Commercial cow's milk supplementation affects synaptic proteins and behavioral patterns in mice

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Abstract

Cow's milk, a bioactive cocktail with essential nutritional factors widely consumed during early childhood development, has been associated with allergic responses, immune cell activation and typical autistic behaviors in children. In order to investigate whether cow's milk consumption regulates autistic behavior, we used BALB/c mouse as experimental model because they were described by low sociability, significant stereotypes and restrict interest features. The major aims were focused in to investigate whether milk supplementation triggers mechanisms that interfere with behavioral patterns in genetically predisposed mice. Animals received orally commercial cow's milk or water (controls), three times per day for one week. Milk consumption affected the distribution of Drebrin and Shank-3, both post-synaptic proteins, in the cerebral cortex. By immunohistochemistry and histomorphometry, we found that Drebrin^{+} and Shank-3^{+} cells significantly decreased in the cortex. No significant changes were observed in cortical cells expressing Synapotophysin (pre-synaptic protein). The milk intake enhanced the number of Iba-1^{+} cells in the cortical brain and cerebellum, and the number of Purkinje cells expressing nitric oxide synthase 2 (NOS2), suggesting neuroinflammatory and oxidative stress signals, respectively. These histological changes were correlated with disturbances on behavioral patterns after milk consumption. Mice supplemented with milk amplified pre-existing atypic movements, including significant stereotypies, restrict interest and low sociability. In addition, Global Motility Test revealed that milk-treated mice moved in repeated paths on the border of open field arena, exploring 40% less its perimeter than control group. On the other hand, milk consumption did not interfere with velocity, total distance explored, motor functions, fine motor skills or sensibility in hind limb paws of BALB/c mice. In conclusion, we suggest that milk consumption in genetically predisposed mice amplificated atypic behaviors involving mechanisms linked to synapse, neuroinflammation and oxidative stress regulation.

Introduction

Cow's milk is a bioactive cocktail containing microRNAs, hormones, carbohydrates, lipids and vesicles that surpass the intestinal epithelial barrier reaching the systemic circulation. Moreover, it is a critical modulator of the consumer's microbiota and generates biological effects in different tissues and cell types [1-3] . Widely consumed during early childhood, cow´s milk components such as lactose, casein and purified phospholipids [4,5] may cause allergic and non-allergic inflammatory responses.

Possible correlations between milk consumption, neuroinflammation and development of neurodegenerative diseases, neuropsychiatric disorder or atypical behaviors have been reported [6-8]. Mice sensitized with β-lactoglobulin developed anxiety- and depression-like behaviors and astrogliosis [7]. The immunization with bovine casein promoted demyelination in mice and cross-reactivity between cow ´s casein and myelin or neuronal antigens in patients with multiple sclerosis [8]. Moreover, cow's milk regulated macrophage polarization and extracellular matrix synthesis [9-11].

BALB/c mice can be considered in interesting model to study triggers that potentialize atypical behaviors. These mice naturally have low sociability, self-aggressive behaviors, and stereotypies when compared

with neurotypical C57BL/6 mice [12,13]. Although it is very difficult to correlate behavioral changes with morphological and biochemical data, the distribution of synaptic proteins reveals some evidence. In this context, Shank-3, Drebrin (both post-synaptic proteins) and Synaptophysin (pre-synaptic protein) have been investigated in distinct experimental conditions. Shank-3 downregulation is correlated behavioral patterns in autism spectrum disorder (ASD), anxiety and depression [14]. On the other hand, dysfunctions of Synaptophysin/Debrin synapses were associated with Alzheimer's disease, schizophrenia and ASD [15]. However, the effects of milk consumption on both types of post-synaptic targets were never studied.

In the current work, we thought to evaluate the effects of cow´s milk consumption on pre and post-synaptic proteins related with some atypical behaviors pre-existing in BALB/c. Our proposed was based on the premise that cow's milk can trigger molecular and cellular mechanisms that amplify behavioral patterns observed in neurodevelopmental disturbances, including stereotypies, sociability and restrict interest.

