Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2019.118

RESEARCH ARTICLE

Immunohistochemical Evaluation of Calretinin in the Periaqueductal Gray Matter of Rats Treated with Monosodium Glutamate

Aleksandra Krawczyk, Jadwiga Jaworska-Adamu* and Karol Rycerz

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033, Lublin, Poland
*Corresponding author: jadwiga.jaworska@up.lublin.pl

ARTICLE HISTORY (18-499)

Received: December 20, 2018
Revised: June 22, 2019
Accepted: June 25, 2019
Published online: November 23, 2019

Key words:
CR
MSG
Neurons
PAG

ABSTRACT

Calretinin (CR) protects neurons against uncontrolled ions influx during glutamate induced toxicity. CR-positive cells are present in the periaqueductal gray matter (PAG) which is composed of dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) parts. The aim of the study was to assess distribution, morphology and morphometry of CR-immunoreactive (CR-IR) neurons in PAG of adult rats treated with monosodium glutamate (MSG). For 3 consecutive days the animals received physiological saline solution (group C), 2 g/kg BW (group I) or 4g/kg BW (group II) of MSG s.c. Brain slices of PAG were immunohistochemically stained for CR using a specific antibody in peroxidase-antiperoxidase method. In PAG of all animals CR-IR neurons were morphologically and morphometrically analysed under a light microscope. The density of CR-IR and all cells, the digital immunostaining intensity and the surface area of CR-IR cells bodies were measured. Microscopic analyses revealed the presence of CR-IR neurons in PAG in all animals. In the control these neurons were mainly localised in dm, dl and vl of PAG. Less number of CR-IR neurons was observed in dm and vl of PAG in group I of rats, while in group II they were present throughout the whole PAG. At the same time the overall number of cells was reduced. The analyses demonstrated an increase in the digital immunostaining intensity of CR-IR neurons of PAG under the influence of MSG. The obtained results may indicate the loss of CR-IR neurons in PAG of rats receiving MSG.

©2019 PVJ. All rights reserved

To Cite This Article: Krawczyk A, Jaworska-Adamu J and Rycerz K, 2020. Immunohistochemical evaluation of calretinin in the periaqueductal gray matter of rats treated with monosodium glutamate. Pak Vet J, 40(1): 93-97. http://dx.doi.org/10.29261/pakvetj/2019.118

INTRODUCTION

Periaqueductal gray matter (PAG) is a part of the midbrain located between the third and fourth brain ventricles along the aqueduct of Sylvius (cerebral aqueduct) (Menant *et al.*, 2016; Ajayi, 2017). In rats PAG consists of four longitudinal parts: dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) (Barbaresi *et al.*, 2012; Ajayi, 2017). Each of them connects the ascending and descending paths of the multiple central nervous system (CNS) regions, performing different functions. PAG participates, inter alia, in the modulation of pain, defence responses (immobilisation, fight or flight, jumping), vocalisation, regulation of respiratory and cardiovascular functions (Barbaresi *et al.*, 2012; Menant *et al.*, 2016; Ajayi, 2017).

Calcium (Ca^{2+}) affects the proper functioning of neural networks. Their concentration depends, inter alia, on the presence of buffering systems in the cytoplasm, including the EF-hand family proteins. They contain calretinin (CR) which has six calcium-binding domains. CR also demonstrates sensory properties that a play role in the synaptic plasticity (Schwaller, 2014). CR-positive neurons occur in many CNS areas in rats, such as hippocampus or PAG, and they belong to inhibiting interneurons network (Tóth *et al.*, 2010; Barbaresi *et al.*, 2012).

