Journal of Neuroimmune Pharmacology
https://doi.org/10.1007/s11481-023-10089-4

RESEARCH

Prenatal SARS-CoV-2 Spike Protein Exposure Induces Autism-Like Neurobehavioral Changes in Male Neonatal Rats

Mumin Alper Erdogan[1] · Miray Turk[2] · Gizem Dinler Doganay[2,3] · Ibrahim Halil Sever[4] · Bahattin Ozkul[5] · Ibrahim Sogut[6] · Ebru Eroglu[7] · Yigit Uyanikgil[7] · Oytun Erbas[8]

Received: 28 May 2023 / Accepted: 17 October 2023

Abstract

Recent research on placental, embryo, and brain organoids suggests that the COVID-19 virus may potentially affect embryonic organs, including the brain. Given the established link between SARS-CoV-2 spike protein and neuroinflammation, we sought to investigate the effects of exposure to this protein during pregnancy. We divided pregnant rats into three groups: Group 1 received a 1 ml/kg saline solution, Group 2 received 150 µg/kg adjuvant aluminum hydroxide (AAH), and Group 3 received 40 µg/kg spike protein + 150 µg/kg AAH at 10 and 14 days of gestation. On postnatal day 21 (P21), we randomly separated 60 littermates (10 male–female) into control, AAH-exposed, and spike protein-exposed groups. At P50, we conducted behavioral analyses on these mature animals and performed MR spectroscopy. Subsequently, all animals were sacrificed, and their brains were subject to biochemical and histological analysis. Our findings indicate that male rats exposed to the spike protein displayed a higher rate of impaired performance on behavioral studies, including the three-chamber social test, passive avoidance learning analysis, open field test, rotarod test, and novelty-induced cultivation behavior, indicative of autistic symptoms. Exposure to the spike protein (male) induced gliosis and neuronal cell death in the CA1-CA3 regions of the hippocampus and cerebellum. The spike protein-exposed male rats exhibited significantly greater levels of malondialdehyde (MDA), tumor necrosis factor alpha (TNF-α), interleukin-17 (IL-17), nuclear factor kappa B (NF-κB), and lactate and lower levels of brain-derived neurotrophic factor (BDNF) than the control group. Our study suggests a potential association between prenatal exposure to COVID-19 spike protein and neurodevelopmental problems, such as ASD. These findings highlight the importance of further research into the potential effects of the COVID-19 virus on embryonic and fetal development and the potential long-term consequences for neurodevelopment.

Keywords COVID-19 · SARS-CoV-2 · Spike protein · Neurodegeneration · Neuroinflammation · Autism spectrum disorder

Introduction

The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a worldwide impact (Lu et al. 2020; Cucinotta and Vanelli 2020). Neurological and mental symptoms are also observed in COVID-19 patients, ranging from headaches to cognitive and mood abnormalities, in addition to respiratory-related symptoms, as reported by a growing body of research (Hampshire et al. 2021; Xiong et al. 2020). Although the

✉ Mumin Alper Erdogan
alpero86@gmail.com

[1] Faculty of Medicine, Department of Physiology, Izmir Katip Celebi University, Izmir, Turkey

[2] Graduate School, Department of Molecular Biology-Genetics and Biotechnology, Istanbul Technical University, 34469 Istanbul, Turkey

[3] Faculty of Science and Letters, Department of Molecular Biology and Genetics, Istanbul Technical University, 34469 Istanbul, Turkey

[4] Faculty of Medicine, Department of Radiology, Demiroğlu Bilim University, Istanbul, Turkey

[5] School of Medicine, Department of Radiology, Istanbul Atlas University, Istanbul, Turkey

[6] Faculty of Medicine, Department of Biochemistry, Demiroğlu Bilim University, Istanbul, Turkey

[7] Faculty of Medicine, Department of Histology and Embryology, Ege University, Izmir, Turkey

[8] Faculty of Medicine, Department of Physiology, Demiroğlu Bilim University, Istanbul, Turkey

Published online: 27 October 2023

exact molecular and cellular mechanisms underlying the impact of SARS-CoV-2 on the central nervous system (CNS) are still unclear, the presence of SARS-CoV-2 spike protein and transcripts in post-mortem brains suggests direct CNS infection by the virus. Furthermore, recent studies have revealed that radiolabeled S1 subunit of SARS-CoV-2 spike protein (S1 protein) can cross the blood–brain barrier when injected intravenously, indicating that S1 protein in the brain parenchyma may impact cognitive processes and contribute to COVID-19 patients' neurological or psychiatric symptoms (Rhea et al. 2021).

The potential neurodevelopmental impact of prenatal exposure to SARS-CoV-2 is a major concern, as even a slight increase in the risk of adverse neurodevelopmental outcomes could have a significant public health impact due to the large number of individuals globally exposed to the virus (Volkow et al. 2021; Lins 2021; Lopez-Diaz et al. 2021; Sakurada et al. 2020; Figueiredo et al. 2021; Okechukwu 2021). This is especially relevant given the staggering number of COVID-19 cases worldwide, with over 59 million people in the United States alone and approximately 300 million individuals worldwide diagnosed with the disease. Moreover, the virus has affected over 155,500 pregnant women in the United States (Centers for Disease Control and Prevention 2021).

Autism spectrum disorder (ASD) is a condition that affects an individual's social communication, interaction, and behavior. The etiology of ASD is not fully understood, but evidence suggests that genetic and environmental factors may play a role in its development (American Psychiatric Association 2013; Hallmayer et al. 2011).

One area of research that has gained attention in recent years is the role of neuroinflammation in the development and progression of ASD. Neuroinflammation is a complex process involving activation of immune cells in the brain and release of inflammatory molecules. While some inflammation in the brain is a normal response to injury or infection, chronic or excessive inflammation can be harmful and contribute to neurodegeneration and neurological disorders (Vargas et al. 2005; Onore and Careaga 2019).

Studies have shown that individuals with ASD often have increased levels of inflammatory markers in their blood and cerebrospinal fluid, suggesting that neuroinflammation may be a feature of the disorder. Additionally, postmortem studies have revealed microglial activation (a type of immune cell in the brain) in the brains of individuals with ASD (Bilbo and Schwarz 2012; Estes and McAllister 2015).

While the exact mechanisms linking neuroinflammation and ASD are not fully understood, researchers have proposed several potential pathways. One theory is that prenatal exposure to inflammation (such as maternal infection) may alter fetal brain development and increase the risk of ASD. Other researchers have suggested that environmental toxins or disruptions in the gut microbiome could trigger immune activation and contribute to neuroinflammation in individuals with ASD (Song et al. 2021a; Siniscalco and Antonucci 2022).

Despite the growing evidence linking neuroinflammation and ASD, it is important to note that not all individuals with ASD have evidence of inflammation, and not all cases of inflammation lead to ASD (National Institute of Mental Health 2023). Further research is needed to better understand the complex relationship between neuroinflammation and ASD and to identify potential targets for treatment or prevention.

In addition to the potential role of neuroinflammation, recent studies have also explored the relationship between ASD and the spike protein of SARS-CoV-2, the virus responsible for the COVID-19 pandemic. The spike protein is a key component of the virus that allows it to enter human cells and cause infection (Vargas et al. 2005; Li et al. 2009; Masi et al. 2017).

One study published in the journal Molecular Autism in May 2021 reported that the spike protein could interact with certain proteins in the brain that are implicated in ASD, potentially leading to neuroinflammation and neuronal damage. The study was conducted in mice and used a synthetic version of the spike protein, rather than the actual virus (Bauman et al. 2014; Hsiao et al. 2012; Onore et al. 2014).

It should be emphasized that the results of this study have not yet been replicated in humans, and further investigation is necessary to confirm these outcomes. Moreover, it remains unclear whether individuals with ASD are more susceptible to severe COVID-19 infection, or whether COVID-19 infection itself could play a role in the development or worsening of ASD symptoms (Chauhan et al. 2011; Rossi and Navarro 2021).

Overall, the relationship between the spike protein of SARS-CoV-2 and ASD is an area of ongoing research, and more studies are needed to fully understand the potential implications. As with any new research findings, it is important to interpret them with caution and to await further validation before drawing definitive conclusions or making clinical recommendations (Baig et al. 2020; Wadman 2021; Valdespino-Gomez et al. 2022; Kinnunen et al. 2022).

In light of the growing body of research indicating a connection between SARS-CoV-2 infection and neurological symptoms, we conducted a study to examine the potential impact of a synthetic version of the SARS-CoV-2 spike protein on the development of autism spectrum disorder (ASD) in offspring born to mothers exposed to the protein during pregnancy. Our research aimed to shed further light on the complex relationship between the virus and the development of neurological conditions, particularly ASD.

Materials and Methods

Animals

The experimental procedures used in the current study were approved by the Animal Ethics Committee (04220903), and rats from the Experimental Animal Laboratory of Science University were utilized. All experiments were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (U.S.).

Adult Wistar rats, consisting of 18 females and 6 males weighing 220 ± 10 g, were housed in standard plastic cages under controlled conditions with a 12-h light/dark cycle and maintained at a constant temperature of 22 ± 2 °C.

Production and Purification of S1-Fc of Spike Protein from SARS-CoV-2

From Addgene, a plasmid encoding the whole S1 protein of the spike protein (amino acids M1 to R682) was bought (#164220), together with Fc and His affinity tags. In Expi293 Expression Medium (A1435101, Gibco, Grand Island, NY, USA), Expi293 cells were grown at 37 °C with 8% CO2 in a humidified incubator. At 3×10^6 cells/ml, cells were subcultured, and cell viability was maintained over 90% throughout the cell culture procedure. For transfection, a calculated amount of cells (3×10^7 cells/30 ml) were added to warmed Expi293 Expression Media in a single-use, sterile PETG Erlenmeyer flask (781011, Nest Scientific, USA) that was 125 ml in size. The final volume was then adjusted to 30 ml. 30 µg of the S1-FcHis coding plasmid and 3 µl of the PEI transfection agent were used to transfect the cells. After five days of incubation, the protein that was released into the medium was collected and filtered before being purified. Binding/washing buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) was used to dilute the media in a 1:1 ratio. The media was then put onto a 1 ml MabSelect SuRe (11003493, Cytiva, Uppsala, Sweden) column and eluted with 100 mM glycine, pH 3. Proteins were concentrated using an Amicon® Ultra-15 Centrifugal Filter Unit (UFC903024, Millipore, Burlington, MA, USA), buffer switched to PBS, and then aliquoted and kept at -80 °C. Proteins were run on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and then detected using an anti-RBD antibody to determine the purity of S1-FcHis (40592-T62, SinoBiological, Beijing, China). SDS-PAGE analysis was used to detect molecular weight and purity simultaneously (Fig. 1).

Study Design

To assess the potential effects of COVID-19 Spike Protein exposure during pregnancy, three groups of six female rats each were randomly assigned. Group 1 (Normal Control), Group 2 (Adjuvant Aluminum Hydroxide, AluHydrox™, InvivoGen, San Diego, CA, USA), and Group 3 (COVID-19 Spike Protein and Adjuvant Aluminum hydroxide). Throughout the study, the behavior and health of every animal were monitored daily using the Scove Systems™, Izmir, Turkey. Female rats were paired with a fertile male (three females/one male) for two to three days during their oestrus cycle, and their vaginal plaque was examined to assess mating. After mating, the male rats were removed from their cages.