Material And Methods

Animals and milk supplementation

Six-week-old male BALB/c mice were obtained from the colony bred at the Federal University of Rio de Janeiro, Brazil. The experimental protocols were approved by local Animal Ethics Committee of Federal University of Rio de Janeiro, Brazil (protocol number: 071/19) in accordance with the guidelines provided the Brazilian College of Animal Experimentation. Mice were randomly separated in two groups. Experimental animals were supplemented with 200 µL three times/day (600 µL/day) of commercial cow's milk by oral gavage. Controls receipt 200 µL of water by gavage during the same time. This study **was carried out in compliance with the ARRIVE guidelines. Each experimental group was composed by 8 mice.**

Behavioral assays

Prior to behavior and motor assays, all animals were acclimated to the testing rooms and to the testing platforms over five consecutive days. Each day they rested during 1 hour inside every new room and subsequently adapted inside the testing platforms: 4 minutes inside open-field; 10 minutes inside the three chambered test platform without the novel colored cubic toy and without the same cage mouse; 15 minutes inside the social interaction and social play behavior box with same cage animals; 40 minutes alone inside the acrylic cage for stereotyped motor behavior filming; and for the ladder rung walk adaptation they needed just to walk freely over the metal rods for 5 minutes or complete 3 circuits. These tests were performed on sound-isolated rooms and on alternative days, to avoid emotional distress and autonomic-freezing responses.

Social interaction, social play and social retraction – Each group of mice at a time were placed inside an open arena 90cmx 45cm x 7cmbox filled with nesting material and they were recorded, 5 frames/second during 15 minutes, from an webcam situated 60 cm above the box. Attitudes of social interaction and social play such as anal sniffing, nose sniffing, pouncing and pinning were recorded for each animal, and

the seconds spent on these behaviors were quantified over the total filmed time. Social retraction which means the condition of non-interaction plus isolation from group of a vigil and not moving mouse was also counted and the percentage of time over the total filmed time was likewise quantified.

Stereotyped motor behavior – Each mouse was individually recorded during 15 minutes, 5 frames/second, from a webcam positioned 60 cm in front of a translucid acrylic cage with 10cmx10cmx15cm to monitor stereotyped motor attitudes such as paw flapping, persistent self-grooming, circling and jumping. We quantified the time of each persistent stereotyped motor behavior and summed all time spent on these over the total filmed time.

Three chamber test – To assess sociability and interest in social or spatial novelty we performed this experiment using one testing mouse (control or milk treated) which would be presented at the same time to (1) a cage containing a mouse already recognized by the testing mouse which is native from the same cage and from the same group; and (2) a cage containing a never presented before colored cubic toy. To check the sociability and social-spatial interest, we counted the time spent by the testing mouse near the cage with the already known same cage mice or with the never presented colored cubic toy (near understood as 5.0 cm from the testing mouse nose tip pointing to one of these cages edge direction) or the time spent by the testing mouse touching, sniffing and exploring one of these cages. This experiment was recorded for 10 minutes by a webcam, 5 frames/second, positioned 1.5m above the cage, and we quantified the exploration time over the total recorded time.

Basso mouse scale, BMS – The test was performed inside a 90 cm circular open-field acrylic platform (EP 154B *Insight Equipamentos Ltda-EPP, BR*). Each animal was evaluated for four minutes by two observers in a double-blinded manner, and were graded from 0 to 9 [16], 0 representing total hind-limb locomotor impairment with paraplegia and 9 normal hind-limb locomotor function. Their performance inside the open-field arena was also recorded with a webcam positioned 1.50m above the platform, 5 frames/second for 2 minutes. Using *ImageJ 1.x* we quantified the percentage of the explored perimeter from the start of tout side movement to the open field center. For such, three points were selected: 1, the center of the open-field where the animal was initially located (*O*); 2, the first point where the animal touched and started to explore the perimeter of the open-field (I); 3, the farthest angular point from the initial perimeter exploration point (*F*). The angle formed by these three points (*FÔI*) is the angular distance from the open-field perimeter. We quantified the percentage of this exploration angular distance over the total angular perimeter (360º) and used the same software to track the mice route over the platform.

Ladder rung walk test – The test was carried out inside a horizontal straight 50 cm ladder platform of 3 mm diameter metal rods spaced each other from a 5mm gap. This catwalk like platform has 90 spaces for metal rods insertion and using the online software RANDOM.ORG: True Random Number Service available at https://www.random.org we randomly selected 60 entrances for the metal rods insertion, creating a random pattern and gaps between the metal rods. This test is used to evaluate fine motor control of mice limbs. These same movements were analyzed to identify possible disturbances in the sensibility of paws, including protopathic sensibility. Each mouse was filmed by a 5 frames/second

webcam during three u-turn routes over the ladder platform. We quantified the percentage of correct steps over the metal rods and divided this number by the total steps number (total steps number = correct steps over the metal rods + slips between the metal rods gaps).

