Alteration of Circadian Rhythms in 2D2 Transgenic Mice

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1,2 **Huiru Xue**
BC 1 **Xiuli Cao**
AEF 2 **Meini Zhang**

1 Department of Physiology, Shanxi Medical University, Taiyuan, Shanxi, P.R. China
2 Department of Neurology, First Hospital of Shanxi Medical University, Taiyuan, Shanxi, P.R. China

Corresponding Author: Meini Zhang, e-mail: meinizhang611@hotmai.com
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Background: Several immunological functions are dependent on circadian rhythms. However, there are still relatively few studies about circadian rhythms in neuromyelitis optica spectrum disorders (NMOSD) and 2D2 transgenic mice. We explore whether 2D2 mice have abnormalities in circadian rhythms and the potential underlying molecular mechanism.

Material/Methods: We first observed the wheel-running motion of the control and 2D2 mice using wheel-running measurements. The cytokine levels were also analyzed using enzyme-linked immunosorbent assay (ELISA), and the results of clock gene expressions in the suprachiasmatic nucleus (SCN) were investigated using real-time polymerase chain reaction (real-time PCR).

Results: The wheel-running rhythm in 2D2 mice differed from that of the controls. The TNF-α and IL-10 rhythms were disrupted in 2D2 mice. Additionally, the rhythm of the clock genes, Per1 and Per2, and expression in the SCN of 2D2 mice were also changed.

Conclusions: The results presented here indicate that alteration of circadian rhythms in 2D2 mice affects behavior and immune function, and the potential molecular mechanism might be the Per1 and Per2 expression disorders in the SCN. 2D2 mice might be a suitable model for studying circadian disruption in NMOSD.

MeSH Keywords: Behavior • Circadian Clocks • Circadian Rhythm • Cytokines • Mice, Transgenic • Neuromyelitis Optica

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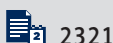
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Background

Neuromyelitis optica (NMO) is an inflammatory autoimmune demyelination disease of the central nervous system, which selectively damages the optic nerve and spinal cord and has high rates of recrudescence and disability [1]. The discovery of the aquaporin-4 (AQP-4) antibody has led to recognition that NMO is an independent disease entity distinct from multiple sclerosis (MS) [2], and the recognized spectrum of disease has expanded gradually in recent years. In 2015, the International NMO Diagnostic Team (IPND) developed a new diagnostic standard for neuromyelitis optica spectrum disorders (NMOSD), which further stratified the diagnosis of NMOSD into AQP-4-IgG-positive and -negative [1]. However, despite continuous improvement in testing technology, it is still unable to detect AQP4 antibody in some NMOSD patients. Other antibodies, such as myelin oligodendrocyte glycoprotein (MOG) antibody, can be detected in the AQP-4-IgG-negative NMOSD patients. The double-transgenic 2D2 mice, born with MOG-specific T cells and demyelinating MOG-specific antibodies in their immune repertoire, represent the AQP-4-IgG-negative NMOSD model [3–6].

A circadian rhythm is a roughly 24-h cycle determining periodicity in various physiological processes of living beings. It plays an important role in animal behavioral and physiological phenomena and disease states. This rhythm is regulated by endogenous networks of clock gene activity and is responsive to environmental cycles (especially light) [7,8]. The influence and importance of the circadian clock for some autoimmune and inflammatory disorders, for example MS, rheumatoid arthritis (RA), and inflammatory bowel disease (IBD), have been investigated [9–12]. A recent clinical study also found that sleep disturbance was associated with fatigue in NMOSD patients [13]. Although it has not been investigated in depth, these reports indicate that changes in the daily rhythms may be correlated with the disease course in NMOSD. In addition, it is thought that the optic nerve, third ventricle, and hypothalamus are the specific intracranial areas involved in NMOSD [1]. Because light signals are passed by the retina–optic nerve–hypothalamic tract to the master clock in the ventral region of the suprachiasmatic nucleus (SCN) [7,14], there is the possibility of significant impact on circadian rhythms of NMOSD and 2D2 mice. Further, if we can better understand the phenomenon and mechanism of the daily rhythms disruption in the 2D2 mouse model, it might help to find new treatments and would improve quality of life of NMOSD patients.