Between days 10–14 of pregnancy, Group 1 rats were given 1 ml/kg of 0.9 NaCl saline (Braun Medical Inc., Bethlehem, PA, USA), Group 2 rats were given 150 µg/kg of adjuvant aluminum hydroxide, and Group 3 rats were given

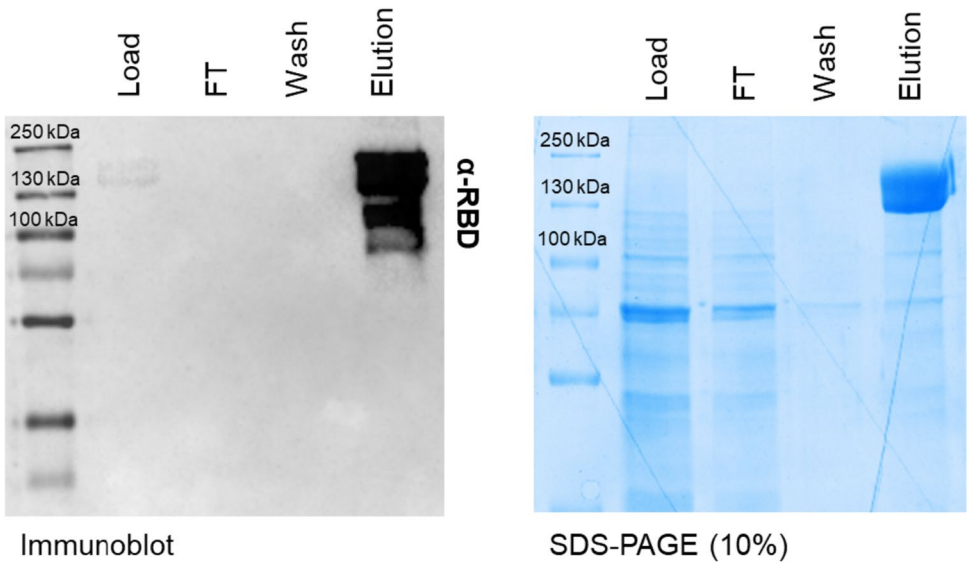

Fig. 1 Immunoblot and SDS-PAGE results of purified S1-Fc domain of Spike protein

150 µg/kg of adjuvant aluminum hydroxide and 40 µg/kg of COVID-19 Spike protein. The number of pups per dam was limited to 9 on the day of birth to ensure consistent maternal care. The dams were allowed to rear their own litters until weaning on postnatal day 21 (P21). At P21, 60 littermates (10 male and 10 female controls, 10 male and 10 female Adjuvant Aluminum hydroxide-exposed, and 10 male and 10 female Spike protein and Adjuvant Aluminum hydroxide-exposed) were randomly separated and housed in cages by sex and study group with unlimited access to standard food and tap water. Adult testing was conducted on P50 using the Scove Systems™, Izmir, Turkey. All behavioral trials were conducted between 10:00 AM and 15:00 PM.

Animals underwent an MR spectroscopy technique after a behavioral test while being given 50 mg/kg of ketamine as anesthesia. At the conclusion of the experiment, all animals underwent sacrification (cervical dislocation) under anesthesia using (100 mg/kg, Ketasol, Richterpharma AG Austria)/xylazine (50 mg/kg, Rompun, Bayer, Germany) and had their brains taken for biochemical and histological examination.

Behavioral Tests

Three-Chamber Sociability and Social Novelty Test

Three-chamber sociability and social novelty test was conducted, with some adjustments to prior descriptions (Erbas et al. 2018; Moy et al. 2004; Ellegood and Crawley 2015). The test consisted of a Plexiglas cage measuring 40 cm × 90 cm × 40 cm, with three identical Sects. (40 cm × 30 cm × 40 cm) created inside. Rats were given a pre-test session of five minutes to familiarize themselves with the testing environment on the first day. The following day, a different rat was introduced into a small plastic cage with mesh-like openings in one side chamber, while the third compartment remained empty to assess the rats' sociability. The test rat was then placed in the central chamber, and its behavior was recorded for 10 min to determine the time spent in each area (sociability test). The rat's head and two front paws entering the chamber were considered an entry. The testing area was cleaned with a 70% alcohol solution between each test and then dried with paper towels to eliminate any remaining odor from the previous rat.

Open Field Test

For the open field test, a box measuring 50 cm × 50 cm × 40 cm was utilized (Erbas et al. 2018). The rats were placed gently in the center of the box at the start of the test and allowed to freely explore the area for five minutes. Each rat's spontaneous activity level was observed for 5 min, and the total number of ambulations (floor divisions crossed using all four paws) was recorded. To eliminate any odor cues, the floor of the box was cleaned with a 70% alcohol-water solution and dried with paper towels between each rat.

Novelty-Induced Rearing Behavior

To assess novelty-induced rearing behavior, animals were immediately transferred from their home cages to a clear Plexiglas cage (50 cm × 50 cm × 40 cm) (Erbaş et al. 2013). The frequency of rearing behavior, defined as the number of times the animal stood on its hind limbs with its forelimbs against the walls of the observation box or free in the air, was recorded for five minutes. Two blinded observers tracked each rat individually. The arena was cleaned with 70% alcohol to eliminate olfactory cues before introducing a new animal.

Rotarod Test

Animal performance and motor coordination were evaluated using the Rotarod test device. The device comprises of a spinning rod, a power supply, and a location where the rat may fall safely below the moving rod. Prior to doing the actual research, all the animals were trained on this equipment to ensure appropriate performance. Three days of training were completed, using an accelerating regimen that increased the rotations per minute (rpm) from 4 to 40 in 5 min. On the last day of the experiment, photocells automatically recorded the latency to fall, and the cumulative latencies on the rod were examined (Carter et al. 2001; ELBeltagy et al. 2010).

Passive Avoidance Learning (PAL)

As previously mentioned (Erbas et al. 2018), the passive avoidance learning (PAL) test was used to assess the learning and memory abilities of offspring. The rat learns to avoid opening a door that appears to be safer but instead goes into a dark compartment with an electric grid system that shocks it through the use of fear-motivated avoidance tasks in PAL. The PAL box included chambers that were both dark and lit and measured 20 cm × 20 cm × 20 cm. Rats often chose to enter the dark room when they were placed in the illuminated compartment. The guillotine door between the light and dark chambers was opened after a 10-s habituation time in the lit compartment. The door dividing the light and dark chambers was shut when a rat entered the dark chamber. Then, a 1.5 mA electric shock was administered over three seconds, after which the rat was taken out of the chamber's darkness and put back in its cage. The rats were put back into the PAL box after twenty-four hours. Although no shock was administered, the amount of time it took the rat to move from the lit to the dark chamber was timed and recorded. It was possible to measure the delay for up to 300 s. The

rat's memory was measured by the amount of time it took to decide against entering the darkened chamber.

Hippocampus Histopathology

To investigate potential hippocampal injury, the CA1 and CA3 regions of the hippocampus were chosen as target areas. For GFAP staining, our primary areas of interest were CA1-CA3 regions of the hippocampus and the cerebellar cortex, specifically within the intermediate discharge layer. After the behavioral experiments, the rats were euthanized, and their brains were extracted and fixed in 10% formaldehyde in 0.1 M phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) for three days, followed by PBS. The brains were then immersed in 30% sucrose (Fisher Scientific, Waltham, MA, USA) and stored at 4 °C. For immunostaining purposes, sections were prepared at a thickness of 40 µm using a sliding microtome (Leica Biosystems, Wetzlar, Germany). However, for the neuronal counting, we utilized thinner sections at a thickness of 8 µm. The sections were mounted on gelatin-coated slides and stained with cresyl violet (Merck, Darmstadt, Germany). An image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) was used to count the number of surviving neurons in six slices per group.

In order to perform GFAP immunohistochemistry, brain slices were first blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA, USA) for one hour at room temperature, after being treated with 10% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) for thirty minutes to remove any endogenous peroxidase activity. After that, the sections were incubated with primary antibodies against GFAP (Abcam, Inc., Massachusetts, United States; 1/1000) for twenty-four hours at a temperature of four degrees Celsius. In order to identify antibodies directed against rabbit IgG, the Histostain-Plus Bulk kit from Invitrogen, Carlsbad, CA, USA was used. Furthermore, 3,3′ diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) was applied in order to see the end result. After washing each slice in PBS, the Olympus C-5050 digital camera attached to an Olympus BX51 microscope was used to take photographs of the sections. In order to compute the GFAP immunostaining index, GFAP-positive cells in each rat's tissue were counted at a magnification of 40 times in three to four randomly chosen sections. After visualization using, images were captured at 40×using a Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). For quantification, select brain areas were defined as regions of interest (ROIs). Using Image-Pro Express 1.4.5, Media Cybernetics, Inc. Rockville, MD, USA, the density and intensity of GFAP-positive cells within these ROIs were measured. The GFAP immunostaining index was then calculated as the average cell density multiplied by mean staining intensity, normalized to controls. The same investigator carried out each and every histological investigation, and this investigator was blinded to the research groups throughout the whole process.

Systematic Random Sampling and Cell Counting

Following the tissue preparation, sections from target areas, including the CA1 and CA3 regions of the hippocampus and the cerebellum, were obtained. In total, 30 sections were procured from each brain region of interest for each animal. To maintain consistency and minimize biases during the quantification process, we employed a systematic random sampling strategy. From the available sections, every 5th section was selected for analysis, resulting in 6 representative sections from each region of interest for each individual animal.

For the quantification of positive cells:

- In the Hippocampal Region: Cells were identified and counted within three cell breadths of the internal rim of both blades of the hippocampal dentate gyrus, as this region has been demonstrated to be significant for observing changes in cell populations (ELBeltagy et al. 2010).
- In the Cerebellum: Positive cells were quantified within designated regions of the molecular and granular layers, ensuring that these regions were consistent across all selected sections and animals.

Tissue Biochemical Analysis

After decapitation, brains were swiftly collected and kept at 20 °C for further biochemical analysis. The whole brain tissues were homogenized with a glass homogenizer in 5 volumes of phosphate buffered saline (pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA), and the resulting homogenate was then centrifuged (Eppendorf, Hamburg, Germany) at 5000 g for 15 min. The supernatant was collected, and the total protein content in the brain homogenates was determined using Bradford's technique (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a reference (Bradford 1976).

To measure levels of nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α), interleukin-17 (IL-17), brain-derived neurotrophic factor (BDNF), and lactate in the rat brains, enzyme-linked immunosorbent assay (ELISA) kits designed specifically for rats were used. These kits were commercially available and obtained from Biosciences (BD Biosciences, San Jose, CA, USA) and Abcam (Abcam, Cambridge, MA, USA). Brain supernatants were prepared from each animal and measured in triplicate according to the manufacturer's instructions. A microplate reader (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, Waltham, MA, US) was used to measure absorbances and quantify the levels of these proteins and molecules in the brain samples.

This allowed us to gain insights into the effects of the experimental conditions on the rats' brain function.

Measurement of Brain Lipid Peroxidation

Malondialdehyde (MDA), which is a reactive molecule for thiobarbituric acid, was measured as a measure of lipid peroxidation in brain tissue samples (TBARS). The brain tissue samples were briefly treated by adding trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) and the TBARS reagent (Cayman Chemical, Ann Arbor, MI, USA), mixing them, and then incubating them at 100 °C for 60 min in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The samples were centrifuged for 20 min at 3000 rpm with ice chilling between cycles, reading the absorbance of the supernatant at 535 nm. Tetraethoxypropane (Sigma-Aldrich, St. Louis, MO, USA) was used to determine MDA levels, which were then quantified at nmol/gr protein.