Immunohistochemistry

Brain was collected, immediately fixed in 4% buffered formalin for 72 hours at room temperature, and segmented into cortex and cerebellum fragments. Sections were heated in Trilogy™ (Sigma-Aldrich, USA). Peroxidase and nonspecific antibody interactions were inhibited by 3% H_2O_2 and BSA 8% diluted in 0.002% PBS-Tween 20. Primary antibodies: anti-SHANK3 (Sigma-Aldrich, USA), anti-NOS2 and anti-IBA-1 (BD Bioscience, USA). After respective secondary antibody incubation, samples were treated with streptavidin–peroxidase (Sigma-Aldrich, USA), diaminobenzidine (DAB) used as substrate, and samples counterstained with Harris' hematoxylin. Percentage of positive cells was defined as number of marked cells in 500 cells per sample. Morphological analysis was performed using high-power microscopy (Zeiss-Axioplan, Germany) and images were acquired by Evolution MP 5.0 RTV-Color camera (Media Cybernetics, Canada).

Immunofluorescence

Paraffin sections were deparaffinize with 2 washes with xylene for 15 minutes each. After deparaffinization, the sections were submitted to a gradient of ethanol solution (100% to 50%) of 2 minutes each. Then, samples were incubated with PBS 1x/0.05% tween 20 for 30 minutes. After that, the sections were washed with PBS 1x/0.05% tween 20, incubated in citrate buffer at 98^{o}C for 40 minutes and treated with blocking buffer (3% bovine serum albumin, 5% normal goat serum [Sigma-Aldrich, USA] diluted in PBS 1X/0.05% tween 20). After blocking, the samples were incubated with the primary antibodies overnight, washed 3 times with PBS 1X/0.05% tween 20 and incubated with secondary antibodies. Nuclei were counterstained with DAPI (Sigma-Aldrich, USA). Slices were mounted with DAKO Mounting Media and imaged on a confocal microscope (Leica TCS SPE). Primary antibodies: anti-synaptophysin and anti-drebrin (Milipore, USA). Secondary antibodies: anti-Mouse 594 for synaptophysin and Anti-Rb 488 for drebrin (Molecular Probes, USA). After capturing 6-8 images per group of cortical external granular layer, the green and red channels were aligned, and the colocalized puncta were analyzed using the Puncta Analyzer plug-in in NIH ImageJ [17]. Experiments were done in duplicate.

Statistical analysis

The statistical tests were accomplished using t-test, and significance threshold was fixed for $\alpha = 0.05$. Therefore, P values ≤ 0.05 were considered statistical differences. Each experiment was performed using 6 mice per group in three independent assays.

Results

Cow's milk consumption disturbed synaptic proteins in the cerebral cortex of BALB/c mice

The first question was focused in synaptic proteins. The organization and distribution of cells expressing Shank-3, Synaptophysin and Drebrin were investigated in the cerebral cortex of mice supplemented with commercial cow's milk. In controls, Shank-3$^+$ cells were widely distributed throughout the cortical regions mostly expressed by neurons and glial cells (Figure 1A). In contrast, cow's milk supplemented mice showed significant disorganization of Shank-3$^+$ cells in the similar area of the brain (Figure 1B). Approximately 80% of cortical cells were positive to Shank-3 in control mice while only 15% of these cells were marked to Shank-3 in mice that received milk (Figure 1C).

Another synaptic target was analyzed in the same samples. Cortical Synaptophysin$^+$ and Drebrin$^+$ cells were widely dispersed throughout the brain parenchyma, frequently co-localized in the tissue of control mice (Figure 1D). On the other hand, in mice supplemented with cow's milk it was observed a significant reduction of Drebrin immunoreactivity with drastic consequences to co-localization of both synaptic proteins (Figure 1E). These data pointed to Shank-3 and Drebrin, both post-synaptic proteins, as potential molecular target to be studied in experimental models of milk supplementation.

Cow's milk induces neuroinflammation and oxidative signals in BALB/c mice

In order to investigate possible neuroinflammatory reactions after milk consumption and synaptic disorders, cells expressing Iba-1 were localized and quantified in the cortical areas of the cerebrum and cerebellum after milk intake. The distribution of Iba-1$^+$ cells was significantly deturbed in the cerebral cortex of cow's milk-supplemented mice when compared with controls (Figure 2A and 2B). Quantitative analysis revealed that the percentage of Iba-1$^+$ microglial cells was significantly increased in the cerebral cortex of mice that receive cow's milk (Figure 2C).

