The Effect of Exercising on Oxidative Stress Status and Pain in a Valproic-acid Induced Model of Autism

Possible relevance of oxytocin

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In this report we will describe the effect of exercising (6 days, each day 3 separate training phases of 5 minutes each, on an adapted treadmill at 1.0 m/s) on oxidative stress status from the temporal lobe (expressed through 3 main parameters: superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA), as a marker of lipid peroxidation) and pain (as determined through 2 specific behavioural tasks such as hot plate test for the supraspinal acute thermal pain and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain) in a rat valproic-acid induced perinatal model of autism, also trying to emphasize a possible implication of oxytocin in this complex pathological picture. We demonstrated here an increased oxidative stress status, as a result of treadmill exercising, in a VPA rat induced model of autism, as demonstrated mainly by a significant decrease in the specific activity of SOD (for the exercised VPA female rats), as well as a significant decrease of GPX specific activity in the male VPA exercised rats, when compared to non-exercised VPA groups, as well as the fact that the aforementioned series of exercises did not resulted in any changes of the pain perception of this rat models of autism, as studied in 2 pain-related behavioural tasks, independent to the gender of the rats with VPA model of autism.

Keywords: autism, oxidative stress, model, pain, superoxide dismutase, glutathione peroxidase, malondialdehyde

Autism is a neurodevelopmental condition characterized by specific social behaviour deficits, excessive repetitive habits, restrained specific interests and persistence on sameness [1]. Nonetheless, aside from the obvious social impairments other traits of autism are reported, such as impairments in cognitive abilities [2], depressive and anxious features [3] and pain perceiving alterations [4, 5].

In addition, physical exercises were demonstrated to offer a lot of advantages to psychiatrically ill patients [6]. Moreover aside the anxiolitic, antidepressive [7] and increased self-esteem levels [8] there has been shown that people practicing physical training might benefit from preventing or delaying the outbreak of the psychiatric condition itself [9] or be a powerful adjuvant in the psychiatric therapeutic approach. Along with the positive effects that exercise manifests on social withdrawal [7], there is also a lot of evidence pointing out to a possible benefit for the autistic disorder as well.

Also, as studying autism only in humans is a difficult task that implies a lot of ethical issues, lately there were created some rodent model of this disorder, which are replicating most of its autistic pathophysiological features. In this way, one of the classical models of autism in rodents is based on the observation that valproic acid (VPA), an antiepileptic treatment, when applied to pregnant women results in an increased teratogenic risk of autism [10]. Following the same rationale, the Rodier et al. [11] research group induced an animal model of autism, based on the perinatal administration of VPA in an increased dosage (usually 500 or 600 mg/kg), in the 12.5 day of gestation, resulting in a complex range of behavioral and anatomical modifications of autistic-like features in the new-born rats (e.g. from the VPA-treated mothers).

Moreover, oxidative stress is the well-known disbalance between antioxidants and reactive oxygen species (ROS), and frequently occurs as an incriminated factor in most of the neuropsychiatric pathologies [12-19], including autism [20]. Noteworthy for the current context is also the fact that lately the pain conditions have been showed to be associated with changes in the oxidative stress status [21-24].

In fact, pain (e.g. a phenomenon that implies sensory, cognitive and emotional features) is still an event that has many unknown characteristics. Also, reports following pain reactions in patients with autism are not numerous and have contradictory outcomes. Most of them indicate to a diminished pain perception of individuals with autism, this finding being even included among the pathological profile of autistic disorders described in the diagnostic manuals published by the American Psychiatry Association [1].

Although these specific pain symptoms were included in the diagnostic features of autism spectrum disorders, the reports and observations which these conclusions on pain insensibility were based on some design and methodological problems [4], being sometimes fully relayed upon anecdotal observations and clinical reactions [25], with a lot controversies in this area of research and authors [26, 27] stating that these theories are not entirely correct.

