

Neonatal L-glutamine modulates anxiety-like behavior, cortical spreading depression, and microglial immunoreactivity: analysis in developing rats suckled on normal size- and large size litters

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Abstract In mammals, L-glutamine (Gln) can alter the glutamate-Gln cycle and consequently brain excitability. Here, we investigated in developing rats the effect of treatment with different doses of Gln on anxiety-like behavior, cortical spreading depression (CSD), and microglial activation expressed as Iba1-immunoreactivity. Wistar rats were suckled in litters with 9 and 15 pups (groups L_9 and L_{15} ; respectively, normal size- and large size litters). From postnatal days (P) 7–27, the animals received Gln per gavage (250, 500 or 750 mg/kg/day), or vehicle (water), or no treatment (naïve). At P28 and P30, we tested the animals, respectively, in the elevated plus maze and open field. At P30–35, we measured CSD parameters (velocity of propagation, amplitude, and duration). Fixative-perfused brains were processed for microglial immunolabeling with anti-IBA-1 antibodies to analyze cortical microglia. Rats treated with Gln presented an anxiolytic behavior and accelerated CSD propagation when compared to the water- and naïve control groups. Furthermore, CSD velocity was higher ($p < 0.001$) in the L_{15} compared to the L_9 condition. Gln treatment increased Iba1 immunolabeling both in the parietal cortex and CA1 hippocampus, indicating microglial activation. The Gln effect was dose-dependent for anxiety-like behavior and CSD in both litter sizes, and for microglial activation in the L_{15} groups. Besides confirming previous electrophysiological findings (CSD acceleration after Gln), our data demonstrate for the first time a behavioral

and microglial activation that is associated with early Gln treatment in developing animals, and that is possibly operated via changes in brain excitability.

Keywords L-Glutamine · Cortical spreading depression · Brain excitability · Immunoreactivity · Microglia · Anxiety-like behavior

Introduction

Glutamine (Gln) is found abundantly in the mammalian central nervous system, where it participates in a variety of metabolic pathways. In distinct types of neuronal cells, Gln influences the synthesis of the excitatory amino acids, glutamate (Glu) and aspartate, and the inhibitory amino acid, γ -amino butyric acid (GABA; Albrecht et al. 2011). A neuron-glia metabolic interaction, known as the glutamate-glutamine cycle, controls the extracellular concentration of Glu and enables the detoxification of ammonia that crosses freely from the blood across the blood–brain barrier (Daikhin and Yudkoff 2000). Enhanced Gln availability could alter the interactions in the glutamate–glutamine cycle and affect ammonia and Glu levels in the brain. Increased availability of Gln may also affect translocation of Gln into GABAergic neurons and GABA is taken up by astroglia (Holecek 2013).

In humans, Gln supplementation may be beneficial in preterm infants, especially in the reduction of infectious complications (Moe-Byrne et al. 2016). However, the potential effects of early Gln supplementation on brain development and subsequent neuron-glia organization and behavioral outcome have not yet been fully elucidated (De Kievit et al. 2012). In addition, few studies have so far evaluated the effect of Gln supplementation on the excitability-related balance between glutamatergic and GABAergic

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transmission (Lima et al. 2009). These authors reported in developing rats a facilitating effect of Gln (500 mg/kg/day over P7–P27) on the excitability-related phenomenon known as cortical spreading depression (CSD).

CSD is a fully reversible neural response, first described in the rabbit cortex, as a slowly propagating wave of depression of spontaneous neuronal activity produced by electrical, mechanical or chemical stimulation of one point on brain tissue, from which it spreads concentrically to remote cortical regions (Leão 1944). This phenomenon has since been demonstrated in a number of animal studies (Toriumi et al. 2016; Shatillo et al. 2015; Lopes-de-Morais et al. 2014; Guedes et al. 2013). CSD is facilitated under unfavorable lactation conditions (suckling in large litters; Guedes 2011), and this condition can also modulate the effect of other treatments (Guedes et al. 2013). Human studies have demonstrated the occurrence of this depolarizing phenomenon in the setting of a variety of pathological states, including migraine (Lauritzen and Strong 2016), cerebrovascular disorders (Nakamura et al. 1994), epilepsy (Dreier 2011), traumatic brain injury (Hartings et al. 2011), and neurosurgical procedures (Carlson et al. 2016).

Measuring CSD velocity of propagation along the cortical tissue is a reasonable and easy way of estimating the brain susceptibility to CSD. This has been experimentally characterized in our laboratory under conditions of environmental, pharmacological, and nutritional manipulations (Abadie-Guedes et al. 2016; Monteiro et al. 2015; Lopes-de-Morais et al. 2014), including treatment with other amino acids (Frazão et al. 2008). Recent studies have also shown possible relationships between alterations of CSD, and behavioral (Francisco and Guedes 2015) and microglial activation (Soares et al. 2015; Lima et al. 2013, 2014). Also, a relationship between litter size and microglia activation and behavior has been reported (Viana et al. 2013; Tu et al. 2012).