MRI and MR Spectroscopy

After administering an anesthetic agent, all exams were conducted using a 3.0 T clinical MRI/MRS scanner (GE SIGNA™ Pioneer, Piscataway, NJ, USA) in the supine position without the use of contrast material. Rats were sedated using an intraperitoneal ketamine/xylazine combination. To eliminate motion artifacts, a specially made plastic head holder was installed on the scanning table. Using a water-heating pad, the rats' body temperatures were maintained at 37 C during the imaging procedure. Using a 16-channel flex coil, excitation and signal detection were carried out (GE Healthcare, Piscataway, NJ, USA). Scout pictures were obtained, followed by T2 weighted coronal section images and an MRS assessment to wrap up the program.

T2-weighted spin echo imaging was used with specific imaging parameters of TR/TE = 2690/102 ms, gap = 0.2 mm, FOV = 33 × 33 mm^2, slice thickness = 2 mm, 256 × 256 pixel matrix, 175 Hz/pixel bandwidth, number of captures = 2, and a total of 12 slices. The right corpus striatum was investigated using spectroscopy. For 1H-MRS, a multivoxel 3D chemical shift imaging sequence was employed with phase encoding x/y of 24/24, a repetition time of 1000 ms, an echo duration of 35 ms, and a voxel size of 2 × 2 × 4 mm.

Contrasting the spectra (1.3 ppm for lactate) obtained in a 16 µl volume of each rat allowed researchers to determine the lactate concentrations in the right corpus striatum of the various groups.

Using the observed signal of free induction decay, the Fourier-transformed MRS was produced. GE Healthcare software was used to process data from a workstation (GE Healthcare, Piscataway, NJ, USA).

Statistical Analysis

All statistical analyses were conducted using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Prior to conducting the main analyses, we ensured that our data met the necessary assumptions. Levene's test was used to verify the homogeneity of variances, and Shapiro–Wilk's test was employed to confirm the normality of the data distribution.

For variables that satisfied these assumptions, a One-way Analysis of Variance (ANOVA) was applied. When significant main effects were detected through ANOVA, post-hoc comparisons were made using the Tukey's HSD (Honestly Significant Difference) test to identify specific differences between groups.

Results are presented as mean ± standard error of the mean (SEM). A p-value of less than 0.05 was considered statistically significant.

Results

Based on the results presented in the tables, the effects of COVID-19 spike protein and adjuvant aluminum hydroxide on various parameters were investigated in normal male and female rats. The statistical analyses were performed by one-way ANOVA, and the significant differences were determined with p-values < 0.05, < 0.01, or < 0.001, depending on the experiment.

Behavior Analysis Results

Behavioral analysis results showed significant differences between the adjuvant aluminum hydroxide and COVID-19 spike protein with adjuvant aluminum hydroxide groups in male rats. In the sociability test, the spend of time with stranger rat percent was significantly decreased in the COVID-19 spike protein with adjuvant aluminum hydroxide group compared to the adjuvant aluminum hydroxide group ($p < 0.05$). Similarly, in the open field test, the number of ambulations was significantly decreased in the COVID-19 spike protein with adjuvant aluminum hydroxide group compared to the adjuvant aluminum hydroxide group ($p < 0.001$). Novelty-induced rearing behavior and passive avoidance learning latency were also significantly reduced in the COVID-19 spike protein with adjuvant aluminum hydroxide group compared to the adjuvant aluminum hydroxide group ($p < 0.001$) (Table 1).

In female rats, no significant differences were observed in sociability test, sociability index, passive avoidance learning latency, and latency time to fall between the groups. However, in the open field test, the number of ambulations was significantly decreased in the COVID-19 spike protein

Table 1 Behavioral Analysis Results for Male–Female Groups

Sex	Male Groups			Female Groups		
Groups	**Normal Male Group**	**Adjuvant Aluminum hydroxide Male group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group**	**Normal Female Group**	**Adjuvant Aluminum hydroxide Female group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group**
Sociability test: The spend of time with stranger rat percent (%)	61.8 ± 3.3	56.7 ± 2.8	51.2 ± 8.9 *	58.7 ± 0.9	55.4 ± 1.1	60.1 ± 4.2
Sociability Index (stranger/empty)	2.8 ± 0.3	3.04 ± 0.9	2.7 ± 0.8	2.4 ± 0.3	2.1 ± 0.2	2.7 ± 0.4
Open Field Test: Number of ambulation	20.9 ± 4.8	19.6 ± 2.03	7.5 ± 2.9 ***	22.5 ± 1.6	18.1 ± 2.6	16.3 ± 2.6 #
Novelty-Induced Rearing Behavior	19.5 ± 1.7	22.1 ± 1.5	5.5 ± 1.6 ***	24.7 ± 1.9	19.7 ± 3.2	15.9 ± 2.8 #
Passive avoidance learning (PAL) Latency (Sec.)	259.5 ± 18.1	248.2 ± 24.3	37.5 ± 12.4 ***	240.9 ± 26.8	255.7 ± 29.5	225.7 ± 41.6
Latency time to fall (sec.)	264.3 ± 34.8	248.5 ± 48.3	180.3 ± 26.4 ***	286.5 ± 5.9	292.6 ± 7.7	265.2 ± 16.5

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * $p < 0.05$, different from Adjuvant Aluminum hydroxide Male group, *** $p < 0.001$, different from Adjuvant Aluminum hydroxide Male group. # $p < 0.05$, different from Adjuvant Aluminum hydroxide Female group

with adjuvant aluminum hydroxide group compared to the adjuvant aluminum hydroxide group ($p < 0.05$). Similarly, novelty-induced rearing behavior was significantly reduced in the COVID-19 spike protein with adjuvant aluminum hydroxide group compared to the adjuvant aluminum hydroxide group ($p < 0.05$) (Table 1).

In summary, the results suggest that the COVID-19 spike protein with adjuvant aluminum hydroxide may affect the behavior of male and female rats differently. Male rats exhibited a significant reduction in sociability, open field activity, novelty-induced rearing behavior, and passive avoidance learning latency, while female rats showed a significant decrease in open field activity and novelty-induced rearing behavior.

Biochemistry Results

In male rats, the brain levels of TNF-α, IL-17, and NF-κB increased significantly in response to COVID-19 spike protein and adjuvant aluminum hydroxide compared to the control group (TNF-α: $p < 0.01$, IL-17: $p < 0.01$, NF-κB: $p < 0.05$). Moreover, the brain level of MDA and lactate increased, and BDNF decreased significantly in male rats that received COVID-19 spike protein and adjuvant aluminum hydroxide compared to the control group (MDA: $p < 0.01$, lactate: $p < 0.05$, BDNF: $p < 0.01$) (Table 2). In the female rat groups, there were significant differences in MDA, IL-17, and NF-κB levels between the control and COVID-19 spike protein and adjuvant aluminum hydroxide groups (MDA: $p < 0.05$, IL-17: $p < 0.01$, NF-κB: $p < 0.01$). Specifically, MDA levels did not show a significant difference between the control and the aluminum hydroxide group, but there was a significant increase in MDA levels in the COVID-19 spike protein and adjuvant aluminum hydroxide group ($p < 0.05$). Similarly, there was a significant increase in IL-17 and NF-κB levels in the COVID-19 spike protein and adjuvant aluminum hydroxide group compared to the control and aluminum hydroxide groups (IL-17: $p < 0.01$, NF-κB: $p < 0.01$) (Table 2). The brain levels of lactate and BDNF did not show any significant differences among the groups (Table 2).

Histological Findings

Histological analysis results showed that in male rats, the neuronal counts in CA1 and CA3 regions of the hippocampus were significantly decreased in response to COVID-19 spike protein and adjuvant aluminum hydroxide compared to the control group, with $p < 0.05$ (Fig. 2) (Table 3). Similarly, the GFAP immunostaining index in CA1 and CA3 regions of the hippocampus and cerebellum were significantly increased in the COVID-19 spike protein and adjuvant aluminum hydroxide group compared to the control group, with $p < 0.05$ (Figs. 3 and 4) (Table 3). On the other hand, in female rats, there were no significant differences

Table 2 Biochemistry Results for Male–Female Groups

Sex	Male Groups			Female Groups		
Groups	**Normal Male Group**	**Adjuvant Aluminum hydroxide Male group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group**	**Normal Female Group**	**Adjuvant Aluminum hydroxide Female group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group**
Brain MDA level (nmol/gr protein)	2.14 ± 0.2	2.3 ± 0.3	3.08 ± 0.2 *	2.02 ± 0.3	2.4 ± 0.2	2.8 ± 0.2 #
Brain TNF-alpha level (pg/mg protein)	22.6 ± 3.9	24.1 ± 5.5	51.6 ± 4.7 **	23.5 ± 1.7	20.3 ± 2.9	33.5 ± 6.8 #
Brain IL-17 level (pg/g protein)	212.7 ± 15.3	209.1 ± 18.6	388.9 ± 14.5 **	197.5 ± 10.1	205.3 ± 12.9	315.3 ± 18.5 ##
Brain NF-KB level (pg/g protein)	16.4 ± 3.9	20.8 ± 6.3	57.4 ± 8.2 *	15.1 ± 1.8	19.2 ± 2.5	30.5 ± 5.4 ##
Brain Lactate level (mmol/100 g wet weight)	0.94 ± 0.06	1.02 ± 0.4	1.8 ± 0.5 *	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.4
Brain BDNF level (pg/mg protein)	13.4 ± 1.5	15.7 ± 0.9	8.5 ± 1.1 **	11.8 ± 1.2	10.8 ± 0.5	9.3 ± 1.8

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ different from Adjuvant Aluminum hydroxide Male group. # $p < 0.05$, ## $p < 0.01$ different from Adjuvant Aluminum hydroxide Female group

in the neuronal counts in the hippocampal regions and the cerebellum between the control and COVID-19 spike protein and adjuvant aluminum hydroxide groups. However, there was a significant increase in the GFAP immunostaining index in the CA1 and CA3 regions of the hippocampus in the COVID-19 spike protein and adjuvant aluminum hydroxide group compared to the control group, with $p < 0.05$ (Table 3).

The histological analysis of the male rats' cerebellum showed a significant decrease in Purkinje neuron count in response to COVID-19 spike protein and adjuvant aluminum hydroxide compared to the control group and adjuvant aluminum hydroxide group ($p < 0.05$) (Fig. 5). Moreover, the GFAP immunostaining index was significantly increased in the COVID-19 spike protein and adjuvant aluminum hydroxide male group compared to the control group and adjuvant aluminum hydroxide group ($p < 0.05$) (Fig. 4) (Table 3).

In the female rats, there were no significant differences in Purkinje neuron count between the control, adjuvant aluminum hydroxide, and COVID-19 spike protein and adjuvant aluminum hydroxide groups. However, the GFAP immunostaining index was significantly increased in the COVID-19 spike protein and adjuvant aluminum hydroxide female group compared to the control and adjuvant aluminum hydroxide groups ($p < 0.05$) (Table 3).

Overall, these results suggest that COVID-19 spike protein and adjuvant aluminum hydroxide exposure may cause neuroinflammatory changes in the brain, as evidenced by the increased GFAP immunostaining index, and may lead to decreased neuronal counts in male rats. These results also suggest that COVID-19 spike protein and adjuvant aluminum hydroxide administration may cause cerebellar damage, particularly in male rats, as evidenced by the decreased Purkinje neuron count and increased GFAP immunostaining index.

MR Spectroscopy Results

The MR spectroscopy results showed significant changes in lactate levels in male rats that received COVID-19 spike protein and adjuvant aluminum hydroxide compared to the Adjuvant Aluminum hydroxide Male group and normal control group. Specifically, the lactate value increased to 542.4 ± 48.5% of the normal control level (100%) in the COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group, while it remained unchanged in the Adjuvant Aluminum hydroxide Male group (121.9 ± 32.6%) (Fig. 6) (Table 4). On the other hand, there was no statistically significant difference in lactate levels between the Adjuvant Aluminum hydroxide Female group and the COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group. The lactate value was 108.5 ± 23.9% and 115.3 ± 34.8% of the normal control level in the Adjuvant Aluminum hydroxide Female group and the COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group, respectively (Table 4).