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Therefore, in the present context, in this report we will describe the effect of exercising (6 days, each day 3 separate training phases of 5 minutes each, on an adapted treadmill at 1.0 m/s) on oxidative stress status from the temporal lobe (expressed through 3 main parameters: superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA), as a marker of lipid peroxidation) and pain (as determined through 2 specific behavioural tasks such as hot plate test for the supraspinal acute thermal pain and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain) in a rat valproic-acid induced model of autism, also trying to emphasize a possible implication of oxytocin in this complex pathological picture.

Experimental part

Material and methods

For this experiment we used adult Wistar rats males and females (n = 12) acquired from the Victor Babes National Institute of Research and Development, Bucharest, Romania. The rats were housed in a room with controlled temperature and humidity, daily monitored, at circadian cycle of 12h light/12h darkness (7:00-19.00), with free access to water and food. There must be stated that all the animals involved in the experiment were treated according to the existing guidelines of animal bioethics stated in the Act on Animal Experimentation and Animal Health and Welfare Act from Romania. All procedures applied were in conformity with the modern specific legislation [28-34]. Efforts were made to minimize animal suffering and to decrease the number of animals included.

Experimental protocol

A first step of the process was the inducing the autistic features into the rat animal model. As aforementioned, we followed the procedure designed by Rodier *et al.*, 1996 [11] and injected the valproic acid (VPA) in gestant female rats in the 12.5 day of gestation using 500mg/kg dosing. The offspring were weaned on postnatal day 21 and 7 after that we conducted the trail period for selecting the animals which will be the runners. After that, the following groups were established from the runners only: exercised VPA male (n = 3); non-exercised VPA male (n = 3); exercised VPA female (n = 3); non-exercised VPA female (n = 3). The training period for the exercised was for 6 days, each day 3 separate training phases of 5 min each, on an adapted treadmill, at a speed of 1.0 m/s and environmental conditions 21°C, 32% humidity and 750 mmHg atmospheric pressure.

All animals (n = 12) were housed in cages of maximum 3 (the actual groups) and were brought into the experimental room 20 min before the start so they could adapt to the environment.

In the last two days, the behavioural reaction to pain was studied. The selected tests for this were the hot plate test for the supraspinal acute thermal pain (performed in day 7) and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain (in the day 8).

Behavioural procedures

The hot plate test is used to determine the latency response of animals subjected to acute thermal stimuli applied by placing the animal to be tested on a plate that reaches temperature of 55°C.

The administration of Zymosan, a beta-glucan-glucose polymer extracted from the cell wall of *Saccharomyces cerevisiae* or *Candida Albicans*, is used to create a model of persistent inflammatory pain. The number of abdominal constriction responses is quantified during a period of 12 minutes from the induced sterile peritonitis.

At the end of all behavioural testing animals were anesthetized by intraperitoneal injection with a cocktail of ketamine and xylazine 1:1 (ketamine 100mg/kg, xylazine 10 mg/kg) and afterwards rapidly decapitated and the whole brain was removed. The temporal lobes were then collected. Each of the samples was weighed and homogenized with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in bidistilled water (1g tissue/ 10mL bidistilled water). Samples were centrifuged 15 min at 3000 rpm. Following centrifugation, the supernatant was separated and pipetted into tubes.

Biochemical determination

Superoxide dismutase measurement

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD Assay Kit (Fluka, product number: 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units [35].

Glutathione peroxidase measurement

Glutathione peroxidase (GPX) activity was measured using the GPx cellular activity assay kit CGP-1 (Sigma Chemicals). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity [36].

Malondialdehyde measurement

Malondialdehyde (MDA) concentrations were determined by thiobarbituric acid reactive substances (TBARs) assay. 200 µL of supernatant was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (*p*H = 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 20 min. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/mg protein [37, 38].

Statistical Analysis

To compare the differences between one-way analysis of variance (ANOVA) was applied. All results are expressed as the means ± SEM. The criterion for statistical significance was considered at $p < 0.05$.

Results and discussions

Oxidative stress markers

Regarding the oxidative stress markers which we determined, these included superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA), as a marker of lipid peroxidation.