In the cerebellum, Iba-1$^+$ cells were found in both control and cow's milk-supplemented mice (Figure 2D and 2E, respectively). These cells represented approximately 4% of total cerebellar cells in controls and about 10% in milk-treated mice (Figure 2F). These data indicated that cow's milk intake induced an expansion of Iba-1$^+$ microglial cells in both cerebrum and cerebellum, suggesting that milk intake induces neuroinflammatory signals.

Oxidative stress was also investigated in these mice receiving milk. NOS2$^+$ cells were localized in the cerebellum of both control and milk-induced mice. Clearly, Purkinje cells were predominantly positive to NOS-2 in these mice (Figure 2G and 2H). NOS2$^+$ Purkinje cells represented approximately 30% of total cerebellar cells in controls whereas this number increased to 65% in cow's milk supplemented mice (Figure 2I). Together, these data indicate that bovine milk intake affects microglial and Purkinje cell niches in the cerebellum of mice.

Cow's milk intake amplified atypical behaviors in BALB/c mice

Disturbances on synapses, signals of neuroinflammation and oxidative stress in the central nervous system (CNS) led us to investigate the pre-existing atypical behaviors in BALB/c mice and to observe whether milk consumption triggered them. Milk-supplemented mice showed significant reduction of social interaction (Figure 3A) and increased social retraction (Figure 3B), suggesting that milk intake amplified the low sociability in BALB/c mice. These mice that received milk used near of 75% of time in isolation and/or performing non-interactive movements. Meanwhile, controls showed this behavioral pattern only during approximately 25% of time (Figure 3B). Repetitive stereotyped behavior was also severely increased in milk-supplemented mice when compared with control group. Mice receiving cow's milk spent approximately 10% of time performing stereotyped movements whereas controls used only 5% of the experimental time repeating some movements (Figure 3C).

The tree-chamber test revealed that milk-treated mice expended more time interacting with same cage mice than control group (Figure 3D). In control group, mice used near of 25% of time in the chamber containing a familiar mouse, however mice that consumed cow's milk spent approximately 45% of time at the chamber containing familiar mouse (Figure 3D). In contrast, milk consumption induced more social distancing with female BALB/c, but not familiar mice. Controls dedicated about 35% of time in contact with a female BALB/c whereas milk-treated mice spent less than 25% of time in the chamber containing a non-familiar female (Figure 3E). BALB/c mice that received milk spent less time exploring new objects. Control mice receiving milk used about 45% of time exploring a new toy. On the other hand, mice that consumed milk spent only 20% of time, suggesting lower interest for new object (Figure 3F). Altogether, these data indicated that cow's milk amplified atypical behavior patterns in genetically predisposed mice.

Cow's milk consumption affected exploration in open field platform

To evaluate other patterns of behavior after the consumption of cow's milk in mice genetically predispose to atypical patterns, the Global Motility Test was performed and important phenotypes were studied in the circular arena. Mice were individually placed in the center of the arena and the total space could be explored. Control mice moved through practically every free space in the arena (Figure 4A), but mice that received cow's milk showed significantly alteration in patterns of movement and exploration (Figure 4B). Intriguingly, these milk-supplemented mice moved with apparent anxiety-related behavior, preferring the border of arena. They moved in repeated paths and when they returned to the starting point, they explored the neighboring area a little more (Figure 4B).

This Global Motility Test revealed that control mice explored approximately 90-95% of the perimeter while milk-supplemented mice explore only 50% of perimeter at the same arena (Figure 4C). Importantly, milk consumption did not interfere with displacement velocity during the exploration of the arena (Figure 4D). Another relevant data revealed that total wandered distance was similar between both groups of mice (Figure 4E), suggesting that differences between explored areas by controls and milk-treated mice would be consequence of fear, insecurity, anxiety, or any other similar central block after milk intake.

Cow's milk intake did not interfere with motor coordination in BALB/c mice

It was clear that cow's milk consumption was directly associated with atypical behaviors in BALB/c mice, including stereotypies and sociability. However, motor coordination and sensibility tests were carried out to assess grosser damage to motor functions and sensitivity of the hind legs, which could interfere with the findings previously described in animals that ingested cow's milk. To measure motor coordination aspects, controls and milk-supplemented mice were submitted to Motor Function Assessment and Fine Motor Skill Assessment. Both groups presented similar scores referent to motor functions (Figure 5A) and fine motor skills (Figure 5B). The sensibility in paws was also analyzed in mice that received cow's milk. In comparison with controls, cow's milk intake did not interfere with protopathic sensibility (Figure 5C). Moreover, both mice groups have similar sensible areas in hind limb paws (Figure 5D). These data suggested that motor coordination and sensibility in paws were not affected by cow's milk consumption in BALB/c mice.