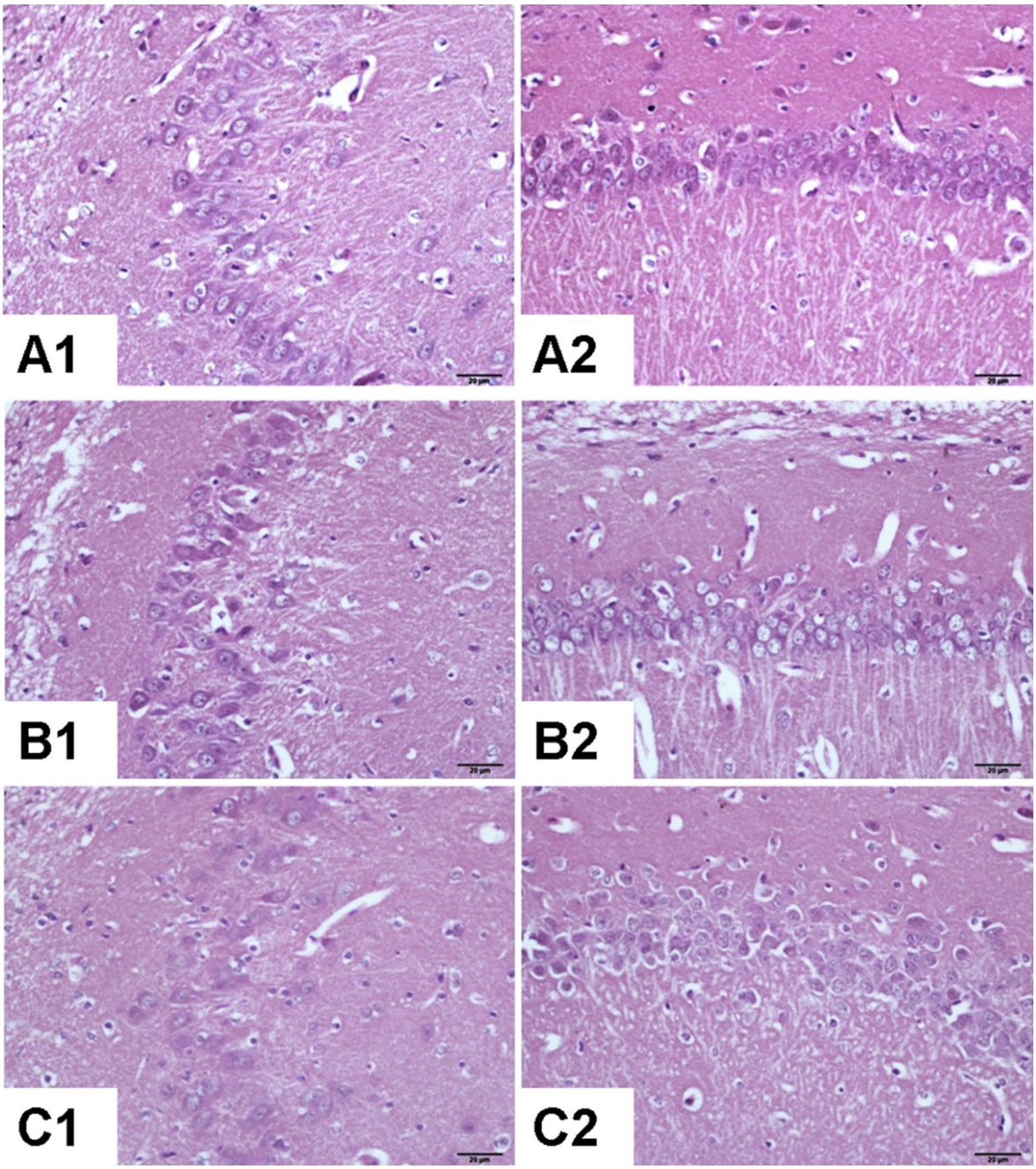

Fig. 2 CA3 and CA1 of hippocampus X 40 magnification. **A1**-**A2**: Normal Control Group Male Rats CA3 and CA1, **B1**-**B2**: Adjuvant Aluminum hydroxide group male rats have nearly normal CA3 and CA1 neuron morphology and count. **C1**-**C2**: COVID-19 Spike Protein and Adjuvant Aluminum hydroxide group male rats have decreased count and dysmorphological changes CA3 and CA1 Neuron (Scale bars for 1 cm = 20 μm)

Statistical analyses performed by one-way ANOVA showed a significant difference in lactate levels between the COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group and the Adjuvant Aluminum hydroxide Male group ($p < 0.001$). However, there was no significant difference in lactate levels between the Adjuvant Aluminum hydroxide Female group and the COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group ($p > 0.05$). These results suggest that COVID-19 spike protein and adjuvant aluminum hydroxide may induce significant changes in lactate levels in male rats, but not in female rats.

Table 3 Histological Analysis Results for Male–Female Groups

Sex	Male Groups			Female Groups		
Groups	**Normal Male Group**	**Adjuvant Aluminum hydroxide Male group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group**	**Normal Female Group**	**Adjuvant Aluminum hydroxide Female group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group**
Neuronal Count CA1	70.2 ± 3.9	68.7 ± 4.2	50.9 ± 2.8 *	65.8 ± 1.8	67.2 ± 1.6	65.5 ± 2.4
Neuronal Count CA3	41.6 ± 1.6	40.8 ± 2.3	28.1 ± 1.5 *	38.1 ± 0.9	36.9 ± 2.1	35.8 ± 1.5
GFAP immunostaining index (CA1)	17.4 ± 1.1	18.5 ± 2.9	32.8 ± 3.7 *	18.5 ± 1.7	17.3 ± 1.3	24.8 ± 0.8 ##
GFAP immunostaining index (CA3)	13.8 ± 2.9	15.4 ± 1.8	24.7 ± 1.5 *	15.1 ± 2.4	14.5 ± 1.9	21.3 ± 2.2 ##
Purkinje Neuron Count Cerebellum	18.9 ± 1.6	17.4 ± 2.5	10.6 ± 0.8 *	19.3 ± 0.6	17.6 ± 1.02	16.5 ± 1.2
GFAP immunostaining index (Cerebellum)	22.3 ± 1.5	21.8 ± 0.9	32.7 ± 1.1 *	23.4 ± 1.1	20.1 ± 2.5	29.8 ± 1.4 #

Results were presented as mean ± SEM. Statistical analyses were performed by one- way ANOVA. *$p < 0.05$ different from Adjuvant Aluminum hydroxide Male group; ## $p < 0.01$; # $p < 0.05$, different from Adjuvant Aluminum hydroxide Female group

Discussion

Our study investigated the potential impact of a synthetic version of the SARS-CoV-2 spike protein on the development of autism spectrum disorder (ASD) in offspring born to mothers exposed to the protein during pregnancy. The results of our study suggest that COVID-19 spike protein and adjuvant aluminum hydroxide may affect various parameters differently in normal male and female rats, with male rats exhibiting more significant behavioral changes, biochemical alterations, and histological damage in the brain compared to female rats.

These results are in line with previous studies that have indicated differences in the immune response between males and females in response to infections and vaccinations. Generally, females exhibit a stronger immune response, which may offer some protection against neuroinflammatory and neurodegenerative diseases (Klein and Flanagan 2016; Mauvais-Jarvis et al. 2020). However, our findings suggest that exposure to COVID-19 spike protein and adjuvant aluminum hydroxide can have more pronounced effects in male rats, potentially overriding these sex differences and leading to more severe neuroinflammatory and neurodegenerative changes.

The behavioral changes observed in our study, such as decreased sociability, open field activity, novelty-induced rearing behavior, and passive avoidance learning latency, are consistent with previous research on neuroinflammation and ASD. Studies have shown that neuroinflammation can lead to cognitive and behavioral impairments in animal models and in humans, including deficits in social interaction, communication, and repetitive behaviors (Vargas et al. 2005; Pardo and Eberhart 2007). Additionally, the histological changes observed in our study, such as decreased neuronal counts in the hippocampus and cerebellum and increased GFAP immunostaining index, are also consistent with previous research on neuroinflammation and neurodegeneration (Heneka et al. 2015; Liddelow et al. 2017).

The increase in lactate levels observed in male rats in our study aligns with previous research on neuroinflammation and neurodegeneration. Heightened lactate levels have been reported in different neurological disorders, such as traumatic brain injury, Alzheimer's disease, and Parkinson's disease, and are believed to reflect a rise in anaerobic metabolism due to mitochondrial dysfunction and energy deficiencies (Tschopp et al. 2018; Chen et al. 2020a).

Our study also contributes to the growing body of research on the potential link between ASD and SARS-CoV-2 infection. A study published in the journal Molecular Autism in May 2021 reported that the spike protein could interact with certain proteins in the brain that are implicated in ASD, potentially leading to neuroinflammation and neuronal damage. However, this study was conducted in mice and used a synthetic version of the spike protein, rather than the actual virus (Han and Criado 2021). Our study adds