Thus, regarding SOD, we could observe a significant decrease of its specific activity in the VPA exercised group, as compared to the non-exercised VPA rats ($p = 0.01$) in the male rats (fig. 1A). However, in the female rats we

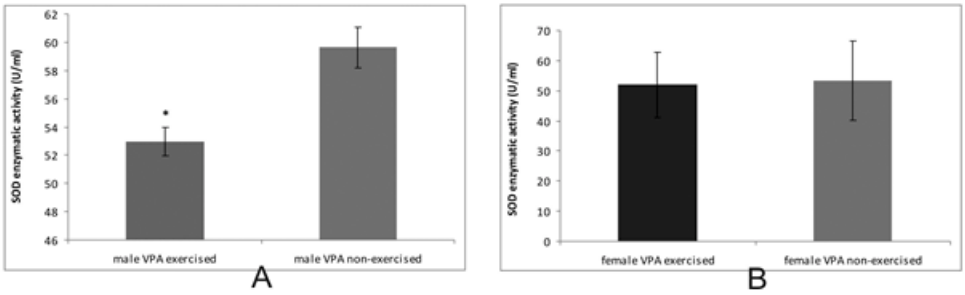


Fig. 1. The effects of exercising or non-exercising on superoxide dismutase (SOD) specific activity from the temporal lobe of rats with VPA-induced model of autism (both genders). The values are mean ± S.E.M. (n = 3 per group). *p = 0.01 vs. non exercised group.

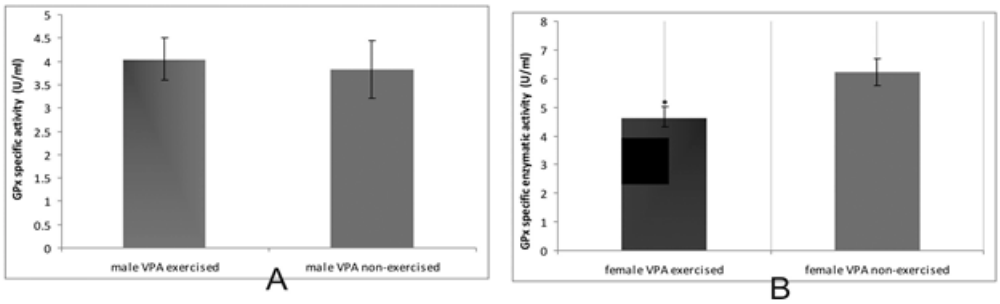


Fig. 2. The effects of exercising or non-exercising on glutathione peroxidase (GPX) specific activity from the temporal lobe of rats with VPA-induced model of autism (both genders). The values are mean ± S.E.M. (n = 3 per group). *p = 0.04 vs. non exercised group

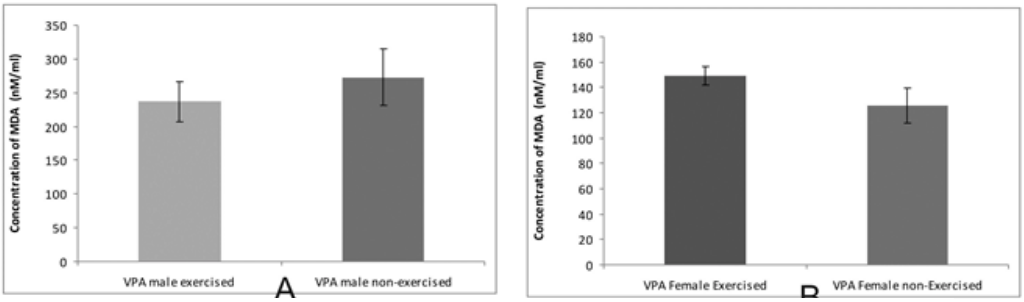
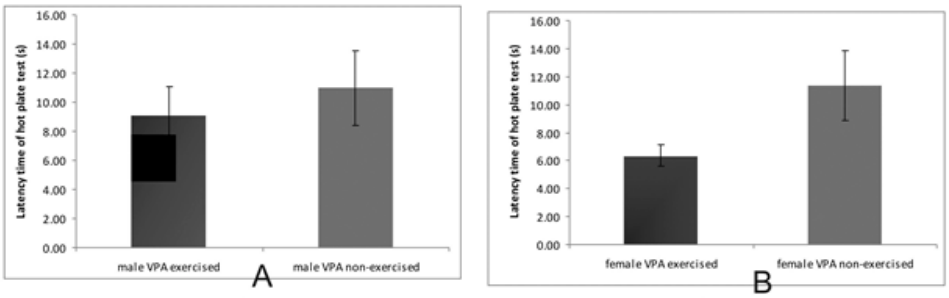