Discussion

For the first time, it was demonstrated that cow's milk intake amplified atypical behaviors in genetically susceptible BALB/c mice. The protocol based on cow's milk supplementation revealed that increase of stereotyped movements, restrict interest and social retraction in these mice would be associated with synaptic dysfunctions (reduction of shank-3^{+} and drebrin^{+} cells in the cerebral cortex), neuroinflammation (increase of Iba-1^{+} cells in the cerebral cortex and cerebellum), and oxidative stress (increase of NOS-2^{+} cerebellar Purkinje cells). These data pointed to specific brain areas, cell types and synapses-related protein potentially targeted to studies involving diet and amplification of atypical behaviors in experimental models.

Neurodevelopmental disorders can be hallmarked by dysfunctions of synaptic proteins, neuroinflammatory reactions and cerebellar oxidative stress. We found that consumption of milk affected two important post-synaptic proteins, Drebrin and Shank-3. Together, disturbances on Shank-3 signaling pathways, GABAergic synapses and microglial cell activation could link our experimental model to pathogenesis of psychiatric disorders, including ASD, depression and anxiety [18-22]. In this context, there is no consensus that cow's milk-free diet is efficient for individuals with atypical behaviors [23]. However, gluten-free/casein-free diets have alleviated ASD symptoms [24].

The disorganization of cellular niches in the CNS can be interpreted as potential targets to elucidate mechanisms involving diets and autistic-like behaviors. In our model, cerebral cortex of mice supplemented with milk was hallmarked by significant reduction of Shank-3^{+} cells. In accordance, Shank-3 stabilizes glutamatergic receptors in post-synaptic densities [25]. Moreover, Shank-3$^{-/-}$ mice exhibited neurodevelopmental disturbances in neurons of prefrontal cortex [26], GABAergic interneuron dysfunctions [18], and social deficits followed by repetitive stereotyped behaviors [27]. For the first time, it

is plausible to propose that cow's milk intake disorganizes niches of Shank-3$^+$ cells and consequently GABAergic and glutamatergic responses in the brain of mice genetically predisposed to autistic-like symptoms.

Consistently, cerebral niches of pre and postsynaptic proteins (synatoptophysin and drebrin, respectively) were also disturbed in mice supplemented with cow's milk. Drebrin fluorescence intensity, but not synatoptophysin, were significantly reduced in the cerebral cortex of mice receiving milk. Drebrin is an actin-binding protein present in postsynaptic glutamatergic neurons whose concentration in dendritic spines organizes cytoskeleton and morphology of the cells. Dysfunctions were associated with neurodegenerative diseases and psychiatric disorders, including Alzheimer's disease, schizophrenia and ASD [28]. It is pertinent to propose that the reduction of Shank-3 and Drebrin in the CNS of milk-supplemented mice can be directly associated with behavioral changes amplified in these mice.

The increase of Iba-1$^+$ cells was also correlated with behavioral symptoms and atypical patterns. The cerebral cortex and cerebellum of milk-supplemented mice were marked by abnormal distribution of Iba-1$^+$ microglial cells. The higher numbers of these cells suggested that milk intake induced pro-inflammatory signals in the CNS. Consistently, the activation and/or increase of Iba-1$^+$ microglial cells is frequently observed during neuroinflammatory responses [29,30].

Gliosis and neuroinflammation have been described in patients and murine models of neurodevelopmental disorders, such as ASD [33,34]. The cerebellum of BTBR T+tf/J (BTBR) mice, a relevant experimental model to study ASD [35,36] is characterized by elevated numbers of Iba-1$^+$ cells and NOS2 expression correlated with stereotypies [37,38]. Our data corroborated with these findings. The cerebellum of milk-supplemented mice was also characterized by increased numbers of Iba-1$^+$ microglial cells and NOS2$^+$ Purkinje cells. In addition, the increased numbers of cerebellar NOS2$^+$ Purkinje cells in milk supplemented mice suggested that cow's milk can induce oxidative stress in this area of the brain. In other experimental conditions, this hypothesis is reinforced [39,40]. These data incite a hypothesis that early damages outside of the CNS can be a trigger to initiate behavioral changes.

Our data indicated that mice receiving cow's milk by oral gavage spent more time performing stereotypies, social retraction with non-familiar mouse and contacting a familiar mouse in three chambers test. On the other hand, these mice supplemented with milk showed even less sociability, including low affinity by same lineage female, and less time exploring new object than controls, suggesting restrict interest. Importantly, milk consumption did not interfere with motor parameters. These behavioral parameters were associated with significant histological and physiologic disturbances, such as reduction of cells expressing synaptic proteins after cow's milk intake. Our data pointed to important cell targets potentially linked to atypical behavioral patterns, and perhaps, an interesting experimental model to study mechanisms that amplify behavioral symptoms in genetically predisposed individuals.

