Life Sciences 93 (2013) 388–392

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# Neonatal treatment with monosodium glutamate lastingly facilitates spreading depression in the rat cortex

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ARTICLE INFO

*Article history:*
Received 10 April 2013
Accepted 10 July 2013

*Keywords:*
Food flavoring agent
Brain development
Brain electrophysiology
Glutamatergic system
Rat

ABSTRACT

*Aims:* Monosodium glutamate (MSG) is a neuroexcitatory amino acid used in human food to enhance flavor. MSG can affect the morphological and electrophysiological organization of the brain. This effect is more severe during brain development. Here, we investigated the electrophysiological and morphological effects of MSG in the developing rat brain by characterizing changes in the excitability-related phenomenon of cortical spreading depression (CSD) and microglial reaction.

*Main methods:* From postnatal days 1–14, Wistar rat pups received 2 or 4 g/kg MSG (groups MSG-2 and MSG-4, respectively; $n = 9$ in each group), saline ($n = 10$) or no treatment (naïve group; $n = 5$) every other day. At 45–60 days, CSD was recorded on two cortical points for 4 h. The CSD parameters velocity, and amplitude and duration of the negative potential change were calculated. Fixative-perfused brain sections were immunolabeled with anti-IBA-1 antibodies to identify and quantify cortical microglia.

*Key findings:* MSG-4 rats presented significantly higher velocities (4.59 ± 0.34 mm/min) than the controls (saline, 3.84 ± 0.20 mm/min; naïve, 3.71 ± 0.8 mm/min) and MSG-2 group (3.75 ± 0.10 mm/min). The amplitude (8.8 ± 2.2 to 11.2 ± 1.9 mV) and duration (58.2 ± 7.1 to 73.6 ± 6.0 s) of the negative slow potential shift was similar in all groups. MSG-treatment dose-dependently increased the microglial immunolabeling.

*Significance:* The results demonstrate a novel, dose-dependent action of MSG in the developing brain, characterized by acceleration of CSD and significant microglial reaction in the cerebral cortex. The CSD effect indicates that MSG can influence cortical excitability, during brain development, as evaluated by CSD acceleration. Data suggest caution when consuming MSG, especially in developing organisms.

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## Introduction

The increased incidence of obesity in modern society appears to be a consequence of the association between organic factors (genetic and hormonal predisposition) and several exogenous factors, including overconsumption of a fat-rich diet and a sedentary lifestyle, facilitated by the excessive use of modern electronic media that reduce daily physical activity, such as remote controls and electronic games. The fact that the incidence of obesity is also increasing in childhood is of great concern (Dachs, 2007).

In the last decade, a new and challenging hypothesis has linked obesity, hyperphagia and growth hormone (GH) deficiency to the consumption of elevated amounts of the amino acid glutamate (GLU) (Hermanussen and Tresguerres, 2003a,b). Supraphysiological doses of GLU are toxic to neuronal cells (Hermanussen and Tresguerres, 2005). A broadly used model for studying this issue in experimental animals consists of treating the animals with repeated subcutaneous administration of monosodium glutamate (MSG). MSG is a neuroexcitatory amino acid used as a flavoring agent; it can be harmful to the central nervous system if consumed in great amounts (Nemeroff et al., 1978). Brain lesions and obesity have been reported in adult mice and monkeys previously treated with MSG early in life (Abraham et al., 1971).

During nervous system development, the activation of glutamate receptors may play important roles in naturally occurring neuron death as well as various neurodegenerative disorders. Over-activation of the glutamate ionotropic receptors leads to excitotoxic cell death and can induce apoptosis or necrosis depending on the intensity of receptor activation (Johnston, 2005). These alterations likely influence electrical activity in the brain (Sadeghian et al., 2012). Therefore, we investigated the effect of previous treatment with MSG on the electrophysiological phenomenon known as "cortical spreading depression" (CSD) in the brains of weaned young rats.