Fig. 3. The effects of exercising or non-exercising on malondialdehyde (MDA) specific activity from the temporal lobe of rats with VPA-induced model of autism (both genders). The values are mean ± S.E.M. (n = 3 per group)



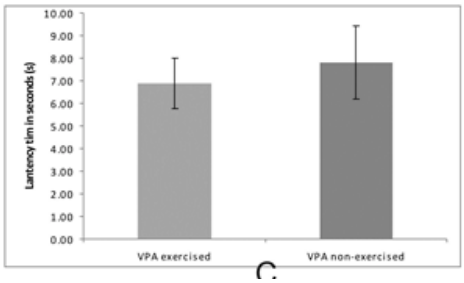
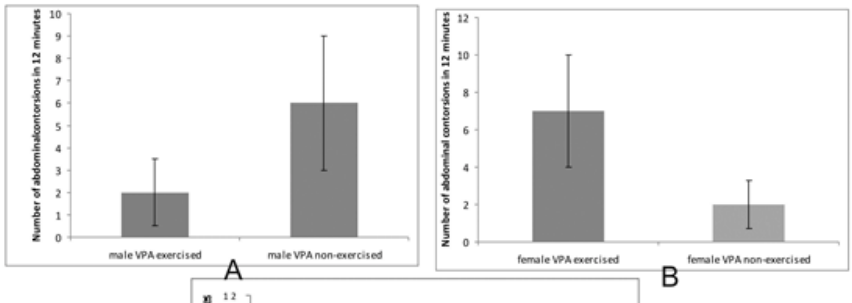


Fig. 4. The effects of exercising or non-exercising on latency time (as expressed in seconds) from the hot-plate task in rats with VPA-induced model of autism (both genders). The values are mean ± S.E.M. (n = 3 per group and n = 6 in the figure 5C – both genders combined).



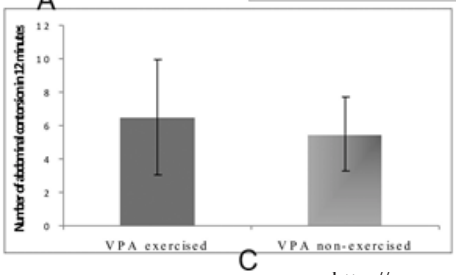


Fig. 5. The effects of exercising or non-exercising on number of abdominal contortions in 12 min from the intra abdominal Zymosan administration test in rats with VPA-induced model of autism (both genders). The values are mean ± S.E.M. (n = 3 per group and n = 6 in the figure 6C – both genders combined)

could not observe any difference regarding the SOD specific activity when comparing VPA exercised rats vs. non-exercised VPA group (fig. 1B).

Somehow similar results were also observed in the case of the second antioxidant enzyme GPX, with the difference that the significant differences between VPA exercised rats (a significant decrease) and the non-exercised VPA group, were seen in the female rats ($p = 0.04$) (fig. 3B), with no significant differences observed for the males groups of rats (fig. 2A).

In the same way, when it comes to the levels of MDA from the temporal lobe, we could observe that there was no significant modification in the concentration of this lipid peroxidation marker in both the male and female groups, when we compared VPA exercised rats vs. VPA-non exercised animals (fig. 3A and B).