Declarations

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Authors' Contributions

Lemos and Oliveira designed the research project. Lemos, Prins Carpi-Santos and Neumann performed the experiments. Lemos, Prins, Martinez and Neumann analyzed the behavioral tests. Lemos, Luisetto, Mello-Coelho and Oliveira analyzed histological data. All authors reviewed and edited the manuscript

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Figures

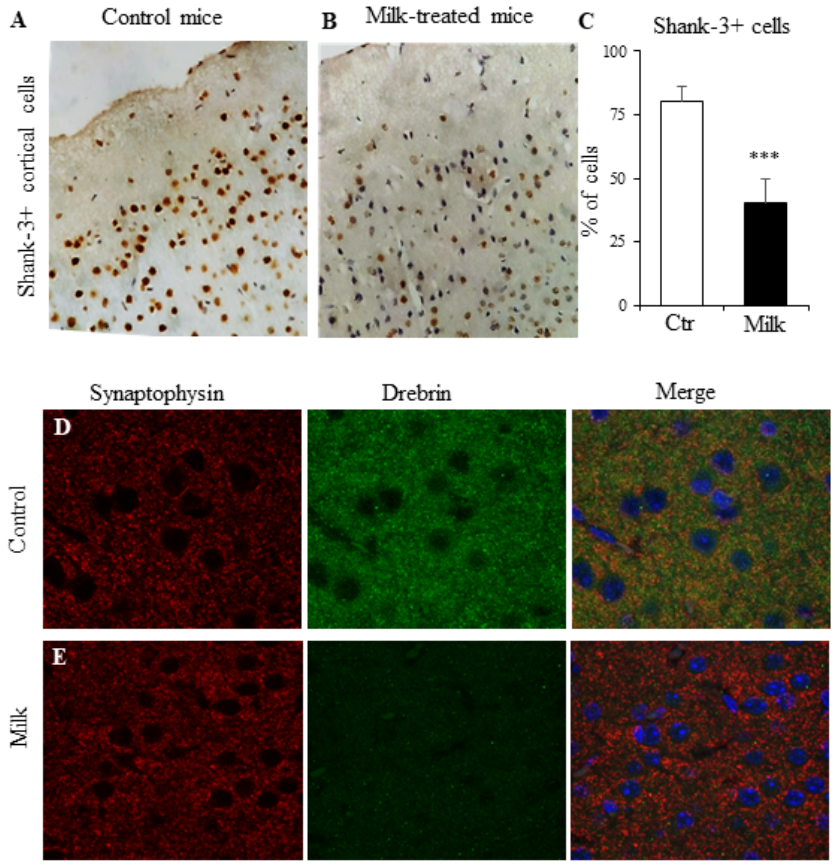

Figure 1

Histological analyses of cerebral cortex of BALB/c mice supplemented with cow's milk.
Immunohistochemistry staining localizes Shank-3+ cells in the cerebral cortex of control (A) and milk-treated mice (B). The bar graphs indicate the percentage of cortical cells expressing Shank-3. White bars represent mice supplemented with water (controls) and black bars indicate milk-treated mice values (C). Immunofluorescence microscopy revealed Synaptophysin+ cells (red) and drebrin+ cells (green) in the

cortex of control (D) and experimental group (E). In both, merge represents an overlay of images (preferentially orange in D and red in E). These data are representative of three independent experiments. (*) p<0.05. (***) p<0.001.