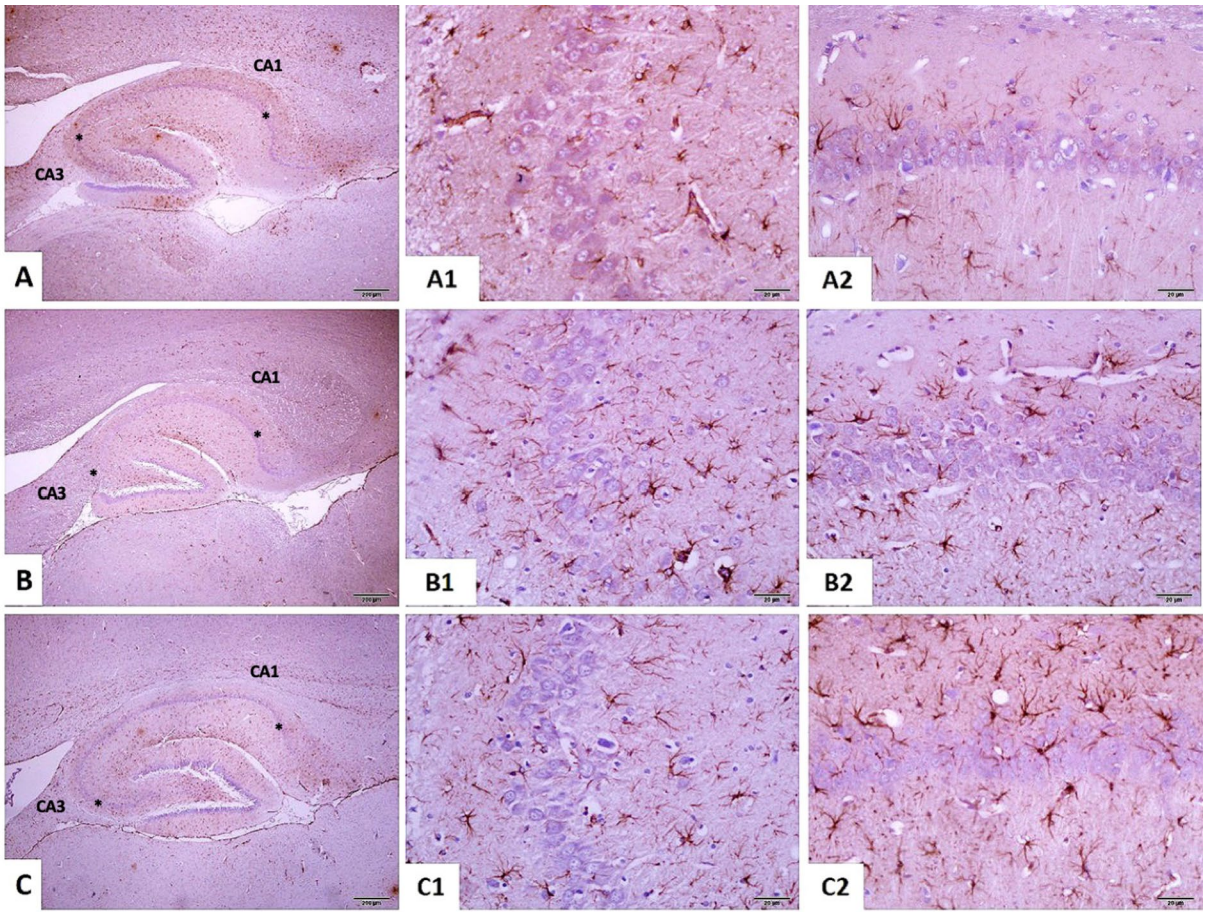

Fig. 3 CA3 and CA1 of hippocampus X 4 and X 40 magnification. Astrogliosis was characterized by GFAP immunostaining (Brown staining). **A**-**A1**-**A2**: Normal Control Group male Rats CA3 and CA1, **B**-**B1**-**B2**: Adjuvant Aluminum hydroxide group male rats have slightly increased glial activity CA3 and CA1. **C**-**C1**-**C2**: COVID-19 Spike Protein and Adjuvant Aluminum hydroxide group male rats have manifest increased glial activity CA3 and especially CA1 (Scale bars for 1 cm = 20 and 200 μm). Regions exhibiting increased glial activity are denoted with an asterisk (*) symbol in the figure for clear identification

to this literature by demonstrating the potential impact of COVID-19 spike protein and adjuvant aluminum hydroxide exposure on the development of ASD-like behaviors and neuroinflammatory changes in normal male and female rats.

It is important to note that our study has some limitations. First, our study was conducted in normal rats, and it is unclear if these findings would generalize to rats with preexisting conditions or to humans. Second, our study used a synthetic version of the spike protein, rather than the actual virus, and it is unclear if the effects observed in our study would be similar to those of natural infection. Third, our study did not assess the long-term effects of COVID-19 spike protein and adjuvant aluminum hydroxide exposure, and it is unclear if these effects would persist or worsen over time.

Our study provides evidence that exposure to the SARS-CoV-2 spike protein may induce neuroinflammatory responses and neurobehavioral changes in mice, including cognitive deficits and anxiety-like behavior. These findings are consistent with previous studies that have reported similar effects of the spike protein on neuronal synapses and brain development (Chen et al. 2020b; Varma et al. 2021; Olajide et al. 2022; Steinman 2020).

Our study also highlights the potential sex differences in the neuroinflammatory and neurodegenerative effects of COVID-19 spike protein and adjuvant aluminum hydroxide exposure. Sex differences in immune response to infections and vaccination have been reported in previous studies, with females generally showing a stronger immune response and potentially being more protected against neuroinflammatory and neurodegenerative diseases (Chen et al. 2020b; Varma et al. 2021). However, our study suggests that exposure to the COVID-19 spike protein and adjuvant aluminum hydroxide may override these sex differences, leading to more severe neuroinflammatory and neurodegenerative changes in male rats.

Moreover, our study also found that the hippocampal neurons were particularly vulnerable to the spike

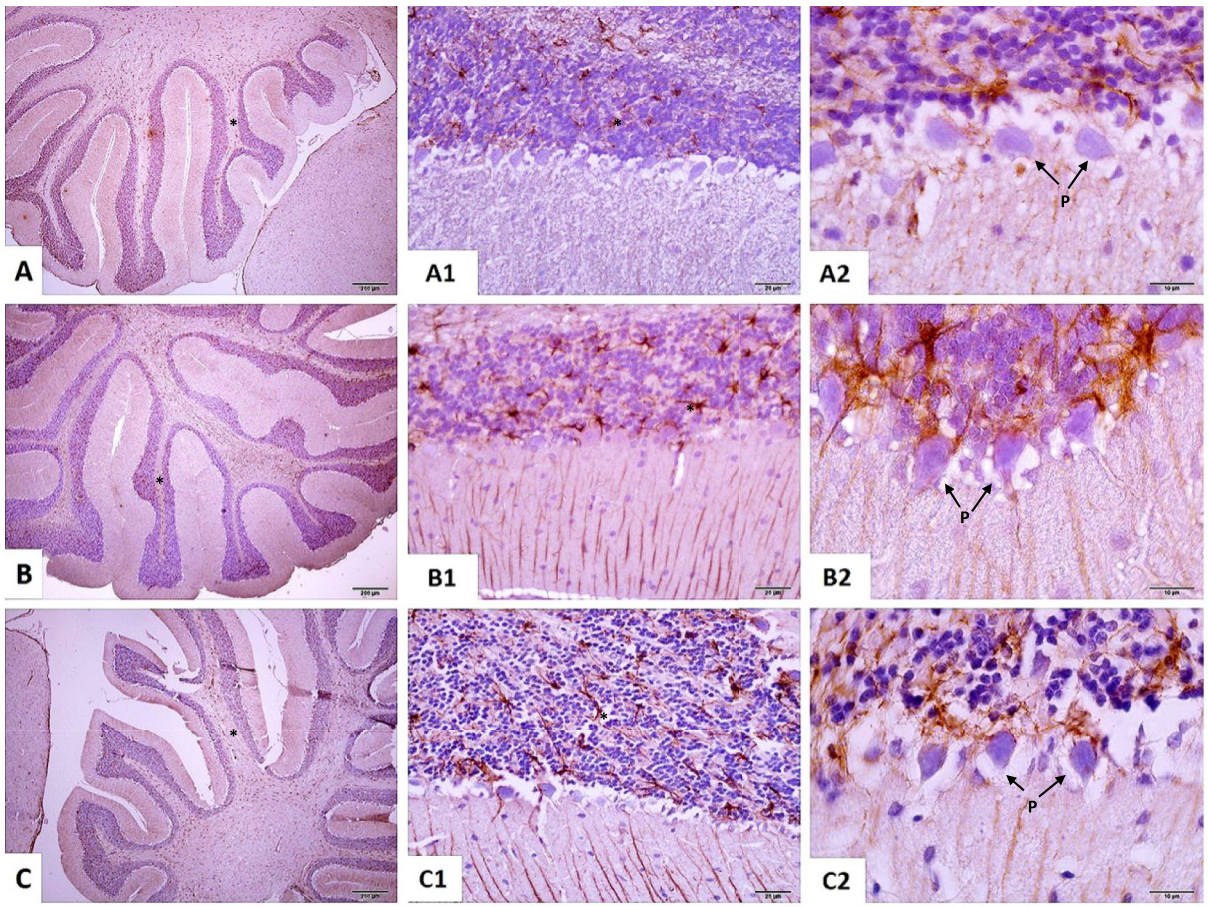

Fig. 4 Cerebellum X 4, X 40 and X 100 magnification. Astrogliosis was characterized by GFAP immunostaining (Brown staining). **A**-**A1**-**A2**: Normal Control Group male Rats; **B**-**B1**-**B2**: Adjuvant Aluminum hydroxide group male rats have slightly increased glial activity; **C**-**C1**-**C2**: COVID-19 Spike Protein and Adjuvant Aluminum hydroxide group male rats have slightly increased glial activity and decreased count & dysmorphological changes Purkinje Neuron. (Scale bars for 1 cm = 10, 20 and 200 µm). Purkinje neurons (cells) have been clearly marked with the 'p' symbol. Area(s) showcasing increased glial activity have been highlighted with the '*' symbol

protein-induced neurotoxicity, which supports the findings of a recent study that reported non-cell autonomous hippocampal neuronal death following exposure to the spike protein (Oh et al. 2022). Our results also raise concerns about potential risks for pregnancy infections and COVID-19 babies, given the potential for neurovascular unit and brain vasculature damages (Rasile et al. 2022; Shook et al. 2022).

Recent research has shown that COVID-19 spike protein can induce neuroinflammation and neurotoxicity, leading to cognitive deficits and anxiety-like behavior in animal models (Song et al. 2021b; Jakhmola et al. 2021). One possible mechanism for these effects is the activation of NF-κB signaling pathway by the spike protein, which has been shown to regulate inflammation and oxidative stress in the brain (Munoz and Ammit 2010; Natarajaseenivasan et al. 2021). NF-κB activation has also been linked to decreased levels of brain-derived neurotrophic factor (BDNF), a key protein for neuronal survival and plasticity (Cunha et al. 2010; Dong et al. 2021).

In addition to NF-κB and BDNF, other inflammatory markers such as interleukin-17 (IL-17) have been implicated in the neurological effects of COVID-19 spike protein. IL-17 is a pro-inflammatory cytokine that is involved in the pathogenesis of several neurological disorders, including multiple sclerosis and Alzheimer's disease (Kebir et al. 2017; Zenaro et al. 2017). Recent studies have shown that IL-17 levels are elevated in COVID-19 patients and may contribute to the neurological symptoms associated with the disease (Yan et al. 2020; Delorme et al. 2020).

Moreover, COVID-19 spike protein has been shown to alter brain metabolism, leading to increased levels of lactate. Lactate is a byproduct of glycolysis that is normally metabolized in the brain, but elevated levels have been associated with neuroinflammation and oxidative stress (Mergenthaler et al. 2013; Barros and Deitmer 2010). A recent study found