Hot-plate test

Regarding the hot-plate latency time (as expressed in seconds) of male autistic animal model induced by administering valproic acid to the gestant female [11], the results showed a graphical tendency of decrease latency time in the exercised male autistic rats ($n = 3$), when compared to non-exercised male autistic rats ($n = 3$), which is however not statistically significant ($p = 0.601366$) (fig. 4A). The same situation repeats itself in the case of female autistic rat models, with a graphical (Figure 4B) decrease being registered among those with physical training ($n = 3$), as compared to those which did not follow a training phase ($n = 3$) in the latency time ($p = 0.13093$).

If the groups are analyzed by making no difference between genders, results indicate again to a non-significant difference between exercised ($n = 6$) and non-exercised animals ($n = 6$) in regard to the latency time ($p = 0.114548$) in the hot plate test (fig. 5C).

Zymosan administration test

As mentioned above, for recording the visceral pain we intraperitoneally administrated Zymosan and followed the writhing reactions for 12 min in both VPA rat exercised and VPA non-exercised rats, that resulted in a non-significant difference ($p = 0.89$) (fig. 5A). Also, even if we analyse the groups separated according to sex differences, no statistically significant values could be highlighted (fig. 5B and 5C). Therefore, according to these results no difference of perceiving visceral pain can be differenced between exercised and non-exercised rats with a VPA-induced model of autism.

We demonstrated here an increased oxidative stress status, as a result of the 6 days exercising on the treadmill, in a VPA rat induced model of autism, as demonstrated mainly by a significant decrease in the specific activity of SOD (for the exercised VPA female rats), as well as a significant decrease of GPX specific activity in the male VPA exercised rats, when compared to non-exercised VPA groups, as well as the fact that the aforementioned series of exercises did not resulted in any changes of the pain perception of this rat models of autism, as studied in 2 pain-related behavioural tasks (hot plate test for the supraspinal acute thermal pain and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain), independent to the gender of the rats with VPA model of autism.

Regarding the connections between autism and exercising, previous studies suggested a reduction in the repetitive pattern of certain behaviours, which are characterising this disorder [39]. Also, considering the sedentary preference of individuals suffering from autism, new methods have been developed for determining them to practice physical exercise in a convenient environment such as a virtual reality, as the one provided by astrojumper which brings promising effect not only in regard to physical training but also an improvement in the irregular movement patterns [40].

In fact, as our group previously reviewed [41] children with autism could also have pervasive gross motor deficits including decreased visual-motor affected coordination and postural impairments in static and dynamic balance [42, 43]. Even more, there is also a seriously high obesity which is present in patients with autism (30.4%, as compared with 23.6% in control children) [44].

In addition, there are also meta-analyses performed in this area of research, demonstrating quite clearly that cardiovascular exercise interventions led to a 37% improvement in the patho-physiology of autism [45].

Also, as mentioned above, exercises related to jogging, roller-skating, hydrotherapy and exergames (e.g. video games that are also a form of physical exercise-please see the above example related to astrojumper) are confirmed to decrease the frequency of stereotypical behaviours [46], aggressive or self-injurious behaviours or hyperactivity [47]. Other related examples are including swimming, jogging, cycling, weight training, walking and horseback riding [48].

In regards to the pain manifestations in the autistic pathology, as also mentioned before [49], recent studies pursuing the opioid hypothesis and designed to sustain the increased resistance to pain of individuals diagnosed with autism, demonstrated actually the opposite: that patients with autism are sensitive to pain [50]. In this way, the difference between subjects without the psychiatric issues and those suffering from autism are modified behavioural reactions and emotional responses that are difficult to be evaluated in a clinical examination. But there is proof that although there are not obvious physical reactions in children and adolescents with autism, manifested as increased levels of stress response and physiological outcomes to venipuncture (e.g. a medical procedure known for its painful response), proving that there is a need of evaluating pain differently in the case of these particular patients [50].

Also, although several studies indicated impairments in expressing painful experience through facial activity in individuals suffering from autism, along with impaired verbal capacities to express painful symptoms, there are studies that contradicts the incapacity of children with autism to facially encode the feeling of pain (as described by [51]).

Therefore, these contradictory results from the current literature are indicating the need of studying further the link between pain and autism.