As the immunity reaction takes part in the pathogenesis of NMOSD and 2D2 mice, we hypothesized this may directly or indirectly disrupt the central clock gene expression in the SCN, then leading to disease. In the present study, we first observed the wheel-running motion of the control and 2D2 mice using wheel-running measurements. The levels of cytokine that displayed

circadian rhythms were also assayed to assess immune functionality in the serum of the 2 groups. Finally, we investigated the results of clock gene expressions in the SCN in order to explore the intrinsic cause of behavioral and immune disorders.

Material and Methods

Animals

2D2 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and wild-type C57BL/6 mice were obtained from the Animal Research Center of Shanxi Medical University. All procedures in the present study were approved by the Shanxi Committee on Ethics of Animal Research. All mice in these studies were group-housed at 22~24°C and were fed and watered ad libitum. The male and female 2D2 transgenic mice were mated with C57BL/6 wild-type mice (1: 1). Genomic DNA was isolated from the tails of baby mice and analyzed using PCR, as previously described [15].

Two groups of mice were used: the experimental group, consisting of the positive baby mice from 2D2 transgenic mice (n=24); and control group, consisting of the C57BL/6 mice (n=24).

Wheel-running behavior

All experimental and control mice, 8–10 weeks old (20–22 g), were placed on wheels in order to obtain wheel-running measurements. They were first maintained on a 12 h/12 h light/dark cycle (LD) for 1 week and then kept in constant darkness (DD) for 2 weeks (n=10/group). The data were recorded every 5 min using the VitaView system (Mini Mitter, Bend, OR, USA), as previously described [16].

Serum and tissue collection

Serum and SCN tissue were collected from mice at Zeitgeber time (Zt)4, Zt10, Zt16, and Zt22 (n=6/time point/group, Zt0=the time of lights on at 6 a.m.), as described in previous literature [11]. Retro-orbital blood of mice was collected. Serum was isolated after they were placed at room temperature for 1 h and then centrifuged at 1000×g for 10 min and stored frozen at –80°C until tested for tumor necrosis factor α (TNF-α) and interleukin-1 (IL-10) levels. The SCN tissue was extracted from mice and stored at –80°C until RNA isolation. Procedures for serum and SCN tissue preparation have been previously described [17,18].

Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of TNF-α and IL-10 in mice were analyzed using the Mouse TNF-α and IL-10 ELISA Kit (Elabscience, Shanghai, China), as previously described [19–21].

The sensitivities of the assays were 18.75 pg/ml for TNF-α and 9.38 pg/ml for IL-I0. The intra- and inter-assay coefficients of variation were <10% for all assays.

Real-time polymerase chain reaction (real-time PCR)

RNA was isolated from the SCN tissue using the Eastep® Super Total RNA Extraction Kit (Promega, shanghai, China) and samples were amplified on the Stratagene MX 3005 P instrument. Approximately 0.2 μg of total RNA were reverse-transcribed into cDNA using the GoScript™ Superscript Reverse Transcriptase System (Promega, shanghai, China). The Taqman primers and probes for mouse GAPDH, Per1, and Per2 were predesigned, and their mRNA expression was determined with GoTaq® qPCR Master Mix (Promega, Shanghai, China). Data were processed using the comparative threshold cycle (CT) ($2^{-\Delta\Delta CT}$) method, as previously described [22].

Statistics

For measuring the wheel-running activity, the RNA expression and cytokine levels, mean ± standard deviation (SD) was computed for each time point. Between the groups, the data were compared using the 2-tailed t test. One-Way ANOVA was used to analyze the data from different time points in each group. P-values less than or equal to 0.05 were considered statistically significant. We analyzed the data obtained from wheel-running using ActiView Biological Rhythm Analysis. Prism GraphPad 5 statistical and graphic program (GraphPad, San Diego, CA, USA) was used for statistical analysis and creating graphics.