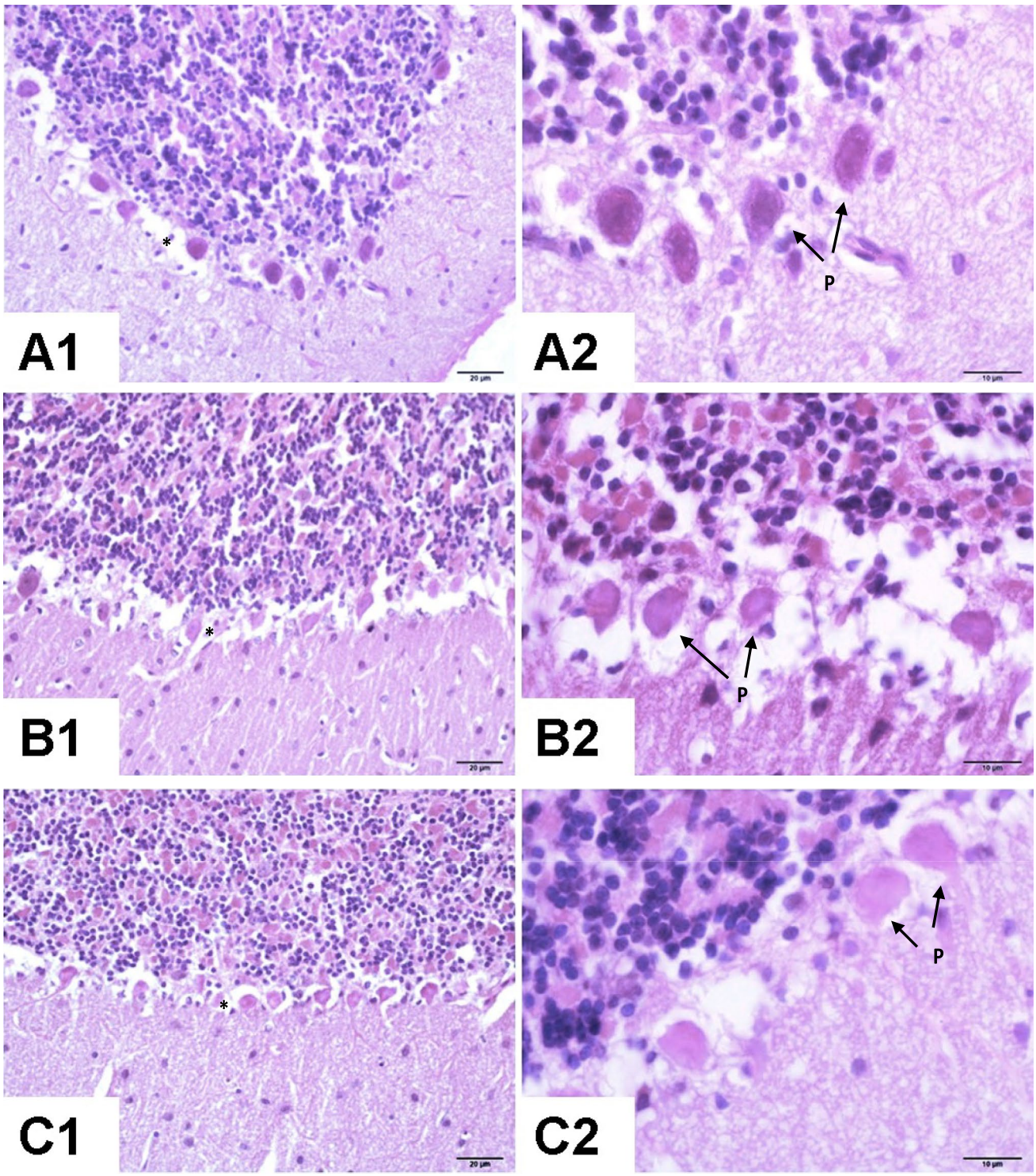

Fig. 5 Cerebellum X 40 and X 100 magnification. **A1**-**A2**: Normal control group male rats; **B1**-**B2**: Adjuvant Aluminum hydroxide group male rats nearly normal purkinje neuron morphology and count; **C1**-**C2**: COVID-19 Spike Protein and Adjuvant Aluminum hydroxide group male rats decreased count and dysmorphological changes in purkinje neurons. (Scale bars for 1 cm = 10, 20 μm). Purkinje neurons (cells) have been clearly marked with the 'p' symbol. Area(s) showcasing increased neuronal decrease have been highlighted with the '*' symbol

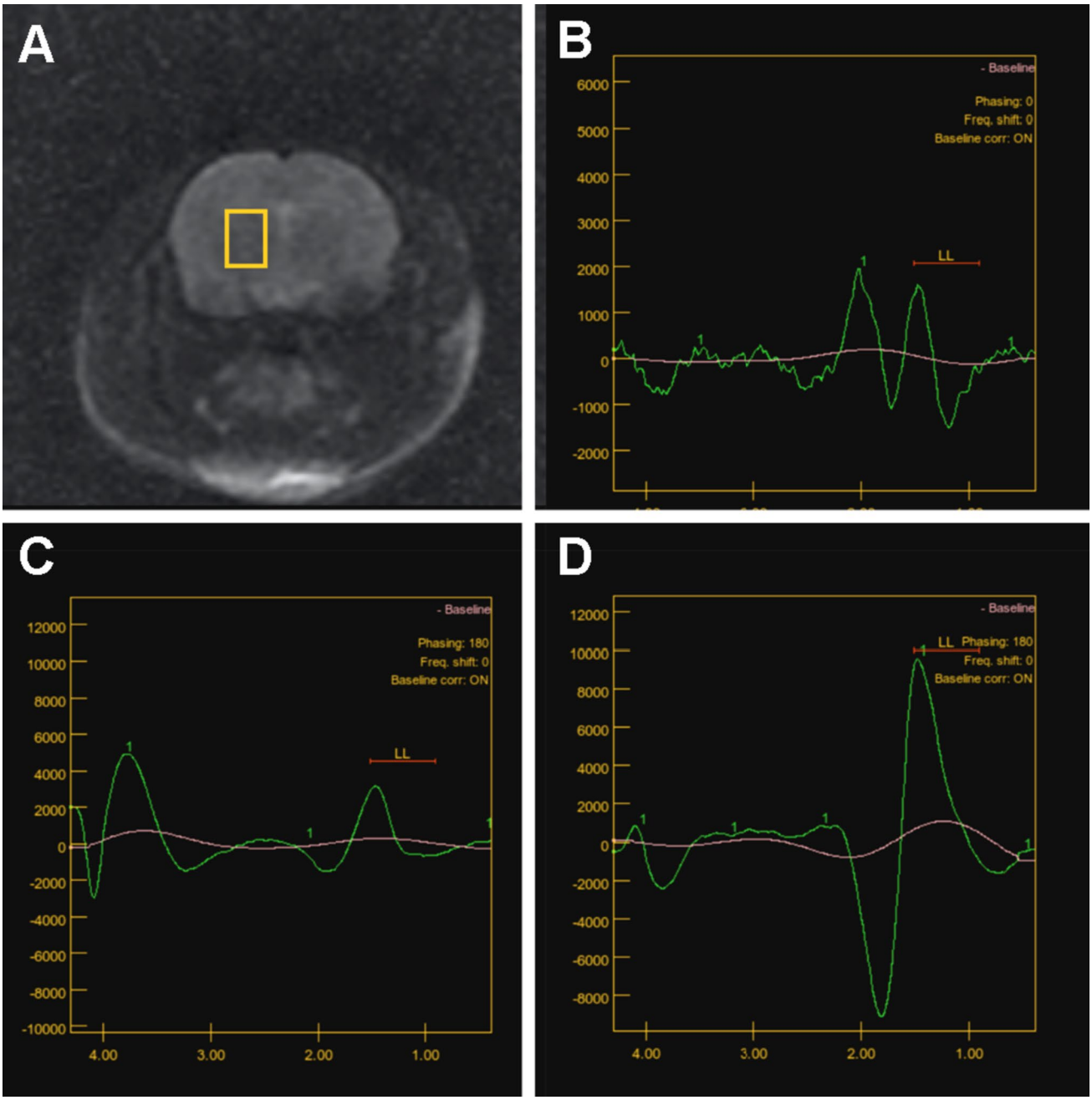

Fig. 6 MR spectroscopy. **A**: MR spectroscopy chosen area (Yellow box), **B**: Normal Control Group, **C**: Adjuvant Aluminum hydroxide group, **D**: COVID-19 Spike Protein and Adjuvant Aluminum hydroxide group

that COVID-19 patients with neurological symptoms had higher levels of lactate in their cerebrospinal fluid, suggesting that the spike protein may induce metabolic changes in the brain (Pilotto et al. 2021).

In addition to the primary findings, it's essential to highlight some molecular pathways potentially affected by the exposure to the SARS-CoV-2 spike protein, both in terms of up-regulation and down-regulation. One of the central pathways that have gained attention in the context of COVID-19 and neuroinflammation is the activation of the toll-like receptor (TLR) signaling cascade. TLRs play a pivotal role in the innate immune response and have been linked to the viral recognition and subsequent inflammatory response in the CNS. In particular, TLR3 and TLR7 have been shown to be activated in response to RNA viruses like SARS-CoV-2 (Totura and Baric 2012; Bortolotti et al. 2021; Manik and Singh 2022).

Toll-Like Receptors (TLRs) are pivotal components of the innate immune system, recognizing pathogen-associated

Table 4 MR Spectroscopy Results for Male–Female Groups

Sex	Male Groups			Female Groups		
Groups	**Normal Male Group**	**Adjuvant Aluminum hydroxide Male group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Male group**	**Normal Female Group**	**Adjuvant Aluminum hydroxide Female group**	**COVID-19 Spike Protein and Adjuvant Aluminum hydroxide Female group**
MR spectroscopy Lactate value (LL) (% of Normal Control) (1.3 ppm)	100	121.9 ± 32.6	542.4 ± 48.5 ***	100	108.5 ± 23.9	115.3 ± 34.8 ns

Results were presented as mean ± SEM. Statistical analyses were performed by one- way ANOVA. *** $p < 0.001$ different from Adjuvant Aluminum hydroxide Male group. ns (non-significant) $p > 0.05$ different from Adjuvant Aluminum hydroxide Female group

molecular patterns (PAMPs). Evidence suggests that the SARS-CoV-2 spike protein can interact with TLRs, potentially leading to the activation of NF-κB. This subsequent activation could enhance the production of a myriad of pro-inflammatory cytokines and chemokines, fostering an environment of neuroinflammation (Totura and Baric 2012; Choudhury and Mukherjee 2020).

Up-regulation of the TLR pathway can initiate downstream signaling, leading to the activation of NF-κB and interferon regulatory factors, culminating in the release of pro-inflammatory cytokines such as IL-6, TNF-α, and interferons (Akira et al. 2006). This surge in inflammatory markers can contribute to a neuroinflammatory state, which has been associated with many of the behavioral and cognitive symptoms observed post-infection.

On the contrary, some pathways seem to be down-regulated post-exposure. The SARS-CoV-2 spike protein has been shown to suppress the PI3K/Akt/mTOR pathway (Li et al. 2021), a pivotal signaling cascade for cell growth, proliferation, differentiation, and survival. This down-regulation can impact neuronal survival and plasticity, potentially contributing to the observed histological changes in brain regions like the hippocampus and cerebellum.

Additionally, given the interaction of the spike protein with ACE2 receptors, there's a potential disruption in the renin-angiotensin system (RAS). This interaction might lead to a down-regulation of the Mas receptor pathway, crucial for anti-inflammatory actions, vasodilation, and neuroprotection (Costa et al. 2020; Gheblawi et al. 2020). The ACE2 receptor, known to facilitate SARS-CoV-2 cellular entry, plays a critical role within the Renin-Angiotensin System (RAS). Binding of the SARS-CoV-2 to ACE2 could disrupt the equilibrium of the RAS, potentially escalating inflammatory responses and contributing to neuroinflammation. This disruption might have significant implications for brain regions where the RAS plays pivotal roles in neuroprotection (Gheblawi et al. 2020).

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is integral to the signal transduction of cytokines and growth factors. It's postulated that the SARS-CoV-2 spike protein might modulate this pathway, potentially inhibiting intracellular antiviral responses. Moreover, activation along this pathway could amplify the production of neuroinflammatory cytokines, contributing to the neurological manifestations observed in some COVID-19 patients. (Zhang et al. 2020; Feldmann et al. 2020; Stebbing et al. 2020).

To provide a comprehensive understanding of these pathways, we've included a diagrammatic representation that illustrates the potential up-and-down regulated pathways upon exposure to the SARS-CoV-2 spike protein (Fig. 7).