To test whether the CSD facilitating effect of early treatment with Gln is dose-dependent, we used three different doses of Gln during the brain development period. Furthermore, we investigated the Gln effect on anxiety-like behavior and Iba1-immunoreactivity of microglia. Considering that unfavorable lactation conditions can modulate both CSD and the brain effects of amino acids (Francisco and Guedes 2015), as well as microglia (Tu et al. 2012) and behavior (Viana et al. 2013), we analyzed the Gln effects on rats suckled in normal and large litters.

Materials and methods

Animals

The Wistar rat pups of this study ($n = 117$ males) were handled in accordance with the norms established by the

National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA). All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our University (Process no. 23076.010667/2012-84). Animals were housed in polypropylene cages (51 cm \times 35.5 cm \times 18.5 cm) in a room maintained at 22 ± 1 °C with a 12:12 h light:dark cycle (lights on at 6:00 a.m.). The newborn male and female rats, born from distinct dams, were pooled and assigned to be suckled under favorable or unfavorable conditions, represented, respectively, by normal size litters, with nine pups (L_9 groups; $n = 65$ males), and large size litters with 15 pups (L_{15} groups; $n = 52$ males). On postnatal day (P) 25, both groups were weaned and switched to the maternal lab chow diet (Purina Ltd.) with 23% protein. Only the male pups ($n = 117$) were used in this study.

L-Glutamine treatment

From P7 to P27, three groups of male pups received per gavage three doses of Gln (Sigma, St. Louis, MO, USA): 250 mg/kg/day (Gln250 group; 14 L_9 and 13 L_{15} pups), or 500 mg/kg/day (Gln500; 14 L_9 and 8 L_{15} pups), or 750 mg/kg/day (Gln750; 15 L_9 and 11 L_{15} pups). Two additional control groups received no gavage (naive group; 11 L_9 and 10 L_{15} pups) or distilled water (vehicle; 11 L_9 and 10 L_{15} pups). The gavage volume of the amino acid solutions or of distilled water ranged from 0.5 ml/day (in the second week of life) to 1.0 ml/day (in the third and fourth week of life).

Body weights

The rats were weighed at P7, P14, P21, and P30–35 (when the electrophysiological recordings were performed).

Elevated plus-maze test

The elevated plus-maze test was conducted on P28. The cross-shaped apparatus consisted of four arms (two closed arms and two open arms, each measuring 49 cm long \times 10 cm wide) raised 55 cm above the floor. A central squared platform (10 \times 10 cm wide) connected the open and closed arms. For each 5-min session, under dim light and in a sound-attenuated room, the rat was initially placed in the central platform facing an open arm. A video camera recorded the behavioral activity of the animal. The recorded activity was stored in a computer and subsequently analyzed with the software ANYmaze™ (version 4.99 m). After each test, the arms and the central platform were cleaned with a paper cloth soaked with 70% ethanol. The following parameters were analyzed: total distance traveled, number of entries into the open arms, and time spent in the open arms. We considered that the animal

entered one open or one closed arm when its four paws entered the arm.

Open field test

On P30, the rats were placed in the center of a circular arena (diameter 100 cm and height 52 cm). The apparatus was placed in a room with a dim light and sound-attenuation. Rats were positioned in the center arena and their movements were captured using a digital camera for 5 min. Between each test, the open field was wiped with a paper cloth soaked with 70% ethanol. The recorded activity was stored in a computer and subsequently analyzed with the software ANYmaze™ (version 4.99 m). The following parameters were considered: total distance traveled, the number of entries in the central zone, and the time spent in central zone.

CSD recording

On the day of CSD recording (P30–35), the animal was anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose injected intraperitoneally. Three trephine holes were drilled on the right side of the skull, aligned in the frontal-to-occipital direction and parallel to the midline. One hole (2 mm in diameter) was positioned on the frontal bone and used to apply the stimulus (KCl) to elicit CSD. The other two holes (2 mm in diameter) were positioned on the parietal bone and used to record the propagating CSD wave. CSD was elicited at 20-min intervals by a 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole drilled at the frontal region. Rectal temperature was continuously monitored and maintained at 37 ± 1 °C by means of a heating blanket. The DC slow potential change accompanying CSD was recorded for 4 h using two Ag–AgCl agar–Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. The CSD velocity of propagation was calculated from the time required for a CSD wave to pass the distance between the two cortical electrodes. In the two cortical recording places, the initial point of each DC-negative rising phase was used as the reference point to calculate the CSD velocities. In addition, amplitude and duration of the CSD waves were calculated, as previously reported (Mendes-da-Silva et al. 2014).