Results

Circadian rhythm was altered in 2D2 mice

The system continuous recorded from VitalView (Figure 1A, 1B) showed that the 2 groups of mice were actively running on wheels after turning off the lights and they rested after the lights were switched on. Additionally, there was a periodic movement of about 24 h in the LD environment. The endogenous wheel-running circadian rhythm of the control group, whose mean free-running period was 23.36±0.31 h, persisted in the DD environment. However, the endogenous wheel-running circadian rhythm of 2D2 mice was not very clear; the mean free-running period was significantly shorter (22.25±0.23 h, $P<0.05$), and the magnitude of free-running significantly decreased (13300±1952 counts/day, $P<0.001$) in the experimental group compared to the control group (28260±2633 counts/day) in the DD environment (Figure 1C, 1D).

Rhythmic TNF-α and IL-10 were disrupted in 2D2 mice

Several cytokines demonstrated rhythmic patterns. In the control group, the minimum release point of the proinflammatory cytokine TNF-α was at Zt16 and secretion was time- or sleep-dependent (Figure 2A). Production of the anti-inflammatory cytokine IL-10 was higher during the night when the mice were active than during the day (Figure 2B). As the effects of the 2 cytokines are reversed, their secretory rhythms were also different (Figure 2). However, both cytokine serum levels were significantly altered in 2D2 mice. There was a statistically significant difference between the 2 groups at Zt16; however, sleep-related changes in the TNF-α production of 2D2 mice was less clear than that in the controls (Figure 2A). IL-10 levels in 2D2 mice peaked later during the rest phase (Zt10), which differed from the rhythm pattern of the control group, but this difference was not statistically significant (Figure 2B).

Per1 and Per2 expression were changed in SCN tissue of 2D2 mice

SCN tissue is the pacemaker for circadian rhythms in mammals. The expression of genes GAPDH, Per1, and Per2 was analyzed in control and 2D2 mice using real-time PCR. Figure 3A shows that the reference gene expression of GAPDH was stable during the course of the 24-h period ($P>0.1$). In contrast to GAPDH, Per1 and Per2 were rhythmically expressed in control mice (Figure 3B, 3C); their gene expression was the highest at Zt10 ($P<0.05$), showing a decrease after an initial increase. Moreover, Per1 and Per2 expression demonstrated less distinct temporal patterns among the time points studied in 2D2 mice compared to the controls. The expression of the Per1 gene during the rest phase (Zt10) in the 2D2 group was lower ($P<0.01$), and the expression of the Per2 gene during the active phase (Zt16) in the 2D2 group was higher than that of the control group ($P<0.05$, Figure 3B, 3C).

Discussion

NMOSD is the most common nervous system immune disease in Asia. Based on the observation results of clinical phenomenon and specific damage areas of NMOSD, the rhythmic damage of this disease seems to occur more easily. However, some indicators are not easy to collect from patients; thus, the 2D2 mouse model has created a convenient condition for our basic research. In this report, behavioral and immune disorders and disruption of Per1 and Per2 gene expression in the SCN were found in 2D2 mice.