CSD has been characterized electrophysiologically in laboratory animals (Leão, 1944, 1947) and humans (Dohmen et al., 2008; Fabricius

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http://dx.doi.org/10.1016/j.lfs.2013.07.009

et al., 2008) as a fully reversible response of the cerebral cortex. In animals, CSD can be produced by electrical, mechanical or chemical stimulation of one point of the tissue and then spread concentrically to remote cortical regions (Leão, 1944; Gorji, 2001). This response propagates slowly as a "wave" of reduced cortical electrical activity. The neural tissue normally offers resistance to CSD propagation. This resistance can be increased or decreased by experimental manipulations, resulting in lower or higher propagation velocity, respectively (Guedes, 2011; Rocha-de-Melo et al., 2006). Measuring the velocity of CSD propagation along the cortical tissue is a reasonable and simple method for estimating susceptibility of the brain to CSD under clinically relevant conditions known to influence brain excitability (Amaral et al., 2009). Experimental conditions that facilitate or impair the brain's ability to produce and propagate CSD may be helpful to understanding the electrophysiological processes dependent on brain excitability and related diseases, such as epilepsy (Leão, 1944, 1972; Guedes and Cavalheiro, 1997; Guedes et al., 2009).

The present study aimed to investigate possible electrophysiological changes in the developing brain, caused by treatment with MSG, as indicated by CSD propagation in rats. We postulated that the susceptibility of the brain to CSD would be altered in weaned young rats that are treated early in life with MSG. In addition, we examined how early administration of MSG affects microglial cells by analyzing IBA1-immunolabeled cells in the motor area of the cerebral cortex.

## Materials and methods

### *Animals*

Male Wistar rat pups received 2 g/kg or 4 g/kg MSG (MSG-2 and MSG-4, respectively; $n = 9$ for each group) subcutaneously every other day during the first 14 days of life. The groups were compared to two control groups: one injected with saline (group Sal; $n = 10$) and one that did not receive any injection (naïve group; $n = 5$). After weaning, the pups were housed in polypropylene cages (51 cm × 35.5 cm × 18.5 cm; 3–4 per cage) in a room maintained at 22 ± 1 °C with a 12:12-h light–dark cycle (lights on at 7 a.m.) and fed a lab chow diet with 23% protein (Purina do Brazil Ltd.). The animals were handled in accordance with the norms of the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco, Brazil, which complies with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, USA).

### *Body weight*

Body weight was measured on postnatal days 2, 10 and 45–50.

### *CSD recording*

When the animals were 45 to 60 days old, they were submitted to CSD recording for a 4-h period. Under anesthesia (1 g/kg urethane plus 40 mg/kg chloralose, ip), the rat's head was secured in a stereotaxic apparatus (Kopf, USA) and three trephine holes (2–3 mm in diameter) drilled on the right side of the skull (two at the parietal bone and one at the frontal bone). The three holes were aligned in the anteroposterior direction and parallel to the midline.

CSD was elicited at 20 min intervals by applying a cotton ball (1–2 mm diameter) soaked in 2% KCl solution (approximately 0.27 M) to the anterior hole drilled at the frontal region for 1 min. The electrocorticogram (ECoG) and slow direct current (DC) potential change accompanying CSD were recorded simultaneously at the two parietal points on the cortical surface using a pair of Ag-AgCl agar-Ringer electrodes. The electrodes consisted of plastic conic pipettes (5 cm length, 0.5 mm tip inner diameter) filled with Ringer solution and solidified with the addition of 0.5% agar, into which a chlorided silver wire was inserted. The pipettes were fixed in pairs with cyanoacrylate glue so that the interelectrode distance was constant for each pair (4–5.5 mm). Each pair of electrodes was fixed to the electrode holder of the stereotaxic apparatus so that the recording electrodes could be placed gently on the intact dura-mater under the guidance of a low-power microscope without any excessive pressure on the cortical surface. A common reference electrode of the same type was placed on the nasal bones. The velocity of CSD propagation was calculated based on the time required for a CSD wave to cross the distance between the two recording electrodes. The initial point of each negative rising phase was used as the reference point for the measurement of CSD velocities. During the recording session, rectal temperature was maintained at 37 ± 1 °C by a heating blanket. After the recording session was terminated, the anesthetized animal was submitted to euthanasia by bulbar injury, which was carried out by introducing a sharp needle into the cisterna magna, provoking immediate cardiorespiratory arrest.

### *Duration and amplitude of slow potential shifts*

For all CSD episodes, we calculated the amplitude and duration of the negative slow potential shifts of the CSD waves recorded in the two cortical points: 1 and 2 (see inset in Fig. 2).