Immunohistochemistry

Rats treated with vehicle ($n = 6$, of which 3 L_9 and 3 L_{15}), Gln250 ($n = 8$, of which 4 L_9 and 4 L_{15}), or Gln500 ($n = 8$; 4 L_9 and 4 L_{15}) were perfused with 0.9% saline solution

followed by 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline (pH 7.4). After being immersed in the fixative for 4 h, the brains were transferred to a 30% (w/v) sucrose solution for cryoprotection. Longitudinal serial sections (40- μ m thickness) were obtained at -20 °C using a cryoslicer (Leica 1850). Sections were immunolabeled with a polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1) to detect microglia (1:3000; anti-Iba-1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). Free-floating sections were submitted to endogenous peroxidase blocking (2% H_2O_2 in 70% methanol for 10 min) and the sections incubated for 1 h in blocking buffer (BB) solution containing 0.05 M Tris-buffered saline (TBS; pH 7.4), 10% fetal calf serum, 3% bovine serum albumin, and 1% Triton X-100. The sections were then incubated overnight at 4 °C with rabbit anti-Iba-1 (1:1500 diluted in BB solution). After three washes with TBS + 1% Triton, sections were incubated at room temperature for 1 h with biotinylated anti-rabbit (1:500) secondary antibodies. Sections were then rinsed in TBS + 1% Triton and incubated with horseradish peroxidase streptavidin (1:500). The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.33 μ l/ml H_2O_2 . Finally, the sections were mounted, dehydrated in graded alcohols, and coverslipped in Entellan® after xylene treatment. Densitometric analysis was performed on four parallel longitudinal sections for each animal. A Leica DMLS microscope coupled to a Samsung high-level color camera (model SHC-410NAD) was used to obtain digital images from brain sections. Images of the parietal cortex and CA1 hippocampus stained for Iba1 were obtained using a 20 \times microscope objective. In each section, photomicrographs of four fields within the parietal cortex (layer 2) and two fields of CA1 hippocampal region were analyzed, using the Image J software (National Institutes of Health, USA, version 1.46r), which is a worldwide used open tool for the analysis of scientific images (see Schneider et al. 2012 for a historical review). Care was taken to obtain the digital images using the same light intensity. Total immunoreactivity expressed as arbitrary units as well as the percentage of the area occupied by the Iba1-labeled cells were analyzed.

Statistics

Results in all groups are expressed as means \pm standard deviations (SD). Data were compared between groups using ANOVA, including as factors lactation conditions (L_9 and L_{15}) and gavage treatment (naïve, vehicle, Gln250, Gln500, and Gln750) followed by a post hoc test (Holm–Sidak). A p value less than 0.05 was considered significant.

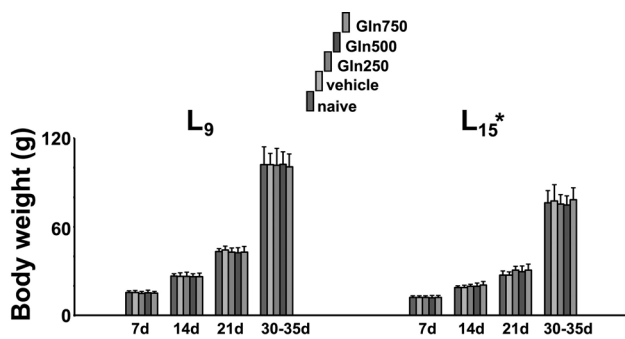


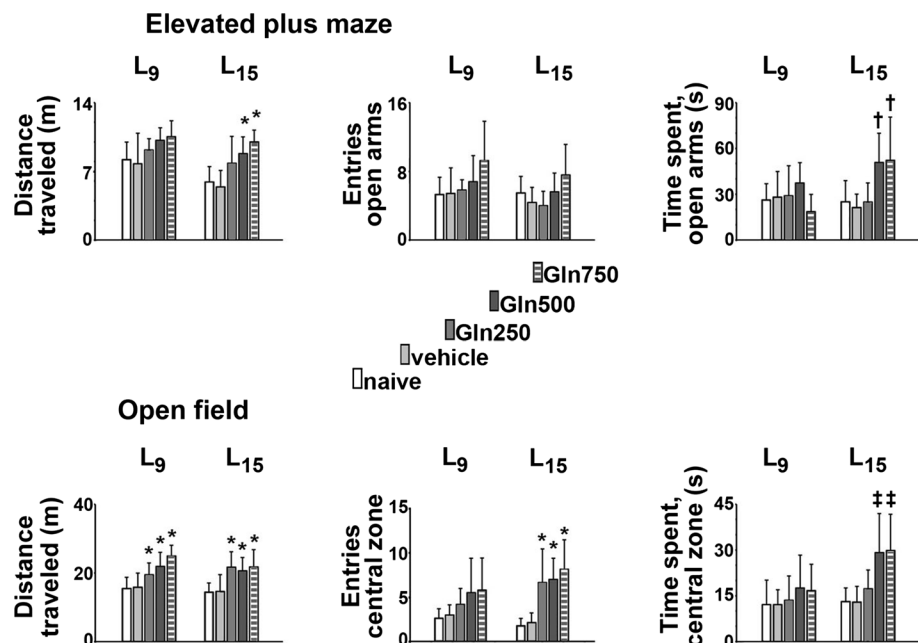
Fig. 1 Body weight of male rats (30- to 35-days old) previously suckled in litters with 9 or 15 pups (respectively, L_9 and L_{15} condition). Gln250, Gln500, and Gln750 are rats treated per gavage from postnatal days 7–27 with L-glutamine at the doses of 250, 500 and 750 mg/kg/day, respectively. Data are mean \pm SD. Asterisk indicate significant difference between L_{15} and the corresponding L_9 groups ($p < 0.05$; one-way ANOVA followed by the Holm–Sidak test)