Calcium homeostasis is necessary to maintain proper neural and Ca^{2+} dependent processes. Disorder of this balance can lead to cell death (Węgierski, 2013). Calcium ions are considered to be the major factor of the neuronal damage in the course of excitotoxicity. This phenomenon is due to excessive or long-term activation of glutamate receptors. Glutamate (Glu) is one of the main stimulatory

neurotransmitters in the mammalian CNS where it participates in the development and proper neural activity. This amino acid is involved, inter alia, in the formation of long-term synaptic potentiation and synaptic plasticity (Ganesan *et al.,* 2013; Bera *et al.,* 2017). Excessive stimulation of Glu-specific receptors contributes to an uncontrolled intracellular increase of calcium ion levels and to activation of catabolic pathways and cell damage. Excitotoxic neuronal death is important in the pathogenesis of many acute CNS pathologies such as stroke, trauma, epilepsy, as well as in chronic neurodegenerative diseases such as Parkinson's, Huntington's or Alzheimer's diseases (Szydłowska and Kamińska, 2008; Lai *et al.*, 2014). Glu is the main component of monosodium glutamate (MSG). When it is taken in excess, it may cause various neurological symptoms (Bera *et al.,* 2017). In rodents MSG-induced brain lesions were reported (Airaodion *et al.,* 2019)

So far, there is no report regarding the effect of MSG on the PAG of rats. Therefore, the aim of this study was to assess the distribution, morphology and morphometry of CR-positive neurons in PAG of adult rats parenterally treated with MSG. Our research may be preliminary to studies in the field of functional neuroanatomy.

MATERIALS AND METHODS

The study was conducted on 15 60-day-old male Wistar rats according to the agreement of the Second Local Ethical Committee in Lublin no. 7/2011. Throughout the experiment animals were kept in cages (20-22°C, 60% air humidity, 12h light/12h dark cycles). The rats were provided with constant access to fodder and water. Animals were randomly divided into three groups of 5 individuals. For 3 consecutive days, rats received s.c. 2 g/kg BW (group I) and 4g/kg BW (group II) of MSG (Sigma-Aldrich, 49621) and physiological saline solution (group C). After 24 h of the last injection, 63-day-old animals were euthanised. Their brains were fixed in 10% formalin, embedded into paraffin blocks, and cut in the frontal 4µm-thick sections.

Immunohistochemical analyses: The indirect peroxidase-antiperoxidase immunohistochemical reactions were performed on the deparaffinised and hydrated brain sections containing PAG from every animal. Antibodies and reagents used in staining were from Sigma-Aldrich (St. Louis, Missouri, USA) and were diluted in 0.5M Tris buffered saline according to producer's recommendations. All slices were incubated at room temperature in 3% H_2O_2 for 30 min, next in the goat serum (G9023, 1:10) for 20 min. The primary rabbit anti-calretinin antibody (C7479, 1:1000) was used for the slices for 16h at 4°C. Subsequently, slices were incubated in the secondary goat anti-rabbit IgG antibody conjugated with the peroxidase-antiperoxidase complex (A9169, 1:400) for 1h at room temperature. Next 3,3'diaminobenzidine tetrachloride was used as a chromogen (30 min, room temperature). The slices were washed in distilled H_2O, counterstained with the Mayer's haematoxylin and mounted in DPX. Positive specificity control was conducted in brain cortex. Negative specificity control was performed by replacing the primary antibody with goat serum. No reaction product was observed in negative control. The stained slices were observed and photographed using the Olympus BX51 microscope with Olympus Color View IIIu digital camera.

Morphological and morphometric analyses: The distribution and morphology of CR-IR neurons of PAG were analysed based on the microscopic observations in 100 slides from every group of rats. The number of CR-immunopositive cells (with brown reaction product) and CR-immunonegative cells (without reaction product) was evaluated in dm, dl, l and vl of PAG of animals from each group in 100 squares of 2.0 x 10^{-2} mm^2 using the Cell^D program. The calculations were made using 20 fields per animal randomly chosen from the imposed grid. The results are shown as the mean density of neurons with CR expression with the standard deviation and the mean density of all cells in the particular parts of PAG with standard deviation. These analyses are to determine if the changes concern cells with CR-expression or whole population of PAG neurons. In the randomly selected 50 CR-IR cells from all parts of PAG in all rats digital immunostaining intensity of the cytoplasm was measured in a square of 1µm^2 area. The results were converted so that the higher values represent a darker colour. In addition, the results were then standardised to remove differences in light exposure by measuring background intensity as was described before (Jaworska-Adamu *et. al.*, 2018) The surface areas of CR-IR cells was measured in 50 randomly selected neurons by manual outlining of their bodies using an interpolated polygon tool. The obtained results were presented as the mean area with the standard deviation. All data were then compared statistically by ANOVA test and the Tukey *post hoc* test. The data that did not meet the condition of normal distribution was compared using the non-parametric Kruskall-Wallis test. The significance factor of all tests was set to α=0.05. The statistical analyses were carried out using the R 3.3.1 program (Free Software Foundation's GNU General Public License, http://www.r-project.org/).