The sleep-wake cycle is a best-known form of circadian rhythm. Sleep and circadian rhythm are closely intertwined, and in most cases they are coordinated to adapt the organism
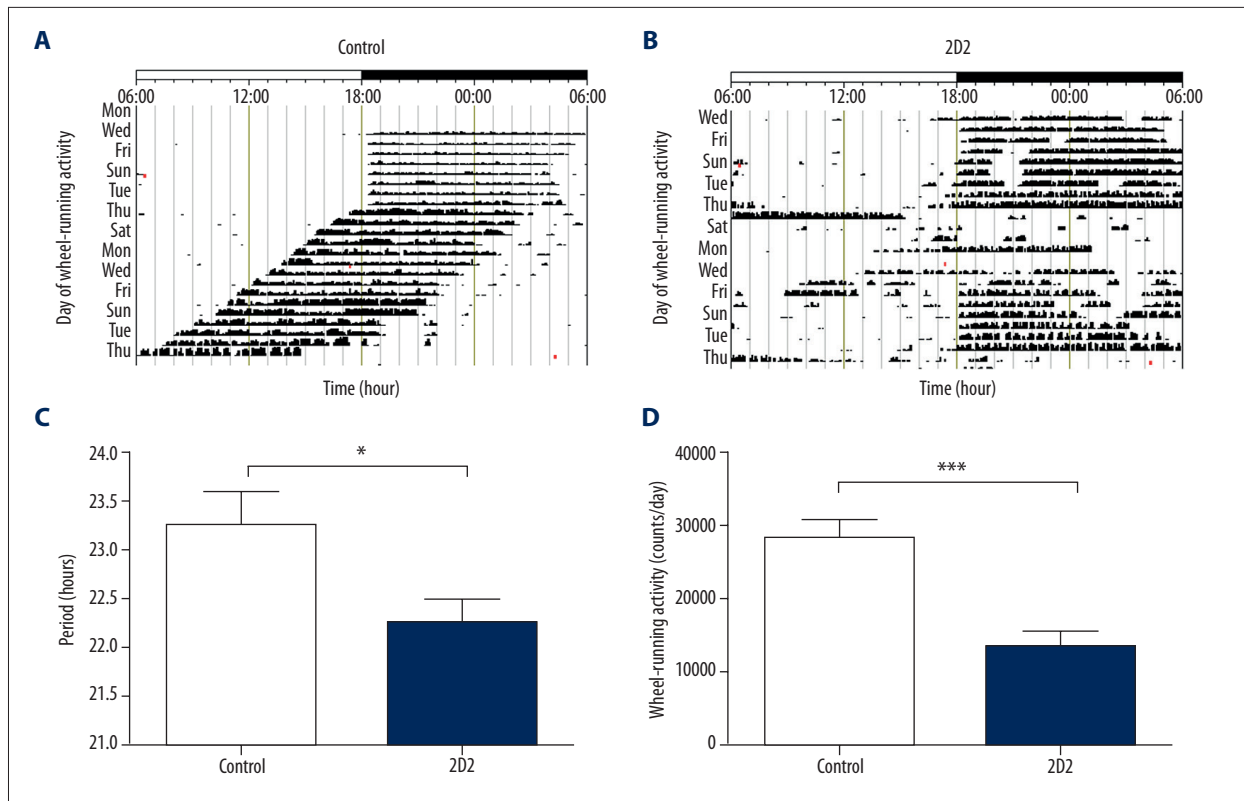
Figure 1. The circadian rhythms of control and experimental (2D2) mice. The circadian rhythm was changed in 2D2 mice. Actograms display wheel-running activity of individual mice from the control and 2D2 groups (n=10/group). The X axis represents time of day and the Y axis indicates the day of wheel-running, which includes a 1-week light/dark cycle (LD) and 2 weeks of constant darkness (DD). The black part indicates the active state, whereas the white represents the resting phase of mice. (**A**) Control group: There is a distinct motion-rest phase boundary. The mice ran on the wheel according to their own rhythm in DD. (**B**) 2D2 group: The mice exhibited disorderly activities in DD. (**C**) Bar graph showing statistical differences in the free-running period in DD between the 2 group (* *P*<0.05). (**D**) Bar graph showing statistically significant differences in the daytime activities of free-running mice in DD between the 2 group (*** *P*<0.001).

to the changing environment. Some previous reports have indicated that sleep abnormalities were common in MS and NMOSD patients [13,23,24]. Other studies reported that the chronotherapy of drug (melatonin) and non-drug (phototherapy) could improve sleep and reduce the risk of disease progression in MS [25,26]. These indicated that sleep and circadian rhythm have a significant impact on neuroimmune diseases. In animal experiments, when the subjects are kept in the DD environment, their free-running rhythms can represent the endogenous rhythms, which removes the environmental interference. Other studies revealed decreased motility and increased sleep fragmentation in experimental allergic encephalomyelitis (EAE), which was a popular animal model of MS [27,28]. In the present study, behavioral experiments showed that the free-running rhythm of 2D2 mice was disrupted, the period was shorted, and the daily activities were decreased in the DD environment. After removing the light, compared with the control group, 2D2 mice lacked the perception of day and night and their physiological functioning was affected.