Results

Body weights

As shown in Fig. 1, L_{15} animals presented with lower ($p < 0.05$) body weights, as compared with those of the L_9 -groups. In the L_9 condition, the body weights of the control groups ranged from 15.4 ± 1.3 g at P7 to 107.8 ± 6.4 g at P30–35, whereas in the L_{15} condition the weights ranged from 12.5 ± 1.0 to 74.3 ± 8.4 g. Treatment with Gln did not alter body weights of animals.

Fig. 2 Behavioral activity, in the elevated plus maze and open field apparatus, of young rats that were previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Gln250, Gln500, and Gln750 are rats treated per gavage from postnatal days 7–27 with L-glutamine at the doses of 250, 500 and 750 mg/kg/day, respectively. Bars represent mean values \pm standard deviation. Asterisk indicates values that are significantly different from naive and vehicle control groups. Plus sign significantly different from control groups and from Gln250. Double plus sign significantly different from the control and from the corresponding L_9 groups ($p < 0.05$; ANOVA followed by Holm–Sidak test)



Elevated plus-maze and open field tests

The effect of Gln treatment on the anxiety-like behavioral activity in the elevated plus-maze, and in the open field tests is shown in Fig. 2. Regarding the elevated plus maze, in the L_{15} condition, Gln500- and Gln750 groups traveled higher distance than the controls and presented with longer time in the open arms than the L_{15} -Gln250, naive, and vehicle groups ($p < 0.05$).

In the open field, the Gln-treated animals of both L_9 and L_{15} condition traveled a higher distance than the corresponding controls. In the L_{15} condition, the Gln-treated animals entered more times in the central zone in comparison with the controls. The Gln500 and Gln750 groups also spent more time in the central zone.

CSD parameters

In all groups, topical application of 2% KCl for 1 min at the frontal cortex usually elicited a single CSD wave, which was recorded by the two electrodes located more posteriorly in the stimulated hemisphere. Figure 3 presents electrophysiological recordings on the cortical surface of five L_9 and five L_{15} animals, illustrating the slow potential change accompanying CSD. The DC slow potential recordings confirmed the presence of CSD after KCl stimulation.

Regarding CSD velocity of propagation, ANOVA indicated intergroup differences [$F(1, 106) = 316.466$; $p < 0.001$], and post hoc (Holm–Sidak) test comparisons

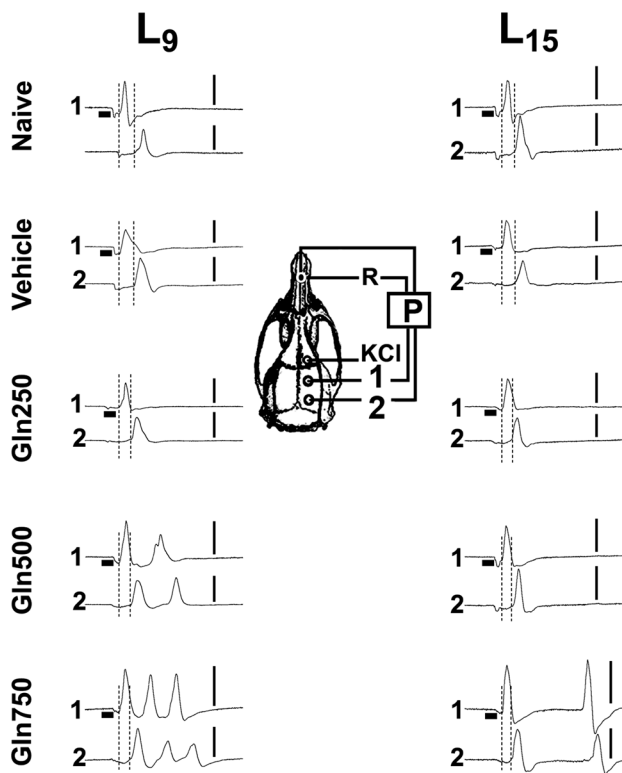


Fig. 3 Slow potential changes (P) during cortical spreading depression (CSD), recorded at two cortical points (1 and 2), in ten developing rats, previously suckled in litters with 9 or 15 pups (respectively, L_9 and L_{15} condition). The diagram of the skull shows the recording positions 1 and 2 from which the traces marked at the left with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones and the application point of the CSD-eliciting stimulus (KCl) are also shown. Gln250, Gln500, and Gln750 are rats treated per gavage with L-glutamine at doses of 250, 500, and 750 mg/kg/day, respectively. The vertical bars indicate 10 mV for P (negative upwards). CSD was elicited in the frontal cortex by chemical stimulation (a 1- to 2-mm diameter cotton ball soaked with 2% KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. The vertical dashed lines indicate the latency for a CSD wave to cross the inter-electrode distance. The latencies were shorter in the L_{15} groups compared with the corresponding L_9 groups. In the groups treated with Gln, the latencies decreased when compared with the respective Naive and Vehicle controls. In both L_9 and L_{15} condition, Gln500 and Gln750 groups displayed shorter latencies than the corresponding Gln250 rats