RESULTS

Evaluation of CR-IR neurons distribution and morphology: Evenly distributed CR-IR neurons in all parts of PAG (dm, dl, l, vl) were demonstrated in all tested rats. A diffuse brown reaction product was localised in both the nuclei and cytoplasm of the examined cells. Nuclear or cytoplasmic immunoreactivity of the tested protein was observed in single neurons. CR-positive cells were characterised by differentiated morphology. In all parts of PAG in the rats from the groups C, I and II the neurons were mainly round or oval. Moreover, fusiform, pyramidal and polygonal cells were observed. In the control and group I of rats, in all parts of the PAG, immunostained processes were observed in some neurons. In group II the immunoreactive for CR initial parts of the processes near the cell bodies appeared sporadically (Fig. 1, 2). In addition, in vl of PAG of group I of rats, a round CR-IR cell with the altered, irregularly shaped nucleus and unevenly distributed cytoplasmic reaction product were observed (Fig. 2 B-I).

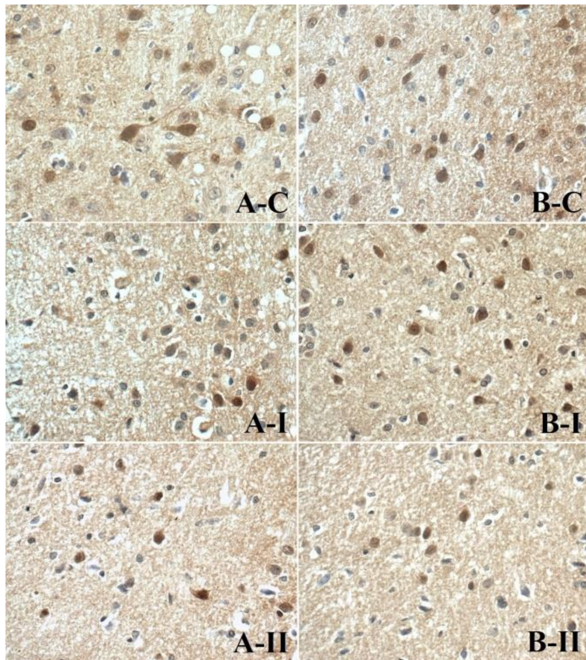

Fig. 1: CR-immunopositive neurons in dm (A) and dl (B) of PAG in rats from group C and from animals treated with 2 g/kg b.w. (I) and 4g/kg b.w. (II) of MSG. Immunoperoxidase labelling for CR; Magnification of objective 40x.

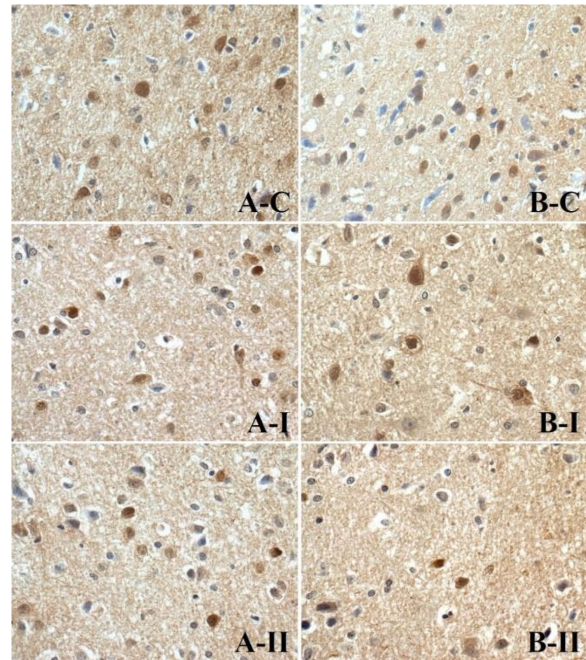

Fig. 2: CR-immunopositive neurons in l (A) and vl (B) of PAG in control (C) and MSG-treated (I, II) rats. Immunoperoxidase labelling for CR; Magnification of objective 40x.