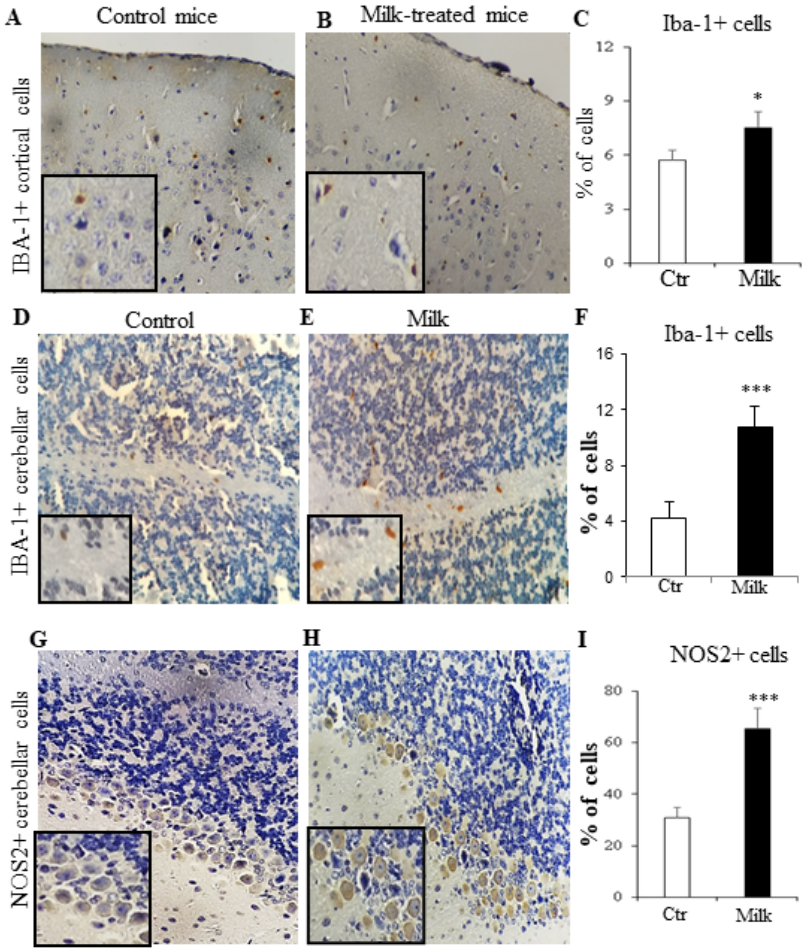

Figure 2

Localization of cells involved with neuroinflammation and oxidative stress in mice supplemented with cow's milk. Immunohistochemistry staining to Iba-1 localizes cortical microglial cells in control (A) and milk-treated mice (B). The bar graphs indicate the percentage of cortical cells expressing Iba-1 (C). In the cerebellum, Iba-1+ microglial cells were localized in controls (D) and milk-treated mice (E). The bar graphs indicate the percentage of cerebellar cells expressing Iba-1 (E). NOS-2+ cells related to oxidative stress were localized in controls (G) and milk-treated mice (H). The morphology and tissue localization of these cells were permissive to conclude that NOS-2+ cells were preferentially Purkinje cells (G and H, inserts). The bar graphs indicate the percentage of NOS-2+ Purkinje cells in the cerebellum of both mice groups (I). White bars represent mice supplemented with water (controls) and black bars indicate milk-treated mice values. These data are representative of three independent experiments. (*) $p<0.05$; (***) $p<0.001$; Amplification: 200X (A, B, D, E, G, H); 400X (inserts).

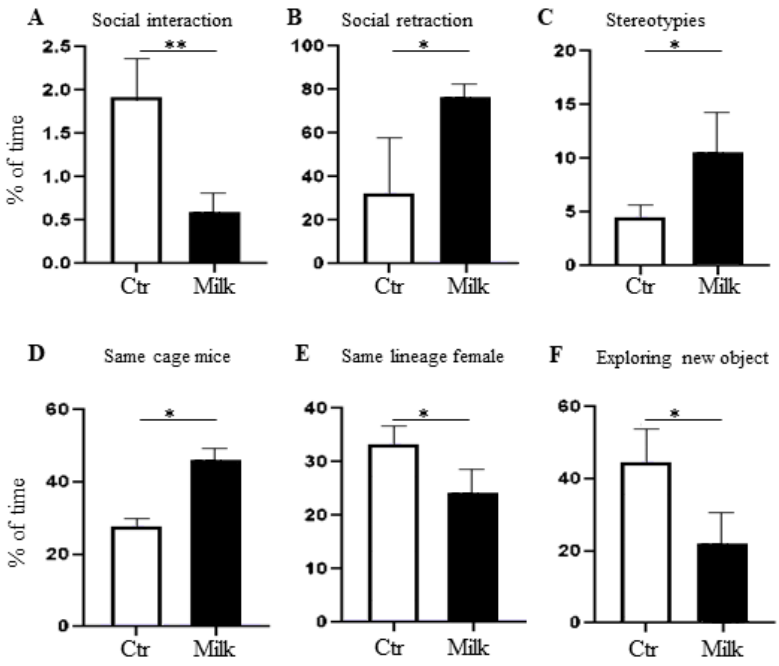

Figure 3

Autistic-like behaviors in BALB/c mice supplemented with cow's milk. Autistic-like behaviors were analyzed in BALB/c mice and the percentage of time spent in social interactions (A), social retraction (B), and stereotyped movements (C). Three chamber tests were used to study preference between same cage (familiar) mouse (D) and female BALB/c (unfamiliar) mice (E). Moreover, restrict interest was also measured by this method (F). White bars represent control mice (supplemented with water) and black

bars indicate milk-treated mice values. These data are representative of three independent experiments. (*) p<0.05. (**) p<0.01.