showed that the velocities were higher ($p < 0.001$) in the L_{15} groups compared to the L_9 groups. Gln treatment significantly increased the CSD propagation velocities [$F(4, 106) = 53.728$; $p < 0.001$] compared with the Naive and Vehicle controls. In both lactation conditions, CSD velocity of the Gln250 animals ($L_9 = 4.12 \pm 0.13$ mm/min; $L_{15} = 4.83 \pm 0.21$ mm/min) was higher than the controls ($L_9 = 3.87 \pm 0.11$; $L_{15} = 4.52 \pm 0.14$ mm/min for the vehicle groups, and $L_9 = 3.85 \pm 0.13$; $L_{15} = 4.61 \pm 0.15$ mm/min for the naive groups), but lower than the Gln500

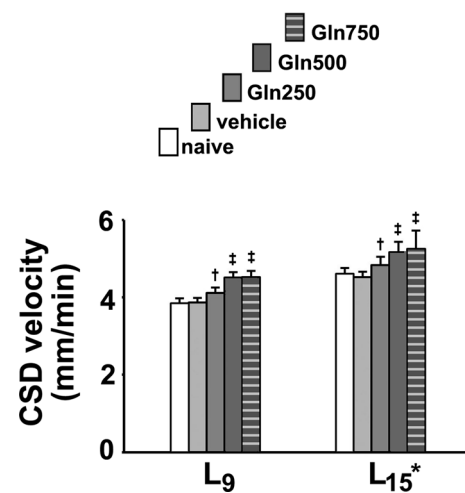


Fig. 4 CSD velocity of young rats (30- to 35-days old) that were suckled in litters with 9 and 15 pups, resulting in two distinct lactation conditions (respectively, L_9 and L_{15} groups). Gln250, Gln500, and Gln750 are rats treated per gavage with L-glutamine at the doses of 250, 500, and 750 mg/kg/day, respectively. Data are mean \pm SD. Asterisk significantly different from the corresponding L_9 groups. Plus sign significantly different from the naive and vehicle control groups. Double plus sign significantly different from the Gln250 and control groups ($p < 0.01$; ANOVA followed by the Holm–Sidak test)

($L_9 = 4.51 \pm 0.14$ mm/min; $L_{15} = 5.17 \pm 0.27$ mm/min) and Gln750 rats ($L_9 = 4.52 \pm 0.16$; $L_{15} = 5.26 \pm 0.46$ mm/min). No difference was found between Gln500 and Gln750 groups in both L_9 and L_{15} condition. The CSD velocities for all groups are shown in Fig. 4.

Data on amplitude and duration of the CSD negative slow potential change are shown in Table 1. ANOVA indicated a main effect of the treatment [$F(4, 87) = 15.025$; $p < 0.001$ for amplitude and $F(4, 96) = 14.194$; $p < 0.001$ for duration]. The Holm–Sidak test indicated that in L_9 condition all groups treated with Gln presented with larger amplitudes and lower durations when compared to vehicle- and naive controls. In this lactation condition (L_9), the Gln750 group had significantly higher amplitude than the Gln250 group. In L_{15} condition, only the Gln750 group showed significantly higher amplitude and shorter duration when compared to the respective controls. The L_{15} vehicle- and naive groups had a shorter duration when compared to the corresponding L_9 .

Iba1 immunohistochemistry

Photomicrographs of Iba1-labeled cells in the parietal cortex and CA1 hippocampus are shown in Fig. 5a, b. The animals of the L_{15} condition had greater immunoreactivity and higher percentage of marked area when compared to the corresponding L_9 condition (Fig. 5c, d). In both lactation conditions,

Table 1 Amplitude and duration of the negative slow potential shifts of CSD in male developing rats (30- to 35-days old) previously suckled in litters with 9 or 15 pups (respectively, L_9 and L_{15} condition)

Group	Amplitude (mV)	Duration (s)
L_9		
Naive ($n = 11$)	10.63 ± 2.20	73.01 ± 5.57
Vehicle ($n = 11$)	10.60 ± 2.54	74.17 ± 6.75
Gln250 ($n = 14$)	14.35 ± 2.13*	68.47 ± 5.52*
Gln500 ($n = 11$)	15.92 ± 2.56*	65.43 ± 5.48*
Gln750 ($n = 14$)	18.10 ± 2.80 [†]	64.40 ± 5.81*
L_{15}		
Naive ($n = 10$)	12.51 ± 3.14	68.54 ± 3.29 [‡]
Vehicle ($n = 10$)	12.86 ± 2.73	69.37 ± 1.83 [‡]
Gln250 ($n = 13$)	14.33 ± 2.96	64.73 ± 4.81
Gln500 ($n = 8$)	15.56 ± 3.69	62.90 ± 3.93
Gln750 ($n = 11$)	16.86 ± 3.39*	60.38 ± 2.85*

