish. Latency in (s) to cross from white to dark side of the aquarium, during the training and test. The left column of each group represents the elapsed time (s) to move from the white side to the black side in the training session, where they receive an electrical shock during training phase. The right column refers to the elapsed time to cross from the white to the black side in the test phase. CSF group consists to the set of results obtained with animals injected with CSF01 and CSF02. It should be considered that the longer the time spent in moving to the other side in the experiment, the more intense the training memory. ** $p < 0.001$.

anti-NMDAR encephalitis evaluating the reduction of the seizure threshold in animals, but epileptic seizures are common during the disease course (Titulaer et al., 2013, 2014; Viaccoz et al., 2014). These epileptic seizures may evolve to life-threatening situations like status epilepticus and coma.

Recent memory deficit is another common symptom in patients with anti-NMDAR encephalitis (Dalmau et al., 2011; Titulaer et al., 2013). The relevance of NMDAR altering the recent memory in zebrafish is demonstrated by experimental studies with MK-801 (a NMDAR antagonist drug) yielding similar results to our experiment with CVMI injection of CSF from patients with anti-NMDAR encephalitis (Blank et al., 2009; Bertoncello et al., 2019). These recent memory deficits have not been observed in control animals, as well as in animals with saline injection.

There was no changes in the locomotor activity, indicating that the animals were not affected negatively by the CMVI (Kizil and Brand, 2011) with injection of saline or patients' CSF. Similarly, the passive transfer experiments in mice with continuous CVMI also reported no changes in the locomotor activity (Planagumà et al., 2015). These results are in accordance to the observation that patients with anti-NMDAR encephalitis may present with movement disorders, but they usually do not have motor impairment.

This was the first study using adult zebrafish as a potential disease model for anti-NMDAR encephalitis. Although the lack of locomotor changes and spontaneous seizures may be limitations of the model, we demonstrated decreased convulsive threshold and recent memory deficit after the CVMI with CSF from patients. Unlike other studies in rodents, our protocol used a single injection of anti-NMDAR positive CSF directly into the zebrafish's telencephalon. The use of zebrafish greatly reduces the amount of CSF required to the experimental model. In addition, it allows experiments with a high number of animals under a relative low-cost compared to rodent models. Moreover, there are well-established protocols to evaluate memory, behaviour and changes of convulsive threshold. Taraschenko et al. (2019) developed a mouse model of anti-*N*-methyl-D-aspartate receptor encephalitis, showing the occurrence of seizures, but not observe memory deficits, anxiety-related behaviour, or motor impairment. In contrast, Carceles-Cordon et al. (2020) observed a significant reduction of prepulse inhibition of the acoustic startle reflex, indicating psychotic-like behaviour, and progressive impairment of memory in mice infused with cerebrospinal fluid (CSF) from patients with anti-NMDAR encephalitis. Although there are differences in the behavioral changes observed in rodent models, our findings demonstrated that zebrafish injected with CSF anti-NMDAR encephalitis also developed memory impairment and a reduction in the seizure threshold, reinforcing the idea that zebrafish is a suitable animal model for studying anti-NMDAR encephalitis. Future studies with zebrafish models may investigate other behavioral changes and molecular interactions after the exposure to CSF of patients with anti-NMDAR encephalitis compared to controls.

Anti-NMDAR encephalitis is a rare and severe condition with a high risk of disability. The development of zebrafish models may provide a simple, reliable, cost-effective platform to investigate new therapeutic strategies in a more tailored approach with less risks or side-effects found in currently used treatments. Further, new specific strategies reducing the binding of pathogenic antibodies to NMDAR may impact the severity of the neuronal dysfunction and clinical disability.

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Declaration of Competing Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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