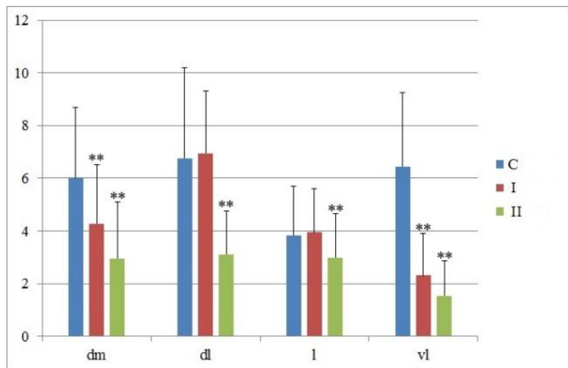

Fig. 3: The mean density with the standard deviation bars of CR-immunopositive cells in dm, dl, l and vl of PAG in control (C) and MSG-treated rats (I, II). Double asterix (**) refers to statistically significant difference between control group and MSG groups (Kruskal-Wallis, P<0.05).

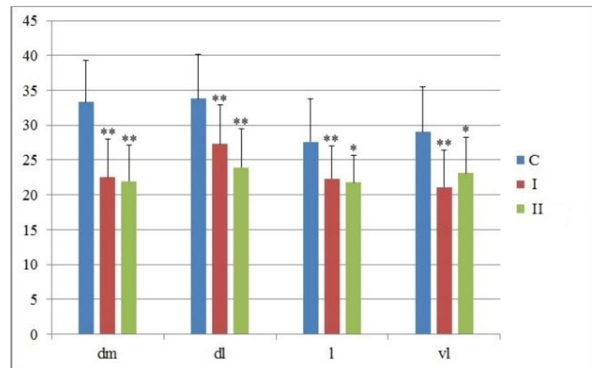

Fig. 4: The mean density with the standard deviation bars of all cells in dm, dl, l and vl of PAG in control (C) and MSG-treated rats (I, II). Single asterix (*) refers to statistically significant difference between control group and MSG groups (ANOVA, P<0,05). Double asterix (**) refers to statistically significant difference between control group and MSG groups (Kruskal-Wallis, P<0.05).

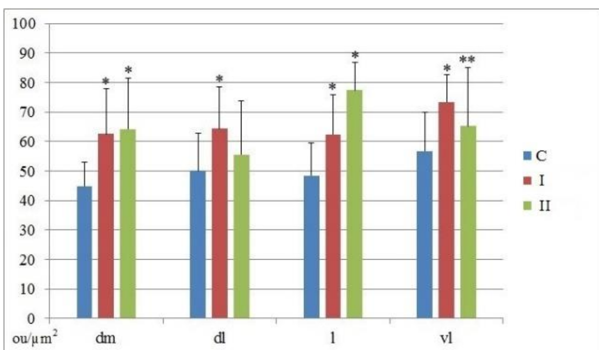

Fig. 5: The mean digital immunostaining intensity with the standard deviation bars of CR-immunopositive cells in dm, dl, l and vl of PAG in control (C) and MSG-treated rats (I, II). Single asterix (*) refers to statistically significant difference between control group and MSG groups (ANOVA, P<0.05). Double asterix (**) refers to statistically significant difference between control group and MSG groups (Kruskal-Wallis, P<0.05).