Gln250, Gln500, and Gln750 are rats treated per gavage from postnatal days 7–27 with L-glutamine at the doses of 250, 500, and 750 mg/kg/day, respectively

Data are mean ± SD

* $p < 0.05$ compared with controls groups in the same suckling condition

[†] $p < 0.05$ compared with Gln250 and controls

[‡] $p < 0.05$ compared with the corresponding L_9 condition (ANOVA plus Holm–Sidak test)

Gln250 and Gln500 animals showed higher immunoreactivity compared to vehicle control, both in cortex and hippocampus. Unlike L_9 condition, the L_{15} animals that were treated with Gln500 showed significantly greater immunoreactivity when compared to the Gln250 group. Regarding the percentage of labeled area, in the L_9 condition Gln250 and Gln500 exhibited higher percentage of labeled area when compared to control, while in L_{15} condition only the Gln500 group was different from the control in the parietal cortex. In the hippocampus, only the Gln500 group of the L_9 condition was statistically different from the control group.

Discussion

In this study, we confirmed our previous results (Lima et al. 2009), which indicated that treatment with Gln during the critical period of the nervous system development accelerates CSD, regardless of the lactation conditions. Furthermore, we could show, for the first time, changes in anxiety-like behavior and microglial immunoreactivity as a consequence of early treatment with Gln. In some instances, the Gln effects were significantly dose-dependent, and in other instances a non-significant dose-dependent tendency was observed.

Body weights were lower in the L_{15} groups compared with the corresponding L_9 groups, confirming the effectiveness of increasing litter size in producing undernutrition, as previously indicated (Francisco and Guedes 2015; Rochade-Melo et al. 2006). In animal models, malnutrition during the critical period of brain development leads to a reduction in the number and/or size of brain cells, in myelin production and in the number of synapses, and changes in neurotransmitter systems (Morgane et al. 2002). Although some effects of malnutrition on the structure and brain metabolism are reversed by nutritional rehabilitation, other effects are not and continue with subsequent electrophysiological, behavioral, and cognitive changes (Levitsky and Strupp 1995; Penido et al. 2012). The treatment with Gln did not alter the animals' body weight (Fig. 1). This is in line with previous reports on Gln treatment per gavage (Lima et al. 2009) and via subcutaneous injection (Ladd et al. 2010).

Our results of anxiety tests reveal a less anxious behavior in the L_{15} , but not L_9 groups treated with Gln as indicated by the longer permanence in the open arms in the elevated plus maze, and by a higher number of entries and more time spent in the central zone in the open field apparatus (Fig. 2). This lower anxiety-like behavior is in line with previous data on animals that were suckled in large litters (Clarke et al. 2013; Bulfin et al. 2011). In the L_9 groups, there was a non-significant tendency for an anxiolytic effect of Gln for all evaluated parameters, except for the time spent in the open arms of the Gln750 group. It is possible that Gln acts at the central nervous system as an anti-anxiety agent by acting as precursor of GABA. Previous studies have already demonstrated an increased GABA level in the brain of rats treated with Gln per gavage (Wang et al. 2007) or subcutaneously (Ladd et al. 2010). This finding could explain the Gln anxiolytic properties described here. An alternative interpretation of the behavioral data could include Gln-induced enhancement of locomotion, as suggested in humans (McCormack et al. 2015), and also as observed after taurine administration in rats (Francisco and Guedes 2015).

The higher CSD velocity from the L_{15} animals confirms the facilitating action of early undernutrition, induced by suckling the pups in large litters. Concerning the mechanisms by which malnutrition facilitates CSD propagation, a larger volume of extracellular space in the brain hinders CSD elicitation and propagation (Mazel et al. 2002). Inadequate nutrient intake early in life increases cell-packing density and reduces the extracellular space, leading to facilitation of CSD. Another important factor that modulates CSD propagation is cortical myelination. Previous work has demonstrated an inverse correlation between the degree of cortical myelination and CSD propagation velocity (Merkler et al. 2009). Nutritional deficiency reduces brain myelination and increases CSD propagation velocity