In the normal sleep-wake cycle, the changes in physical activity, cardiovascular function, and thermoregulation are obvious, but there are also the changes in immune parameters such as the number and function of white blood cells, and the production and proliferation of cytokines are mostly synchronized with sleep. In earlier studies, production of proinflammatory cytokine TNF-α was found to be maximal during nocturnal sleep in humans, and production of anti-inflammatory cytokine IL-10 was increased during the daytime [29–31], probably because TNF-α and IL-10 play different roles in immune and inflammation regulation. As observed in humans, as levels of TNF-α became elevated, IL-10 levels became suppressed in C57BL/6 mice in the present study, but this is slightly different in mice. The time of change was the opposite to that in humans, as mice are nocturnal animals. Recent studies have demonstrated that circadian rhythm disorder results in an upregulation of inflammatory cytokines [12,31]. Inflammatory cytokines were proved to be up-regulated by circadian rhythm disorder in another study [32]. These results are consistent with those of the present
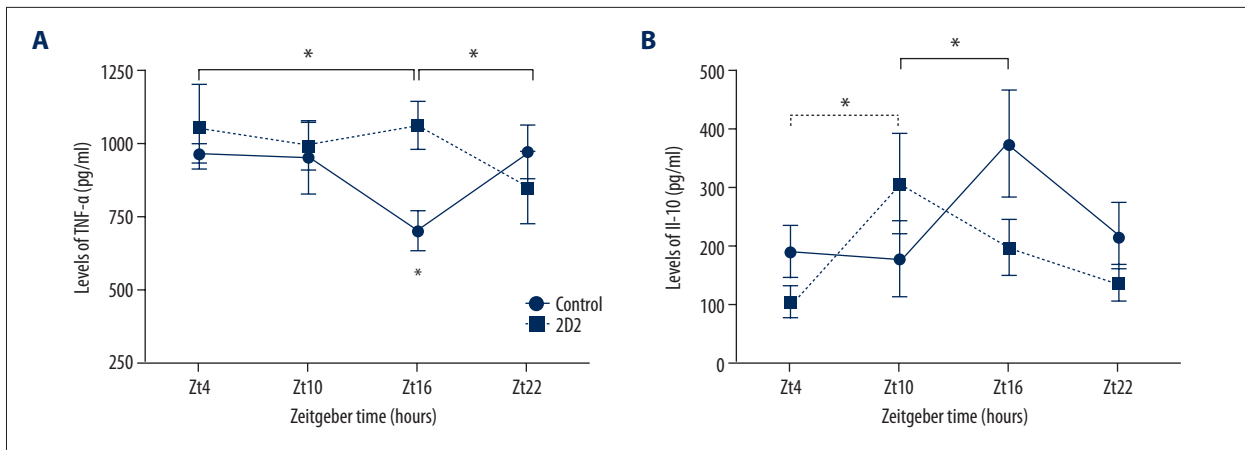
Figure 2. Levels of TNF-α (pg/ml) and IL-10 (pg/ml) in control and experimental (2D2) mice. Serum levels of TNF-α and IL-10 were detectable in each subject at 4 time points using ELISA (n=6/time point/group). Zeitgeber time (Zt) 0 was equal to the time that the lights were on. The serum level of rhythmic cytokine TNF-α (**A**) and IL-10 (**B**) was tested at different time points in the 24-h period in individual control and 2D2 mice. (**A**) TNF-α levels in control mice was significantly suppressed during the active phase (Zt 16); (Zt16 versus Zt4 or Zt22 p<0.05). TNF-α levels in 2D2 mice were elevated at all-time points tested with no significant differences in peak levels. (**B**) IL-10 levels in control mice peaked significantly during the active phase (Zt16) (Zt16 versus Zt10, p<0.05), while IL-10 levels in 2D2 mice peaked later during the rest phase (Zt 10) (Zt10 versus Zt4, p<0.05).
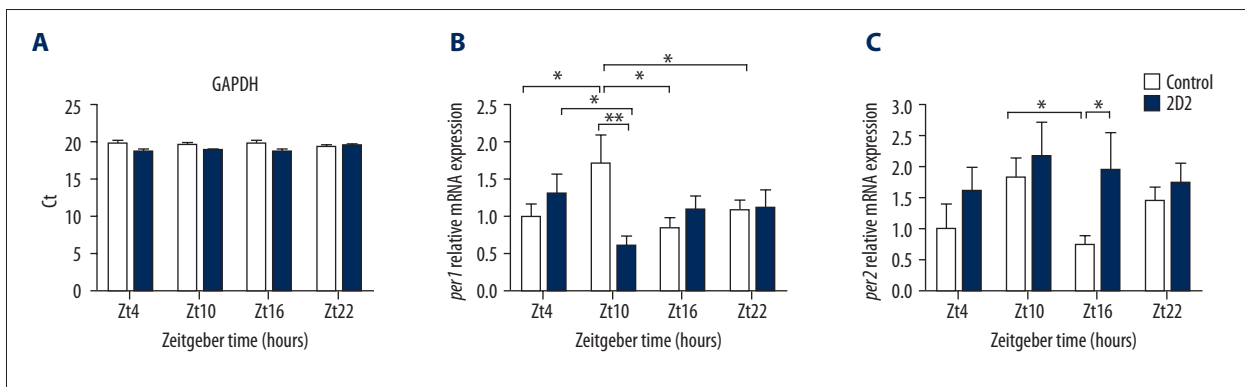