As mentioned, in the present study we showed that 6 days exercising on the treadmill, in a VPA rat induced model of autism, did not resulted in any changes of the pain perception of this rat models of autism, as studied in 2 pain-related behavioural tasks (hot plate test for the supraspinal acute thermal pain and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain), independent to the gender of the rats with VPA model of autism.

In the present study we also showed some signs of increased oxidative stress status in the temporal lobes of these exercised VPA-autistic rats, as demonstrated especially through some specific decrease in the enzymatic activities of SOD and GPX.

Similar aspects regarding the possibility that some types of exercised could increase oxidative stress status were

previously described by our group in both rats (where 5 minutes of exercises on a treadmill, resulted in decreased SOD and increased levels of malondyaldehide) [52], as also in human volunteers, where we showed that untrained students receiving 40 min bout of bicycle exercising exhibited decreased GPX and increased MDA, deficits which however were prevented by the initial administration of vitamin C (12 h before) [53].

Of course, this could be also related to the fact that oxidative stress was previously described as an important factor in the very complex and multifactorial pathological picture of autism, as reviewed for example by [54] or the pro-oxidant effects of VPA administration .

As mentioned in the introduction section, we will also shortly describe here *the possible relevance of oxytocin in this context, as related to autism, exercising, oxidative stress and pain,* since our group also has undergo results in this exciting area of research.

In this way, it is known (with our group also demonstrating this in the past [55]) that oxytocin could exert some antioxidant effects in various regions and situations [56-60]. Still, there are authors also demonstrating some clear pro-oxidant effects for oxytocin administration [61].

Even more, the relation between exercises and oxytocin showed some controversial results as well, with classical studies for example in 1982 demonstrating that plasma oxytocin did not suffer any modifications as response to exercising (*running exercise until exhaustion*) [62] or a study in Hormones Research in 2001, clearly showing that oxytocin from the plasma does not suffer any modifications to the same progressive exercising until exhaustion in professional cyclists [63].

However, very recently this year in 2017 the research group of Broderick et al. [64] demonstrated that actually exercise training (e.g. 8 weeks) and caloric restriction could exert fundamental effects on the cardiac oxytocin natriuretic peptide system in a specific genetic mouse model of diabetes.

In addition, the group of Gutkowska et al. [65] previously showed that exercise (lasting again 8 weeks) training enhances cardiac oxytocin in ovariectomized rats.

In regards to the connections between oxytocin and pain, things are also very recent, as for example right now in 2017 there is an undergoing clinical trial describing the possible usage of intranasal oxytocin in the treatment of persistent pain [66], as based on some reports suggesting analgesic effects of oxytocin [67, 68].

In this way, considering all the aforementioned aspects, our research group is already working in a follow-up study regarding the administration of oxytocin (and especially the intranasal one) [69, 70] on the patho-physiological manifestations related to oxidative stress, pain and exercising from autism (in this VPA-induced animal model, but also in human patients, considering the experience we described above).

Conclusions

In the present paper we showed an increased oxidative stress status, as a result of the 6 days exercising on the treadmill, in a VPA rat induced model of autism, as demonstrated mainly by a significant decrease in the specific activity of SOD (for the exercised VPA female rats), as well as a significant decrease of GPX specific activity in the male VPA exercised rats, when compared to non-exercised VPA groups, as well as the fact that the aforementioned series of exercises did not resulted in any changes of the pain perception of this rat models of autism, as studied in 2 pain-related behavioural tasks (hot plate test for the supraspinal acute thermal pain and the intra abdominal Zymosan administration for eliciting a local inflammatory reaction following responses to inflammatory visceral pain), independent to the gender of the rats with VPA model of autism.

Acknowledgements: Iulia Antioch, Alin Ciobica, Dana Ababei, Radu Lefter and Cezar Honceriu were supported by a research grant PN II PN-II-RU-TE-2014-4-1886 called A complex study regarding the relevance of oxytocin administration in some animal models of neuropsychiatric disorders.

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Manuscript received: 14.02.2017