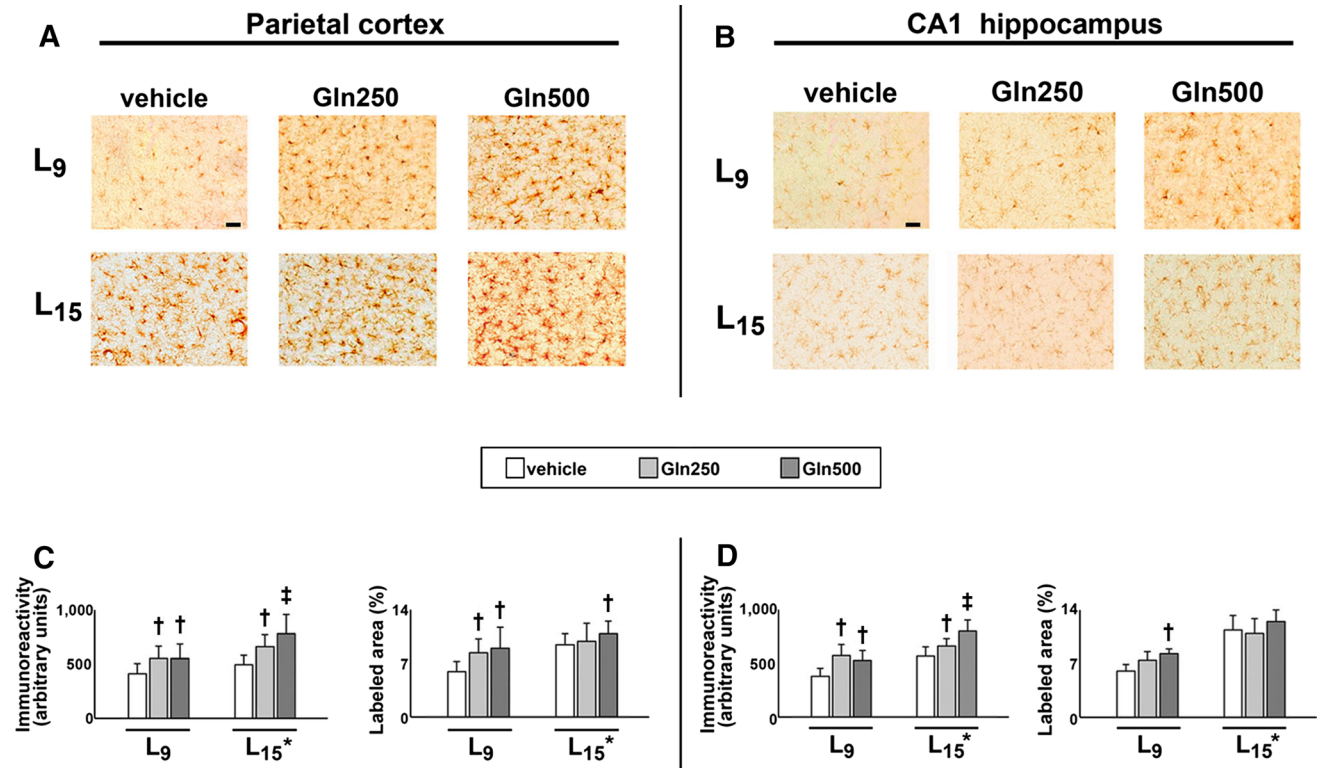


Fig. 5 Low magnification photomicrographs of Iba1-labeled microglial cells in longitudinal sections of the parietal cortex (a) and CA1 hippocampus (b) of six young rats (with 30- to 35-days of life) suckled in litters with 9 and 15 pups, resulting in two distinct lactation conditions (respectively, L_9 and L_{15} groups). Gln250 and Gln500 are rats treated per gavage with L-glutamine at the doses of 250 and 500 mg/kg/day, respectively. Scale bars 20 μ m. The bar graphics show immunoreactivity expressed as arbitrary units and the percent

area occupied by the Iba1-labeled cells of slices of the parietal cortex (c) and CA1 hippocampus (d) of vehicle control ($n = 6$; 3 L_9 and 3 L_{15}), Gln250 ($n = 8$; 4 L_9 and 4 L_{15}) and Gln500 rats ($n = 8$; 4 L_9 and 4 L_{15}). Data are expressed as mean \pm SD. Asterisk $p < 0.05$ compared with L_9 condition. Plus sign $p < 0.05$ compared with vehicle control. Double plus sign $p < 0.05$ compared with the other two groups (ANOVA plus Holm–Sidak test)

(De Luca et al. 1977; Rocha-de-Melo et al. 2006). Furthermore, malnutrition may impair glial function (Morgane et al. 1978) and caloric restriction may suppress induced microglial activation in non-cortical brain areas (Radler et al. 2015; Tu et al. 2012). Furthermore, CSD is facilitated by glial impairment (Largo et al. 1997) and induces potassium currents in microglia (Wendt et al. 2016). In addition, malnourished rats present increased levels of the enzyme glutamic acid decarboxylase (Díaz-Cintra et al. 2007). This condition, in association with reduced brain glutamate uptake (Feoli et al. 2006), enhances extracellular glutamate in the malnourished brain, which might contribute to CSD facilitation (Marrannes et al. 1988).

Our data clearly demonstrate that neonatal treatment with Gln dose-dependently accelerated the propagation velocity of CSD irrespective of the lactation condition (L_9 versus L_{15}). These results are coherent with a previous report (Lima et al. 2009), and reinforce the importance of Gln for the proper electrophysiological functioning of the brain, therefore suggesting that neonatal treatment with Gln

can modulate, at least in part, the brain's ability to propagate CSD. The vehicle group, which was treated per gavage with distilled water, presented CSD features similar to the naive control. In view of this observation, we are compelled to conclude that the assumed causal link between Gln treatment and the here described CSD changes is substantial, and cannot be attributed to the gavage stress.