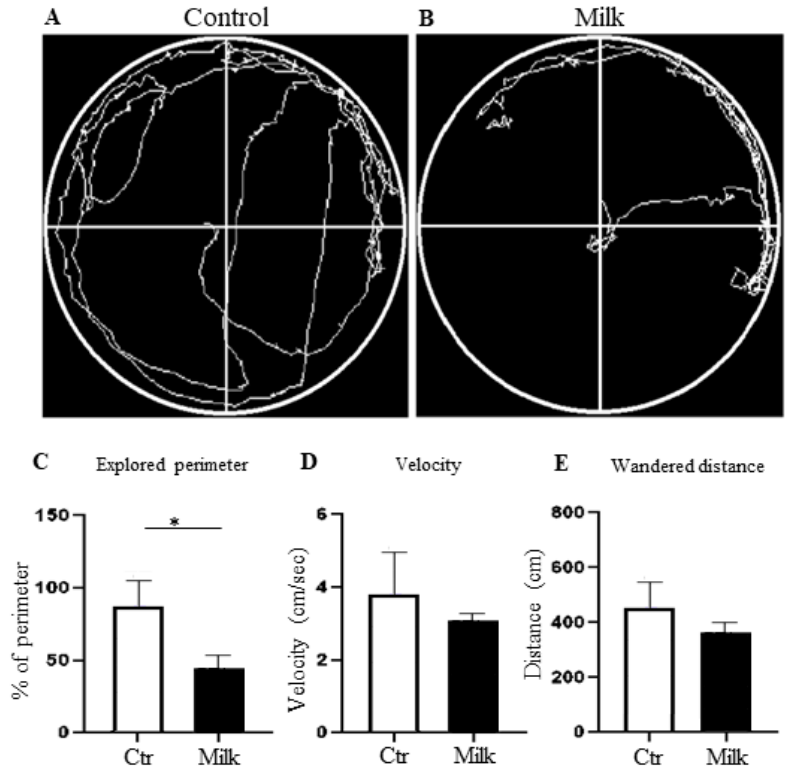

Figure 4

Analysis of motility of BALB/c mice supplemented with cow's milk. The perimeter of circular arena was monitored, and displacement of mice was evaluated in controls (A) and animals supplemented with

cow's milk (B). The global motility test was used to investigate total perimeter (C), velocity (D) and wondered distance during the travel through the arena(E). White bars represent control mice (supplemented with water) and black bars indicate milk-treated mice values. These data are representative of three independent experiments. (*) $p<0.05$.

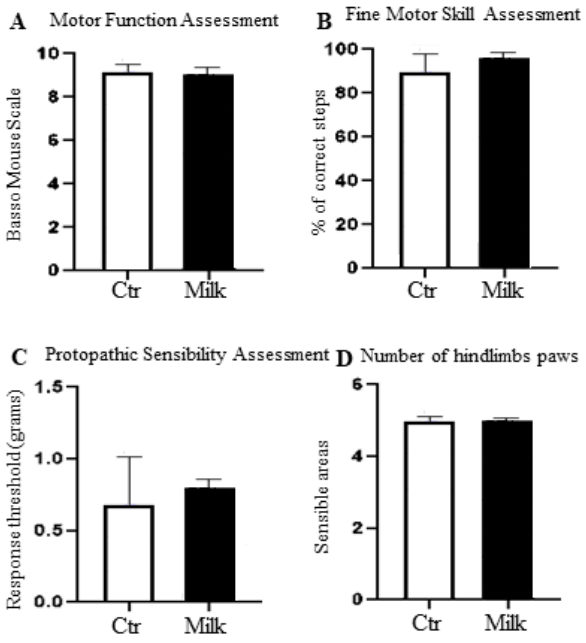

Figure 5

Analysis of motor functions and sensibility in paws of BALB/c mice supplemented with cow's milk. This important tests revealed that milk consumption did not influence in the motor functions (A), fine motor skills (B), protopathic sensibility (C), and numbers of hind limbs paws (D). White bars represent control mice (supplemented with water) and black bars indicate milk-treated mice values. These data are representative of three independent experiments.

Supplementary Files

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