### *Analysis of immunolabeled microglial cells*

An additional group of 12 rats treated with saline ($n = 4$), MSG-2 ($n = 3$) and MSG-4 ($n = 5$) were perfused with 0.9% saline solution followed by 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline, pH 7.4. The brains were removed and immersed in the fixative during 4 h and thereafter transferred to a 30% (w/v) sucrose for cryoprotection. Longitudinal serial sections (40-μm thickness) were obtained at −20 °C with a cryoslicer (Leica 1850). Sections were immunolabeled with a polyclonal antibody against the ionized calcium-binding adapter molecule 1 (IBA-1) to detect microglia (anti-IBA-1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). All chemicals used in this investigation were supplied by Vector Labs (Burlingame, CA, USA) or Sigma–Aldrich (Poole, UK). Free-floating sections were submitted to endogenous peroxidase blocking (2% $H_2O_2$ in 70% methanol for 10 min); then sections were incubated for 1 h in blocking buffer solution (BB) containing 0.05 M Tris-buffered saline (TBS) pH 7.4, 10% fetal calf serum, 3% bovine serum albumin and 1% Triton X-100. Afterwards, sections were incubated overnight at 4 °C with rabbit anti-Iba-1 (1:1,500 diluted in BB solution). After 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 μl/ml $H_2O_2$. Finally, sections were mounted, dehydrated in graded alcohols and after xylene treatment coverslipped in Entellan®. For each animal, densitometric analysis was performed on four parallel longitudinal sections. In each section, we analyzed photomicrographs of four fields within the motor cortex (layer 5) using the software Image J. A Leica DMLS microscope coupled to SAMSUNG high level color camera (model SHC-410NAD) was used to obtain digital images from brain sections. Images from the motor cortex immunoreacted for Iba1 were obtained with a 20× microscope objective. Care was taken to obtain the digital images using the same light intensity conditions. We analyzed the area occupied by the Iba1-labeled cells as well as the immunoreactivity expressed as arbitrary units.

### *Statistical analysis*

Intergroup differences were compared by ANOVA, followed by a post hoc (Tukey–Kramer) test when indicated. Statistical analyses

were performed using Sigmastat® version 3.10. Differences were considered significant when $P \leq 0.05$.

## Results

### *Body weight*

As illustrated in Fig. 1, MSG treatment was associated with a dose-dependent reduction in body weight at 45–50 days of age. The difference was significant ($P < 0.05$) in the group treated with the higher MSG dose (MSG-4) compared to the control rats.

### *Velocity of CSD propagation*

Fig. 2 shows representative electrophysiological recordings (slow DC potential change and ECoG) in one MSG 4, one Sal and one naïve rat. In all groups, 1-min stimulation with 2% KCl at one point of the frontal cortex elicited a single CSD wave that propagated without interruption and was recorded by the two electrodes located more posterior on the surface of the parietal cortex (see stimulation and recording points in the inset of Fig. 2). In each recording point, the ECoG depression and slow potential change confirmed the presence of CSD after KCl application. As a rule, the recovery of the electrophysiological changes caused by CSD took approximately 5 to 10 min, and we maintained a 20-min interval between subsequent stimulations with KCl.

Fig. 3 presents the CSD propagation velocities calculated for all groups. Treatment with 4 g/kg MSG early in life resulted in significantly higher CSD velocities (4.59 ± 0.34 mm/min) compared to the two control groups (saline: 3.84 ± 0.20 mm/min; naïve: 3.71 ± 0.8 mm/min). The MSG-2 group had a mean velocity of 3.75 ± 0.10 mm/min.

### *Duration and amplitude of CSD waves*

The amplitude and duration of the negative slow potential wave, which is the hallmark of CSD, were measured at the two recording points (point 1 and point 2). ANOVA revealed no significant intergroup difference. The mean amplitudes (in mV) for the controls, MSG-2 and MSG-4 groups were, respectively, 8.8 ± 2.2, 9.0 ± 3.8 and 10.7 ± 3.0 for point 1, and 11.1 ± 3.9, 10.0 ± 1.5 and 11.2 ± 1.9 for point 2. The durations (in s) for the controls, MSG-2 and MSG-4 groups were respectively 62.6 ± 10.3, 58.6 ± 7.7 and 58.2 ± 7.1 for point 1, and 73.6 ± 6.0, 63.6 ± 10.4 and 68.8 ± 7.6 for point 2. Data are presented in Table 1.