Although in this study amino acid blood levels have not been monitored, it is reasonable to assume that the present long-term (3 weeks) neonatal Gln treatment might have caused an amino acid imbalance in plasma and tissues (Holecek 2011) and increased its brain concentration (Szpetnar et al. 2016). Thus, it is tempting to hypothesize that the increase in plasma Gln would impair the output of Gln from the astroglia to the bloodstream. The enhanced Gln availability could alter interactions in the glutamate–Gln cycle and affect ammonia and glutamate levels in the brain. This may occur in neurons by activation of the enzyme glutaminase and in astroglia by suppression of another enzyme, Gln synthetase. Enhanced availability of

Gln may also affect translocation of Gln into GABAergic neurons and released GABA into astroglia (Holecek 2013). In fact, glutamate- and GABA-mediated mechanisms are important for the phenomenon of CSD (Holland et al. 2010; Haghiri et al. 2009; Guedes et al. 1992; Marrannes et al. 1988), and glutamate to GABA imbalance may ultimately lead to a hyperexcitable brain (Becerra et al. 2016), which can help in explaining our findings. Another possibility that deserves future investigation would be the evaluation of the effects of Gln on the activation of astrocytes, which seems to be associated with microglia activation (Guan et al. 2016).

Under CSD accelerating and decelerating conditions, microglial immunolabeling has been found to be, respectively, enhanced (Lima et al. 2013, 2014) and reduced (Soares et al. 2015). These data suggest a direct relationship between CSD velocity of propagation and microglia activation. Recent evidence from others suggests that microglia is required for spreading depression (Pusic et al. 2014) and, conversely, CSD activates microglia (Takizawa et al. 2016) and NMDA receptors (Wendt et al. 2016). Our findings are in agreement with those of Lima et al. (2013, 2014), and Takizawa et al. (2016) as we found CSD acceleration and microglial activation in the Gln-treated animals, which suggests a positive modulating role for Gln in the microglial activation in the brain. All of the evidence notwithstanding, a deeper investigation on the possible directions of this relationship is still required.

Microglial cells are the resident immune cells of the CNS. With their highly dynamic processes, they continuously survey the microenvironment, thus being the first cells to be activated in response to injurious tissue demands. These cells are involved in inflammatory responses in the brain, as well as behavioral responses (Viana et al. 2013). It must be remarked that Iba1 immunolabeling is not a specific marker for microglia, as it also labels macrophages, which may infiltrate the CNS and may become involved in inflammatory responses in the brain, as well as behavioral responses (Wohleb et al. 2014).

Microglia help to prevent glutamate-related excitotoxicity by promoting glutamate uptake (Hanisch and Kettenmann 2007). Svoboda and Kerschbaum (2009) reported in vitro apoptosis of microglia as a result of the increase in Gln concentration, suggesting Gln toxicity. They concluded that hydrolysis of Gln and, accordingly, accumulation of ammonium in mitochondria induced the intrinsic pathway of apoptosis. In addition, recent evidence suggests that enhancement of Gln levels by glutaminase inhibition blocks glutamate release from stimulated microglia (Thomas et al. 2014). Data collectively support the Trojan horse hypothesis of Gln toxicity, which involves both astroglia and microglia cells (Albrecht and Norenberg 2006; Svoboda and Kerschbaum 2009).

In the Gln-treated groups, microglia activation has been observed in the parietal cortex and, to a lesser extent in the hippocampal CA1 area (Fig. 5), which suggests a regional difference in microglia activation. This is interesting, as suckling in large litters may increase microglia activation in the hippocampus (Viana et al. 2013) and overnutrition (suckling in litters with 4 pups) enhances microglia activation in hypothalamus and cerebellum (Tapia-González et al. 2011).

In conclusion, the results of this study suggest a brain effect of early Gln treatment, expressed as behavioral (anxiety), electrophysiological (CSD) and microglial alterations in developing rats. Regarding on how the Gln-induced anti-anxiety, pro CSD, and pro microglial activation are related to each other, and how unfavorable suckling conditions can modify this relationship, recent evidence from others indicate that microglia is required for CSD (Pusic et al. 2014), and suckling in large litters increases the brain GABA levels after Gln treatment (Ladd et al. 2010), and modulates CSD (Francisco and Guedes 2015), behavior, and microglia activation (Viana et al. 2013). Furthermore, the relevance of the CSD phenomenon for important neurological diseases has been well established along the last two decades (Gorji 2001; Dreier 2011; Torrente et al. 2014; Kramer et al. 2016). Most discussed mechanisms for this interplay might include Gln action on the GABA-glutamate balance (Holecek 2013; Wang et al. 2007). Future experiments are needed to further clarify the underlying mechanisms, as well as to explore the possibility of such Gln effects on the adult brain.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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