Figure 3. Per1 and Per2 gene expression in control and experimental (2D2) mice. RNA was isolated from the SCN tissue collected from the control and the 2D2 group (n=6/time point/group). Referencing the GAPDH gene, clock Per1 and Per2 gene expression were analyzed using real-time PCR. Ct meant threshold cycle and Zeitgeber time (Zt) 0 was equal to the time that the lights were on. (**A**) Statistical analysis of the differences in the means between the groups demonstrated that there was no significant difference between the time points investigated for GAPDH (*P*=0.1 to *P*=0.9). (**B, C**) Real-time PCR analysis of the mouse Per1 and Per2 gene expression demonstrated a time-dependent expression pattern over the 24-h period in control mice. Per1 analysis showed that the peak expression occurred at Zt10 and was significantly different from the expression at Zt4, Zt16, and Zt10 (* *P*<0.05). Additionally, Per2 expression at Zt10 was significantly different from the expression at Zt16 (* *P*<0.05). In the 2D2 group, Per1 analysis showed that peak expression was at Zt4, which was significantly different from the expression at Zt10 (* *P*<0.05), and the Per2 gene expression did not significantly vary over the 24-h period. Comparisons of means at Zt10 for Per1 expression (** *P*<0.01) and Zt16 in Per2 expression (* *P*<0.05) demonstrated statistically significant differences between the 2 groups.

study. We found that the levels of TNF-α and IL-10 were also altered in 2D2 mice. In both cytokines, the result changed to a less discernable daily rhythm compared to the control group.

The SCN of the hypothalamus is the master pacemaker in mammals that synchronizes the circadian rhythms of different

peripheral organs, in time and space via paracrine secretion, and neural connections [33]. The rhythmic expression of the Per1 and Per2 in the SCN has a crucial role in circadian rhythms [33,34]. The clock genes and their encoded clock proteins form an automatic feedback loop that is a universal molecular mechanism for the operation of the biological clock.

A previous study revealed that the rhythms of mPer1 and mPer2 in the SCN were slightly phase-delayed and were lower for rhythm-splitting mice than for rhythm-unsplit mice [35]. The present data showed that there was disorganized expression of the genes Per1 and Per2 in the SCN of 2D2 mice. That is consonant with behavior disorders and cytokine release. Because there is autoimmune-mediated destruction of hypothalamic and nerve fibers, it is possible that there is direct damage to SCN neurons in NMOSD and 2D2 mice. Therefore, it is reasonable to propose that the disruption of Per1 and Per2 expressions in the SCN could lead to sleep disturbances and immune disorders in 2D2 mice.

This study had several limitations. First, patients with NMOSD have numerous reasons for sleep disruption, including pain, anxiety, depression, and concomitant use of steroids. These factors can also affect the patient's disease progression and are difficult to fully represent in an animal model. This motivates us to study these factors in further research. Second, it is currently unclear whether disruption of circadian rhythms induces the initiation of NMOSD and 2D2 mice, or if it is a potentially modifiable factor for disease exacerbation. This relationship should be investigated in future studies.

Conclusions

In conclusion, the present study provides evidence of alterations of circadian rhythms in the 2D2 mouse model, which provides experimental evidence for the clinical study of circadian rhythm abnormality in NMOSD patients. Better understanding of the mechanism underlying regulation of immunity by the central circadian clock may be beneficial for devising new therapies and improving quality of life for NMOSD patients.

Acknowledgements

Conflict of interest

None.

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