### *Iba1 immunohistochemistry and densitometric analysis*

The specific immunolabeling of the calcium-binding protein Iba1 in the microglial cells enables the analysis of microglial distribution in the cerebral cortex without the interference of the cortical blood vessels (Fig. 4A). Compared to the controls, MSG-treated animals presented higher percentage of labeled area (Fig. 4B), and a higher immunoreactivity as expressed in arbitrary units (Fig. 4C). This MSG effect was directly dependent on the MSG dose, with the highest effect seen in the MSG-4 group.

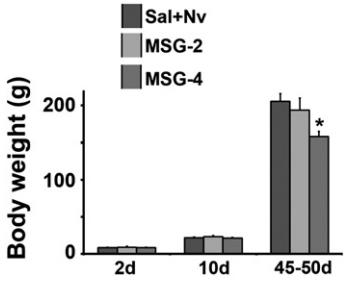


**Fig. 1.** Body weights of rats at 2, 10 and 45–50 days of life. Sal, saline; Nv, naïve, or no treatment; MSG-2, 2 g/kg subcutaneous MSG; MSG-4, 4 g/kg subcutaneous MSG. *$P < 0.05$ compared to control, ANOVA plus Tukey test. Data are presented as mean ± SEM. Groups Sal and Nv were pooled, as their weights were similar.

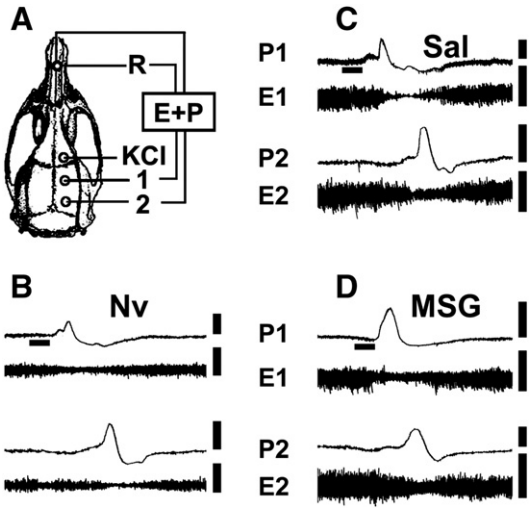


**Fig. 2.** (A) Diagram of the rat skull showing the location of KCl application, the reference electrode and the recording points 1 and 2 (at which the traces in B to D marked with the same numbers were recorded). (B–D) Electrocorticogram (E) and slow potential change (P) recorded during cortical spreading depression (CSD) in three 45- to 50-day-old rats from the untreated group (Nv), saline (Sal) group and group treated with 4 g/kg monosodium glutamate (MSG-4). The horizontal bars in P1 show the period (1 min) of stimulation with 2% KCl necessary to elicit CSD. The vertical bars equal −10 mV and −1 mV, respectively, for P and E (negativity is upwards).

## Discussion

Our data suggested that the neural effects of MSG treatment early in life are important determinants of body weight and documented electrophysiological and microglial alterations in the cerebral cortex. The body weight changes associated with MSG treatment confirmed previous findings (Sun et al., 1991; Zhang et al., 1994; Lobato et al., 2011). Electrophysiologically, we characterized a novel effect of MSG in the developing rat brain.

Our main finding was that 4 g/kg, but not 2 g/kg, MSG treatment early in life increased the brain's ability to propagate CSD when the animals reached 45–60 days of age, as indicated by higher CSD velocities compared to the velocities of the controls. As MSG treatment occurred early in life and CSD acceleration, as well as the microglial reaction was observed after weaning, we postulate that these effects of MSG are permanent, or at least long lasting. In neonatal rats, MSG can cause

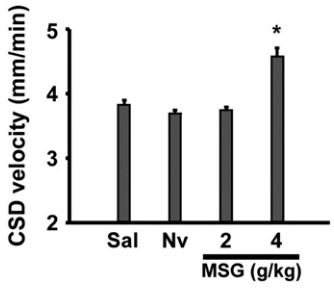


**Fig. 3.** Velocities of CSD propagation in 45- to 60-day-old rats that received previous (during the lactation period) saline (Sal), no treatment (naïve group; Nv) or 2 g/kg and 4 g/kg MSG (subcutaneous injections; MSG-2 and MSG-4, respectively). Data are presented as mean ± SEM of 12 CSD episodes elicited at 20-min intervals by 1-min KCl application during the 4-h recording period. *$P < 0.05$ compared to the other groups, ANOVA plus Tukey test.