One of the more pronounced neurobehavioral symptoms linked with Autism Spectrum Disorders (ASDs) is anxiety

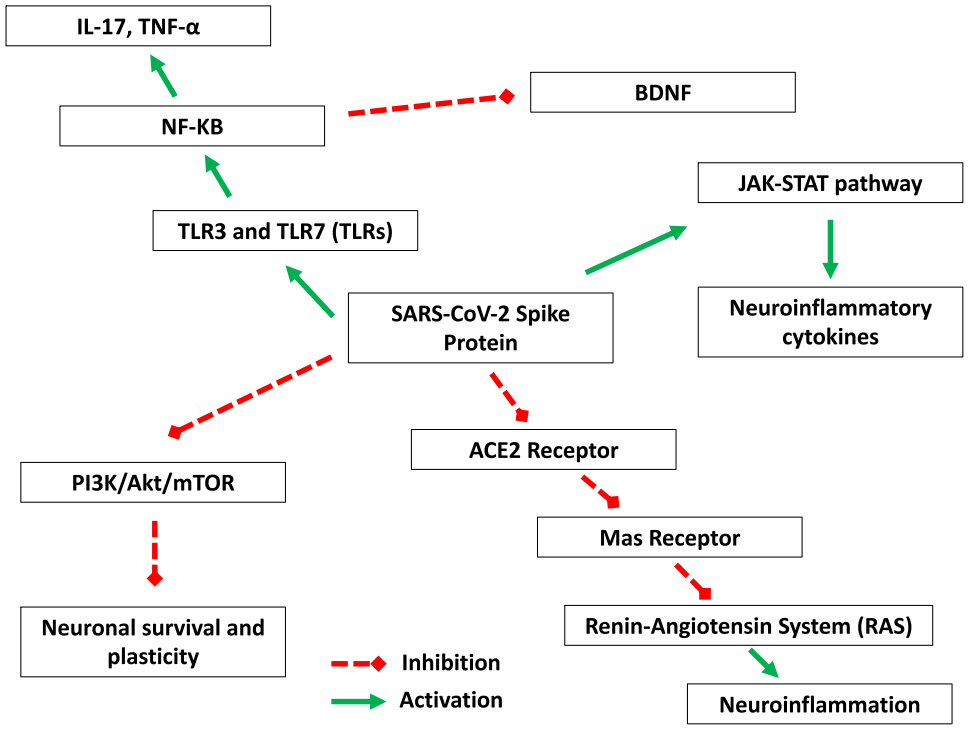

Fig. 7 A comprehensive diagram, which provides a visual representation of the discussed mechanisms and their interactions

(White et al. 2009). ASD individuals consistently display a high prevalence of co-occurring anxiety disorders, a phenomenon well-documented in the literature (Steensel et al. 2011). This co-occurrence has led researchers to believe that there might be shared underlying neurobiological mechanisms.

A common method to evaluate anxiety-like behaviors in rodent models is the open field test (OFT). This test gauges the exploratory behavior and general activity of the rodents in an unfamiliar environment. Anxiety in rodents, similar to humans, can be discerned from the reluctance to explore open spaces or the center of an open field. Rearing behaviors, on the other hand, can be seen as an indicator of exploratory tendencies and can be inversely correlated with anxiety (Sturman et al. 2018). Reduced rearing can be interpreted as increased anxiety-like behavior.

Our findings corroborate the anxiety-ASD link, as animals exposed to the spike protein showed decreased open field and rearing activities, suggesting heightened anxiety-like behaviors. This is in line with the work of Sturman et al. (2018) which provides a foundational understanding of how these tests mirror anxiety manifestations in rodents (Sturman et al. 2018). The association we observed between spike protein exposure and increased anxiety-like behavior could potentially imply a mechanistic link with the neurobehavioral manifestations observed in ASD. This interplay, if further substantiated, could offer insights into the broader spectrum of neurobehavioral changes post SARS-CoV-2 exposure.

The above observations further augment the growing body of evidence indicating that viral components, like the SARS-CoV-2 spike protein, can have profound effects on the CNS, not limited to purely structural changes but extending to behavioral alterations as well. Further studies will be necessary to delineate the exact pathways through which these proteins affect neuronal functioning and behavior, but the preliminary indications, as shown by our results, are both significant and concerning.

Taken together, these findings suggest that COVID-19 spike protein can induce neuroinflammation and neurotoxicity through the activation of NF-κB signaling pathway and altered levels of inflammatory cytokines, BDNF, and lactate in the brain (Hampshire 2021; Xiong et al. 2020; Rhea et al. 2021; Lins 2021; Okechukwu 2021; Centers for Disease Control and Prevention 2021; American Psychiatric and Association 2013). These mechanisms may contribute to the neurological symptoms observed in COVID-19 patients and provide a basis for future research into potential treatments for these symptoms.

Our study adds to the growing body of literature indicating that SARS-CoV-2 infection, and exposure to the spike protein in particular, can have detrimental effects on the nervous system and brain function (Lu et al. 2020; Cucinotta and Vanelli 2020; Pantelis et al. 2021). These findings underscore the importance of continuing to investigate the potential neurological, neuropsychiatric, and neurodevelopmental complications of COVID-19, and developing effective strategies to address the neuroinflammatory and neurotoxic effects of the virus (Theoharides 2020, 2021; Matta et al. 2019).

Conclusion

In this study, we investigated the effects of exposure to the COVID-19 spike protein coupled with adjuvant aluminum hydroxide in male and female rats. Our findings demonstrated that male rats are more susceptible, manifesting notable behavioral, biochemical, and histological alterations in the brain when compared to female rats. Specifically, exposure induced neuroinflammatory changes, resulting in decreased neuronal counts in male rats and observable cerebellar damage in both genders. This research bolsters the evolving discourse on the relationship between ASD and SARS-CoV-2 infection. Importantly, our study underscores the urgency of further exploration into the long-term neurological implications of COVID-19, emphasizing the necessity of crafting effective strategies to mitigate the neuroinflammatory and neurotoxic effects potentially associated with the virus.

Authors' Contributions MAE and OE: conceived and designed research, formal analysis, wrote the manuscript, edited the text, Conceptualization. MAE, MT, GDD, IHS, BO, IS, EE, YU and OE: Data curation, conducted experiments, wrote the manuscript. All authors read and approved the manuscript.

Funding This research was supported by grants from the Scientific and Technological Research Council of Turkey (TUBITAK) under the project number 120Z305; Istanbul Technical University Scientific Research Projects with codes TGA-2022–43373 and TGA-2022–43955; Health Institutes of Turkey (TUSEB) with the grant number 7162/8972; and the TUBITAK 2211C Program. There was no specific grant received from commercial or non-for-profit sectors.

Availability of Data and Material The datasets supporting the conclusions of this article are included within the article and its additional files.

Code Availability Not applicable.

Declarations

Ethics Approval The Animal Ethics Committee of Demiroglu Science University authorized the experimental procedures used in this study (Approval no: 04220903).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare that they have no conflicts of interest.

References

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124(4):783–801

American Psychiatric Association (2013) Diagnostic and statistical manual of mental disorders (5th ed.). https://doi.org/10.1176/appi.books.9780890425596

Baig AM, Khaleeq A, Ali U, Syeda H (2020) Evidence of the COVID-19 Virus Targeting the CNS: Tissue Distribution, Host-Virus Interaction, and Proposed Neurotropic Mechanisms. ACS Chem Neurosci 11(7):995–998. https://doi.org/10.1021/acschemneuro.0c00122

Barros LF, Deitmer JW (2010) Glucose and lactate supply to the synapse. Brain Res Rev 63(1–2):149–159. https://doi.org/10.1016/j.brainresrev.2009.10.002. Epub 2009 Oct 22 PMID: 19852965

Bauman MD, Iosif AM, Smith SE et al (2014) Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. Biol Psychiatry 75(11):332–341. https://doi.org/10.1016/j.biopsych.2013.08.011. Epub 2013 Sep 25 PMID: 24074927

Bilbo SD, Schwarz JM (2012) The immune system and developmental programming of brain and behavior. Front Neuroendocrinol 33(3):267–286. https://doi.org/10.1016/j.yfrne.2012.08.006

Bortolotti D, Gentili V, Rizzo S, Schiuma G, Beltrami S, Strazzabosco G, ... Rizzo R (2021) TLR3 and TLR7 RNA sensor activation during SARS-CoV-2 infection. Microorganisms 9(9):1820

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carter RJ, Lione LA, Humby T, Mangiarini L, Mahal A, Bates GP, Dunnett SB (2001) Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. J Neurosci 21(24):961–966

Centers for Disease Control and Prevention (2021) Data on COVID-19 during pregnancy: Severity of maternal illness, centers for disease control and prevention

Chauhan A, Gu F, Essa MM, Wegiel J, Kaur K, Brown WT, Chauhan V (2011) Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism. J Neurochem 117(2):209–220. https://doi.org/10.1111/j.1471-4159.2011.07283.x. Epub 2011 May 18 PMID: 21501183

Chen CY, Chou YC, Hsueh YP (2020) SARS-CoV-2 D614 and G614 spike variants impair neuronal synapses and exhibit differential fusion ability. BioRxiv 2020–12

Chen Y, Cai F, Wu H, Qian Z (2020a) Mitochondrial dysfunction, oxidative stress and neurodegenerative diseases. Protein Cell 11(11):710–721

Choudhury A, Mukherjee S (2020) In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike protein with ACE-2 receptor homologs and human TLRs. J Med Virol 92(10):2105–2113

Costa LB, Perez LG, Palmeira VA, Macedo e Cordeiro, T., Ribeiro, V. T., Lanza, K., & Simoes e Silva, A. C. (2020) Insights on SARS-CoV-2 molecular interactions with the renin-angiotensin system. Frontiers in Cell and Developmental Biology 8:559841

Cucinotta D, Vanelli M (2020) WHO Declares COVID-19 a Pandemic. Acta Biomed 91:157–160

Cunha C, Brambilla R, Thomas KL (2010) A simple role for BDNF in learning and memory? Front Mol Neurosci 3:1–14. https://doi.org/10.3389/neuro.02.005.2010

Delorme C, Paccoud O, Kas A, Hesters A, Bombois S, Shambrook P, Bodeau N, Stroebel A, Moulin V, Bouvard MP, Guillermier M, Royer PY, Varoquaux A, Etcharry-Bouyx F, Leboyer M, Houenou J (2020) COVID-19-related neuropsychiatric symptoms in patients with enclosed space confinement. Front Psychiatry 10(11):589225. https://doi.org/10.3389/fpsyt.2020.589225. PMID: 33281720; PMCID: PMC7689795

Dong Y et al (2021) COVID-19: Characteristics, comorbidities and acute outcomes in the Republic of Ireland and Northern Ireland. PLoS ONE 16(2):e0247135

ELBeltagy M, Mustafa S, Umka J, Lyons L, Salman A, Chur-yoe GT, ... Bhalla P (2010) Fluoxetine improves the memory deficits caused by the chemotherapy agent 5-fluorouracil. Behav Brain Res 208(1):112–117

Ellegood J, Crawley JN (2015) Behavioral and cognitive mouse assays for autism: a survey of reproducibility and validity. Nat Methods 12(7):523–529

Erbas O et al (2018) Neurobehavioral effects of long-term maternal fructose intake in rat offspring. Int J Dev Neurosci 69:68–79

Erbaş O, Solmaz V, Karakilic A, Kaplan S (2013) Effects of the acute and chronic social isolation stress on the morphological and biochemical parameters in the limbic brain structures of rats. Cytotechnology 65(3):387–94. https://doi.org/10.1007/s10616-012-9487-1. Epub 2012 Oct 12. PMID: 23065195; PMCID: PMC3656673

Estes ML, McAllister AK (2015) Immune mediators in the brain and peripheral tissues in autism spectrum disorder. Nat Rev Neurosci 16(8):469–486. https://doi.org/10.1038/nrn3978

Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, ... Hussell T (2020) Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. Lancet 395(10234):1407–1409

Figueiredo CP et al (2021) SARS-CoV-2-associated cytokine storm during pregnancy as a possible risk factor for neuropsychiatric disorder development in post-pandemic infants. Neuropharmacology 201:108841

Gheblawi M, Wang K, Viveiros A, Nguyen Q, Zhong JC, Turner AJ, ... Oudit GY (2020) Angiotensin-converting enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system: celebrating the 20th anniversary of the discovery of ACE2. Circ Res 126(10):1456–1474

Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, Miller J, Fedele A, Collins J, Smith K, Lotspeich L, Croen LA, Ozonoff S, Lajonchere C, Grether JK, Risch N (2011) Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry 68(11):1095–1102. https://doi.org/10.1001/archgenpsychiatry.2011.76

Hampshire A et al (2021) Cognitive deficits in people who have recovered from COVID-19. E Clin Med 101044

Han J, Criado AE (2021) SARS-CoV-2 spike protein disrupts synapses and induces neural inflammation in rats: Implications for COVID-19-associated neuropathology. Molecular Autism 12(1):50

Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, ... Kummer MP (2015) Neuroinflammation in Alzheimer's disease. Lancet Neurol 14(4):388–405

Hsiao EY, McBride SW, Chow J et al (2012) Modeling an autism risk factor in mice leads to permanent immune dysregulation. Proc Natl Acad Sci U S A 109(21):12776–12781. https://doi.org/10.1073/pnas.1202556109. Epub 2012 May 7 PMID: 22566635

Jakhmola S, Indari O, Kaushik S et al (2021) COVID-19: Pathogenesis, cytokine storm and therapeutic potential of interferons. Cytokine Growth Factor Rev 58:46–53. https://doi.org/10.1016/j.cytogfr.2021.02.002

Kebir H et al (2017) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Commun 8(1):1–17

Kinnunen T, Kieseppä T, Kiviranta H (2022) SARS-CoV-2 infection and autism spectrum disorder. J Autism Dev Disord 52(1):41–48. https://doi.org/10.1007/s10803-021-05123-y

Klein SL, Flanagan KL (2016) Sex differences in immune responses. Nat Rev Immunol 16(10):626–638

Li F, Li J, Wang PH, Yang N, Huang J, Ou J, ... Zhang Q (2021) SARS-CoV-2 spike promotes inflammation and apoptosis through autophagy by ROS-suppressed PI3K/AKT/mTOR signaling. Biochim Biophys Acta (BBA)-Mol Basis Dis 1867(12):166260

Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li XM, Ji L, Brown T, Malik M (2009) Elevated immune response in the brain of autistic patients. J Neuroimmunol 207(1–2):111–116. https://doi.org/10.1016/j.jneuroim.2008.12.002. Epub 2009 Jan 21 PMID: 19157558

Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, ... Barres BA (2017) Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541(7638):481–487

Lins B (2021) Maternal immune activation as a risk factor for psychiatric illness in the context of the SARS-CoV-2 pandemic. Brain Behav Immun Health 16:100297

Lopez-Diaz A et al (2021) COVID-19 infection during pregnancy and risk of neurodevelopmental disorders in offspring: time for collaborative research. Biol Psychiatry 89:e29–e30

Lu R et al (2020) Genomic characterisation and epidemiology of 2019 novel coronavirus: Implications for virus origins and receptor binding. Lancet 395:565–574

Manik M, Singh RK (2022) Role of toll-like receptors in modulation of cytokine storm signaling in SARS-CoV-2-induced COVID-19. J Med Virol 94(3):869–877

Masi A, Glozier N, Dale R, Guastella AJ (2017) The immune system, cytokines, and biomarkers in autism spectrum disorder. Neurosci Bull 33(2):194–204. https://doi.org/10.1007/s12264-017-0100-y. Epub 2017 Mar 20 PMID: 28321847

Matta SM, Hill-Yardin EL, Crack PJ (2019) The influence of neuroinflammation in Autism Spectrum Disorder. Brain Behav Immun 79:75–90

Mauvais-Jarvis F, Bairey Merz N, Barnes PJ, Brinton RD (2020) COVID-19 and sex differences: Mechanisms and biomarkers. Mayo Clin Proc 95(10):2189–2203

Mergenthaler P, Lindauer U, Dienel GA, Meisel A (2013) Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends Neurosci 36(11):587–97. https://doi.org/10.1016/j.tins.2013.07.001. Epub 2013 Aug 20. PMID: 23968683; PMCID: PMC4074855

Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP, ... Crawley JN (2004) Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains. Behav Brain Res 153(1):403–410

Munoz L, Ammit AJ (2010) Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. Neuropharmacology 58(3):561–568. https://doi.org/10.1016/j.neuropharm.2009.11.005

Natarajaseenivasan K, Radhakrishnan N, Hariharan R, Muthusamy R, Madhavan T (2021) Role of nuclear factor-kappaB and its upstream regulators in Alzheimer's disease. Biomed Pharmacother 137:111309. https://doi.org/10.1016/j.biopha.2021.111309

National Institute of Mental Health. Autism spectrum disorder. Accessed 27 Feb 2023. https://www.nimh.nih.gov/health/topics/autism-spectrum-disorders-asd/index.shtml

Oh J, Cho WH, Barcelon E, Kim KH, Hong J, Lee SJ (2022) SARS-CoV-2 spike protein induces cognitive deficit and anxiety-like behavior in mouse via non-cell autonomous hippocampal neuronal death. Sci Rep 12(1):5496

Okechukwu C (2021) Inflammatory cytokines induced by severe acute respiratory syndrome coronavirus 2 infection during pregnancy may alter fetal brain development predisposing the offspring to neurodevelopmental disorders. Nigerian J Exp Clin Biosci 9:58

Olajide OA, Iwuanyanwu VU, Adegbola OD, Al-Hindawi AA (2022) SARS-CoV-2 spike glycoprotein S1 induces neuroinflammation in BV-2 microglia. Mol Neurobiol 1–14

Onore CE, Careaga M (2019) Inflammatory exposures and brain development: Implications for autism spectrum disorders. Brain Behav Immun 86:30–41. https://doi.org/10.1016/j.bbi.2019.06.012

Onore CE, Schwartzer JJ, Careaga M, Berman RF, Ashwood P (2014) Maternal immune activation leads to activated inflammatory macrophages in offspring. Brain Behav Immun 38:220–226. https://doi.org/10.1016/j.bbi.2013.12.011. Epub 2013 Dec 25 PMID: 24370625

Pantelis C, Jayaram M, Hannan AJ, Wesselingh R, Nithianantharajah J, Wannan CM, ... O'Brien TJ (2021) Neurological, neuropsychiatric and neurodevelopmental complications of COVID-19. Aust N Z J Psychiatry 55(8):750–762

Pardo CA, Eberhart CG (2007) The neurobiology of autism. Brain Pathol 17(4):434–447

Pilotto A, Odolini S, Masciocchi S, Comelli A, Volonghi I, Gazzina S, Nocivelli L, Pezzini A, Focà E, Carifi A, Magni E, Sessa M, Gerevini S, Bonacina S, Benussi A, Alimonti D, Facheris MF, Castelli F (2021) SARS-CoV-2 encephalitis is a cytokine release syndrome: evidences from cerebrospinal fluid analyses. Clin Infect Dis ciab096. https://doi.org/10.1093/cid/ciab096. Epub ahead of print. PMID: 33599689; PMCID: PMC7929082

Rasile M, Lauranzano E, Mirabella F, Matteoli M (2022) Neurological consequences of neurovascular unit and brain vasculature damages: potential risks for pregnancy infections and COVID-19-babies. FEBS J 289(12):3374–3392

Rhea EM et al (2021) The S1 protein of SARS-CoV-2 crosses the blood-brain barrier in mice. Nat Neurosci 24:368–378

Rossi F, Navarro X (2021) Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles 10(1):e12055. https://doi.org/10.1002/jev2.12055.PMID

Sakurada et al (2020) Neurodevelopmental disorders induced by maternal immune activation: toward a prevention strategy in the era of the COVID-19 pandemic. Psychiatry Int 1:24–26

Siniscalco D, Antonucci N (2022) COVID-19 in autism spectrum disorder: Does neuroinflammation have a role? Brain Sci 12(1):83. https://doi.org/10.3390/brainsci12010083

Song Y, Ding W, Xiao X, Wu J, Zeng Y, Wu C, Wang J, Wang M (2021a) The spike protein of SARS-CoV-2 interacts with autism-associated proteins, implicating Wnt signaling pathway in COVID-19 and autism. Molecular Autism 12(1):39. https://doi.org/10.1186/s13229-021-00446-8

Song E, Zhang C, Israelow B et al (2021b) Neuroinvasion of SARS-CoV-2 in human and mouse brain. J Exp Med 218(3):e20202135. https://doi.org/10.1084/jem.20202135

Shook LL, Sullivan EL, Lo JO, Perlis RH, Edlow AG (2022) COVID-19 in pregnancy: implications for fetal brain development. Trends Mol Med

Stebbing J, Phelan A, Griffin I, Tucker C, Oechsle O, Smith D, Richardson P (2020) COVID-19: combining antiviral and anti-inflammatory treatments. Lancet Infect Dis 20(4):400–402

Steinman G (2020) COVID-19 and autism. Med Hypotheses 142:109797

Sturman O, Germain PL, Bohacek J (2018) Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. Stress 21(5):443–452

Theoharides C (2020) COVID-19 brain inflammation and autism spectrum disorder. J Child Adolesc Psych 3:1–6

Theoharides TC (2021) Ways to address perinatal mast cell activation and focal brain inflammation, including response to SARS-CoV-2, in autism spectrum disorder. J Pers Med 11(9):860

Totura AL, Baric RS (2012) SARS coronavirus pathogenesis: host innate immune responses and viral antagonism of interferon. Curr Opin Virol 2(3):264–275

Tschopp JF, Bochelen D, Pellerin L (2018) Lactate and the Warburg effect in Alzheimer's disease. In Lactate as a Signaling Molecule (pp. 181–193). Springer

van Steensel FJ, Bögels SM, Perrin S (2011) Anxiety disorders in children and adolescents with autistic spectrum disorders: a meta-analysis. Clin Child Fam Psychol Rev 14(3):302–317

Valdespino-Gomez VM, Valdespino-Castillo PM, Valdespino-Castillo E (2022) Neurological and neuropsychiatric implications of COVID-19: A systematic review. Front Neurol 12. https://doi.org/10.3389/fneur.2021.742756

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57(1):67–81. https://doi.org/10.1002/ana.20315

Varma P, Lybrand ZR, Antopia MC, Hsieh J (2021) Novel targets of SARS-CoV-2 spike protein in human fetal brain development suggest early pregnancy vulnerability. Front Neurosci 14:1440

Volkow ND et al (2021) The healthy brain and child development study-shedding light on opioid exposure, COVID-19, and health disparities. JAMA Psychiat 78:471–472

Wadman M (2021) Brain researchers fret over pandemic's effects. Science 372(6538):14–15. https://doi.org/10.1126/science.372.6538.14

White SW, Oswald D, Ollendick T, Scahill L (2009) Anxiety in children and adolescents with autism spectrum disorders. Clin Psychol Rev 29(3):216–229

Xiong J et al (2020) Impact of COVID-19 pandemic on mental health in the general population: A systematic review. J Affect Disord 277:55–64

Yan CH, Faraji F, Prajapati DP, Ostrander BT, DeConde AS (2020) Self-reported olfactory loss associates with outpatient clinical course in Covid-19. Int Forum Allergy Rhinol 10(7):821–831. https://doi.org/10.1002/alr.22592. Epub 2020 May 28. PMID: 32469165; PMCID: PMC7307983

Zenaro E et al (2017) The role of chemokines in neuroinflammation and neurodegeneration. Cell Mol Life Sci 74(17):3275–3291

Zhang Q, Bastard P, Liu Z, Le Pen J, Moncada-Velez M, Chen J, ... Bolze A (2020) Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. Science 370(6515)