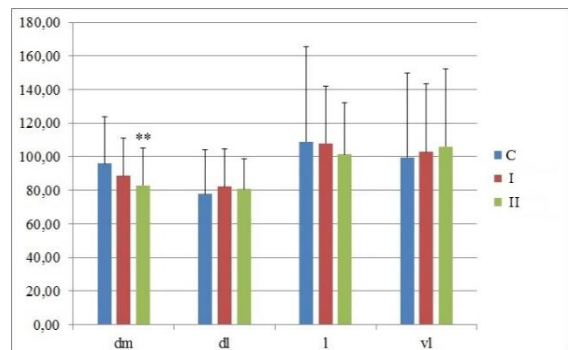

Fig. 6: The mean surface area with standard deviation bars of CR-immunopositive cells in dm, dl, l and vl of PAG in control (C) and MSG-treated rats (I, II). Double asterix (**) refers to statistically significant difference between control group and MSG group (Kruskal-Wallis, P<0.05).

Morphometric evaluation of CR-IR neurons: In the control CR-IR neurons were most abundant in dm, dl and vl of PAG. In group I of animals, the majority of CR-immunopositive cells were found in dl of PAG, while in group II of rats in dm, dl and l of PAG. The morphometric and statistical analyses showed a decrease in the number of immunoreactive CR cells in dm and vl of PAG in group I of animals and in all PAG parts of group II of rats compared to the control (Fig. 3). Furthermore, all cells in the whole PAG in groups I and II of rats were less numerous in comparison to group C (Fig. 4). The morphometric and statistical analyses showed an increase in the digital immunostaining intensity of CR-positive cells cytoplasm in all parts of PAG in group I of rats and in dm, l and vl of PAG in group II compared to the control animals (Fig. 5).

In examined rats, the surface area of the CR-positive neuronal bodies in each PAG part was comparable. CR-positive neurons had an average surface area of 94.6 μm^2. The largest cells were localised in l and vl of PAG of all tested rats. Only in dm of PAG in group II neurons with a smaller surface area were observed in comparison with the control animals (Fig. 6).

DISCUSSION

The present study of rats treated with MSG subcutaneously has shown changes in the number of CR-IR neurons in PAG. Immunohistochemical reactions demonstrated a decrease in CR-IR cells in dm and vl of PAG in the rats treated with 2g /kg BW of MSG (group I) and in all parts of PAG in those administered 4 g/kg BW of MSG (group II). This decrease along with a decrease of the total number of cells, may indicate the loss of CR-immunoreactive neurons. Moreover, round CR-IR cells with altered, irregularly shaped nucleus and unevenly distributed cytoplasmic reaction product were observed. It may indicate on cell damage and death. A lot of experiments have confirmed that MSG causes cell damage and death in many CNS areas in animals (Ganesan *et al.*, 2013; Onaolapo *et al.*, 2016; Kazmi *et al.*, 2017). Pyknotic changes in the Purkinje cells and granular cells of the cerebellar cortex were demonstrated in adult animals receiving MSG *per os* at a dose of 3 g/kg BW for 14 days (Hashem *et al.*, 2011). Whereas a decrease in the cell number, their damage, hypertrophy and vacuolization were observed in the lateral geniculate body of adult rats which were fed with 3g and 6g of MSG added to fodder for two weeks (Eweka and Om'Iniabohs, 2007).

Moreover, our research has shown an increase in the digital immunostaining intensity of CR-IR neurons affected by MSG. This may be due to the increase of intracellular Ca^{2+} ion levels and change in conformation of the protein after Ca^{2+} attachment. In this case the protein changes its immunoreactivity as shown by the immunohistochemical studies (Kuźnicki and Filipek, 1997).

The decrease in the number of CR-IR neurons, the increase in the digital immunostaining intensity of these cells and their altered structures demonstrated in the present study may occur as a result of uncontrolled increase in intracellular Ca^{2+} ion levels due to the excessive stimulation of Glu-specific receptors. Neurotoxicity of MSG may be related to persistent high concentration of Glu in the perineuronal space. As a result, the neurons die due to necrosis or apoptosis. The type of death depends on, inter alia, the intensity of calcium ion influx. Necrosis occurs during persistent depolarisation of cell membranes and rapid penetration of Ca^{+2} ions into the cell. This may lead to metabolic and functional depletion of the cells and to neuronal death (Mattson, 2008). Apoptosis occurs in the course of slow excitotoxicity. This phenomenon is accompanied by oxidative stress with the formation of free forms of oxygen and a disfunction of mitochondria and endoplasmic reticulum (Jiang *et al.*, 2005; Szydłowska and Kamińska, 2008; Hashem *et al.*, 2011; Kazmi *et al.*, 2017). CR-positive cells are very sensitive to the excitotoxic effects of Glu, as was observed in the hippocampus in the course of ischemia or in various models of epilepsy (Huusko *et al.*, 2013). Morphological analyses revealed that these neurons die by necrosis. Evaluation of their ultrastructure showed cytoplasmic degeneration with numerous vacuoles and mitochondrial decay (Tóth *et al.*, 2010).