**Table 1**
Amplitude and duration of the negative slow potential shifts of cortical spreading depression in rats treated with MSG.

| Groups | CSD amplitude (mV) | | CSD duration (s) | |
|---|---|---|---|---|
| | Point 1 | Point 2 | Point 1 | Point 2 |
| Saline + naïve ($n = 10$) | 8.8 ± 2.2 | 11.1 ± 3.9 | 62.6 ± 10.3 | 73.6 ± 6.0 |
| MSG-2 ($n = 8$) | 9.0 ± 3.8 | 10.0 ± 1.5 | 58.6 ± 7.7 | 63.6 ± 10.4 |
| MSG-4 ($n = 7$) | 10.7 ± 3.0 | 11.2 ± 1.9 | 58.2 ± 7.1 | 68.8 ± 7.6 |

MSG-2, 2 g/kg; MSG-4, 4 g/kg. The treatment groups were compared to control groups treated with saline or no treatment (naïve). The two control groups were pooled, as they did not differ. Data are expressed as mean ± standard deviation. No significant differences were observed.

structural, neuroendocrine and biochemical abnormalities (Zhang et al., 1994; Olney, 1969; Beas-Zarate et al., 2001; Gonzalez-Burgos et al., 2001), which raises the question of whether the MSG-induced electrophysiological changes are caused by an MSG-induced brain lesion or the expression of compensatory mechanisms against such lesion, as previously suggested (Ureña-Guerrero et al., 2003).

The central nervous system is an important target organ for the actions of MSG, particularly during brain development. Brain maturation in the rat occurs mainly during the lactation period, a stage in which synaptogenesis is intense, corresponding to the synaptogenic period in the human brain, approximately the first year of life (Dobbing, 1968; Morgane et al., 1978). The relevance of our data for human brain function is based on the fact that a growing part of the human population consumes MSG as a food flavoring, including children. In European and Asian countries, the increasing consumption of MSG is a matter of great concern (Beyreuther et al., 2007), not only due to the increased risk of obesity but also due to the neurotoxic effects that can occur with high consumption of MSG.

The glutamatergic system has been shown to be involved in the regulation of brain excitability in both humans (Stagg et al., 2011) and laboratory animals (El-Hassar et al., 2011). Interestingly, Lopez-Perez et al. (2010) recently demonstrated that MSG-treated rat pups have altered EEG and behavioral reactions, suggesting seizure initiation. In line with this evidence, we observed facilitation of CSD propagation in the group treated with 4 g/kg MSG. This is important to the present discussion because of the postulated relationship between CSD and excitability-related neurological disorders like epilepsy (Guedes et al., 1988; Guedes and Cavalheiro, 1997) and migraine (Vecchia and Pietrobon, 2012).

The mechanisms by which MSG exerts its actions on the nervous system are not yet fully clarified. Hermanussen and Tresguerres (2005) showed increases in GLU levels and the activation of GLU receptors after neonatal administration of 4 mg MSG/g body weight. They also reported important changes in NMDA-R molecular composition with signs of neuronal damage and an increase in glial cell reactivity. This last effect is in line with our immunohistochemical data that showed increased microglial reaction in the cerebral cortex. It is interesting to note that Iba1-positive microglial cells have been shown to increase in the hippocampus under stressful conditions such as restraint (Park et al., 2011) and aging (Viana et al., 2013).