The CR-IR neurons observed in the rats' PAG, in our study, probably belong to the subpopulations of GABA-ergic structures. In many CNS areas, it was shown that CR-positive cells are inhibiting interneurons containing γ-aminobutyric acid (GABA) (del Río and DeFelipe, 1996; Gonchar *et al*., 2008). In the rat PAG neurons expressing GABA demonstrate a distribution similar to CR-IR cells. They are present mostly in dl and vl of PAG (Reichling, 1991). Neurons expressing CR and GABA have a similar size. According to some authors this points out to their colocalisation in the neuronal cells. Moreover, it was shown that in dl and vl of PAG there is 35-39% of CR-positive axonal terminals which form symmetric, GABA-ergic synapses on dendrites and neuronal bodies of PAG inhibiting their activity (Barbaresi *et al.*, 2012). Additionally, it was found that GABA-positive axons containing CR connect to other inhibitory cells (Barbaresi, 2010). Among them, there are mainly neurons expressing CR but also calbindin (CB) occurs. CB-immunopositive neurons form symmetric and inhibitory synapses with the principal neurons (Barbaresi *et al.*, 2013). The loss of CR-IR interneurons can lead to the reversion of inhibition of CB-containing cells. Thus, they may inhibit the activity of the principal neurons. The death of CR-positive cells may interfere with the synchronization of the interneuronal network activity. In turn, this may impair neurotransmission as a result of improper control of plasticity of stimulating signals coming to the main neurons.

The decrease in the number of CR-IR neurons in the rats' PAG observed in our study may lead to inhibition of local principal cell activity and functional impairment of particular PAG parts. These changes will probably interfere with the proper functioning of the PAG and other areas of the brain which are connected to it. Further studies are suggested to determine whether cell death of CR-IR neurons occurs through apoptosis or necrosis. In addition, it is important to determine the effect of MSG on the reactivity of other calcium-binding proteins present in the PAG neurons.

Conclusions: our preliminary study of the effect of MSG administrated subcutaneously on rats' PAG has shown that MSG, especially at high doses, may lead to loss of CR-positive neurons. Cell damage occur probably as a result of uncontrolled increase of intracellular Ca^{2+} ions level due to excessive stimulation of Glu-specific receptors.

Authors contribution: AK designed the study and performed the experiment. AK and JJA collected the tissue and analysed tissue samples. KR analysed the data. All authors interpreted the data. AK wrote the manuscript while JJA and KR corrected and revised the manuscript. All authors approved the final version of the manuscript.

REFERENCES

Airaodion A, Ogbuagu EO, Osemwowa E, *et al.,* 2019. Toxicological effect of monosodium glutamate in seasonings on human health. J Food Sci Nutr 1:1-9.
Ajayi IE, 2017. Neuro anatomical and physiological considerations for the role of the periaqueductal gray in expressing emotions. MOJ Anat Physiol 3:153-5.
Barbaresi P, 2010. Postnatal development of GABA-immunoreactive neurons and terminals in rat periaqueductal gray matter: A light and electron microscopic study. J Comp Neurol 518:2240-60.
Barbaresi P, Mensà E, Lariccia V, *et al.*, 2013. Differential distribution of parvalbumin- and calbindin-D28K-immunoreactive neurons in the rat periaqueductal gray matter and their colocalization with enzymes producing nitric oxide. Brain Res Bull 99:48-62.
Barbaresi P, Quaranta A, Amoroso S, *et al.*, 2012. Immunocytochemical localization of calretinin-containing neurons in the rat periaqueductal gray and colocalization with enzymes producing nitric oxide: a double, double-labeling study. Synapse 66:291-307.
Bera TK, Kar SK, Yadav PR, *et al.,* 2017. Effects of monosodium glutamate (MSG) on human health: a systematic review. WJPR 5:139-44.
del Río MR and DeFelipe J, 1996. Colocalization of calbindin D-28k, calretinin, and GABA immunoreactivities in neurons of the human temporal cortex. J Comp Neurol 369:472-82.
Eweka AO and Om'Iniabohs FAE, 2007. Histological studies of the effects of monosodium glutamate on the lateral geniculate body of adult wistar rats. Electron J Biomed 2:14-8.
Ganesan K, Sukalingam K, Balamurali K, *et al.*, 2013. A studies on monosodium L- glutamate toxicity in animal models- a review. IJPBCS 3:1257-68.
Gonchar Y, Wang Q and Burkhalter A, 2008. Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. Front Neuroanat 1:1-11.
Hashem HE, El-Din Safwat MD, *et al.*, 2011. The effect of monosodium glutamate on the cerebellar cortex of male albino rats and the protective role of vitamin C (histological and immunohistochemical study). J Mol Histol 43:179-86.
Huusko N, Römer C, Ndode-Ekäne XE, *et al.*, 2013. Loss of hippocampal interneurons and epileptogenesis: a comparison of two animal models of acquired epilepsy. Brain Struct Funct doi: 10.1007/s00429-013-0644-1.
Jiang SX, Lertvorachon J, Hou ST, *et al.*, 2005. Chlortetracycline and demeclocycline inhibit calpains and protect mouse neurons against glutamate toxicity and cerebral ischemia. J Biol Chem 280:33811-8.
Jaworska-Adamu J, Krawczyk A, Rycerz K, *et al.*, 2018. Immunoreactivity for calretinin in interneurons of the hippocampal CA1 field and dentate gyrus in adult rats after administration of habanero peppers (*Capsicum chinense Jacq.*). Pol J Vet Sci 21:469-74.
Kazmi Z, Fatima I, Perveen S, *et al.*, 2017. Monosodium glutamate: Review on clinical reports. Int J Food Prop 20:1807-15.
Kuźnicki J and Filipek A, 1997. Różnorodność i wielofunkcyjność białek wiążących wapń (CaBP). Kosmos 46:603-8.
Lai TW, Zhang S and Wang YT, 2014. Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog Neurobiol 115:157-88.
Mattson MP, 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann N Y Acad Sci 1144:97-112.
Menant O, Andersson F, Zelena D, *et al.*, 2016. The benefits of magnetic resonance imaging methods to extend the knowledge of the anatomical organization of the periaqueductal gray in mammals. J Chem Neuroanat 77:110-20.
Onaolapo OJ, Onaolapo AY, Akanmu MA, *et al.*, 2016. Evidence of alterations in brain structure and antioxidant status following 'low-dose' monosodium glutamate ingestion. Pathophysiology 23:147-56.
Reichling DB, 1991. GABAergic neuronal circuitry in the periaqueductal gray matter. In: The midbrain periaqueductal gray matter. Functional, anatomical, and neurochemical organization. (Depaulis A, Bandler R, eds): Plenum Press, New York, USA pp:329-44.
Schwaller B, 2014. Calretinin: from a "simple" Ca2+ buffer to a multifunctional protein implicated in many biological processes. Front Neuroanat 8:1-7.
Szydłowska K and Kamińska B, 2008. Farmakologiczna modulacja funkcji i żywotności astrocytów – jako nowa strategia neuroprotekcji po niedokrwieniu. Kosmos 57:315-29.
Tóth K, Eross L, Vajda J, *et al.*, 2010. Loss and reorganization of calretinin-containing interneurons in the epileptic human hippocampus. Brain 133:2763-77.
Węgierski T, 2013. Mikroskopowe metody rejestracji wewnątrzkomórkowych odpowiedzi wapniowych. Kosmos 62:193-203.