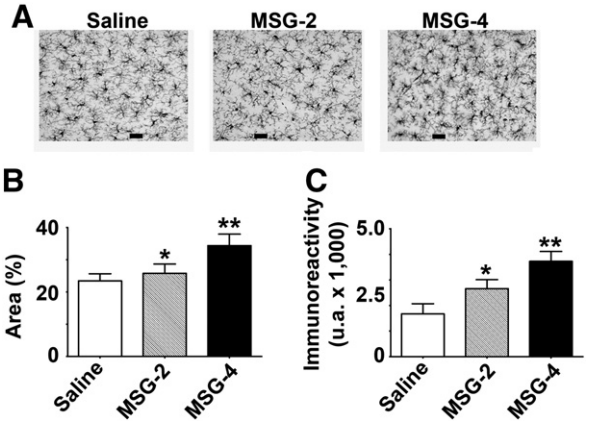


**Fig. 4.** (A) Digital images of Iba1-immunolabeled microglial cells in longitudinal sections through the motor cortex of three rats treated respectively with saline, 2 g/kg MSG and 4 g/kg MSG. Note the darker labeling in the MSG-4 group. Scale bars = 20 μm. (B) Percent area occupied by the Iba1-labeled cells. (C) Immunoreactivity expressed as arbitrary units (a.u.). Data are expressed as means ± standard deviations. *$P < 0.05$ compared to the saline group. **$P < 0.05$ compared to the Saline and MSG-2 groups.

On the other hand, Kaufhold et al. (2002) were able to prevent the adverse effects of neonatal MSG treatment with concurrent administration of a selective and highly potent noncompetitive NMDA-R antagonist, dizocilpine maleate. In general, MSG has been demonstrated to modulate brain excitability (Lopez-Perez et al., 2010), a process that could influence phenomena such as seizures and CSD (Guedes and Cavalheiro, 1997). Effects on neurotoxicity via the p38 pathway (Torres et al., 2006) or increasing brain glutamate levels after MSG treatment (Lopez-Perez et al., 2010) have been discussed but still require corroboration based on robust experimental evidence. However, one cannot exclude the possibility that MSG administration during the postnatal critical period of brain development could also influence other neurotransmitter systems, e.g., the GABAergic system (Ureña-Guerrero et al., 2003, 2009). Furthermore, during development, glutamate may also exert a neurotrophic function, positively influencing neuronal differentiation and circuitry formation (McDonald and Johnston, 1990). This can be relevant for excitability-related phenomena such as CSD.

Although the mechanisms by which MSG accelerates CSD propagation are not yet known, we can speculate based on CSD experiments involving mechanisms that are also proposed as being involved in neural MSG actions. Interestingly, changes in the p38 pathway have been reported in animals with seizures induced by the manipulation of the glutamatergic system (Che et al., 2001). In addition, the accumulation of reactive oxygen species and subsequent release of nitric oxide have been described as a consequence of glutamatergic stimulation (Kostandy, 2012). Such neurotoxic effects seem to be counteracted by the antioxidant molecule ascorbic acid (Hashem et al., 2012), suggesting that the antioxidants potentially exert neuroprotective actions against MSG-induced oxidative stress (Farombi and Onyema, 2006). Notably, all of these mechanisms are known to influence CSD, as demonstrated previously by our laboratory (Guedes et al., 1988; Maia et al., 2009; Monte-Guedes et al., 2011) and by others (Marrannes et al., 1988; Viggiano et al., 2008).

In conclusion, we demonstrated for the first time that the brains of weaned rats treated with MSG during their development are more susceptible (or less resistant) to CSD propagation, without altering other electrophysiological CSD parameters, particularly the amplitude and duration of the negative DC potential typical of the phenomenon. The immunohistochemical data revealed that MSG also induced microglial reaction in the cerebral cortex. We suggest that these MSG actions are related to its excitability effects via synaptic glutamatergic transmission, which ultimately modulates brain excitability. Our findings stress the importance of searching for the molecular mechanisms underlying the actions of MSG in brain development and their electrophysiological expression. As CSD is an energy-demanding phenomenon that involves glutamate, ATP and glucose (Costa-Cruz et al., 2006), and its occurrence involves neuron–glia interaction, we think that the present findings can also be useful for further understanding the CSD underlying mechanisms.

### Conflict of interest statement

None.

## Acknowledgements

The authors thank the following Brazilian agencies for financial support: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

(CAPES-Procad no. 131/2007), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (Facepe—IBPG no. 0085-2.07/09), Instituto Brasileiro de Neurociências (IBN-Net/Finep no. 4191) and Instituto Nacional de Neurociência Translacional (INCT no. 573604/2008-8). R.C.A. Guedes is a Research Fellow from CNPq (no. 301190/2010-0).

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