BIOLOGIJA. 2014. Vol. 60. No. 1. P. 23–31

Effects of N-stearoylethanolamine on anxiety-like behavioral reactions of rats after chronic alcoholization

Oleksandr V. Bondarenko[1]*,

N. M. Hula[2],

M. Yu. Makarchuk[1],

T. M. Horid'ko[2]

[1] Taras Shevchenko National University of Kyiv, Educational and Scientific Centre "Institute of Biology", 2, Academician Glushkov avenue, Kyiv 03022, Ukraine

[2] Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, 9, Leontovicha str., Kyiv, 01601, Ukraine

The effect of N-stearoylethanolamine (NSE) administration (isolated and combined with alcoholization) in the dose of 5 mg/kg on the behavior reactions of rats in the elevated plus maze was investigated. It was revealed that the isolated administration of NSE at the dose of 5 mg/kg rather reduced general locomotor activity of rats in elevated plus maze than increased their anxiety-like behavior in these conditions. It was shown that chronic 30-day alcoholization increased anxiety-like behavior of rats in elevated plus maze but introduction of NSE on the background of alcohol eliminated changes of locomotor and emotional behavior of animals after alcoholization. The presence of significant changes in locomotor and emotional behavior of rats in elevated plus maze after administration of NSE at the dose of 5 mg/kg indicated a redundancy of such dose of the substance and therefore research of the influence on the behavior of smaller doses of NSE is very relevant.

Key words: N-stearoylethanolamine, alcohol, elevated plus maze, anxiety, behavior

INTRODUCTION

There is a large and growing body of clinical and preclinical evidences suggesting an important, albeit complex, relationship between stress, anxiety and alcohol use disorders (Piazza and Le Moal, 1998; Kushner et al., 2000; Roberts et al., 2000; Weiss et al., 2001). It was found that chronic consumption of ethanol can increase the level of endogenous cannabinoid receptor ligands in neuroblastoma cells (Basavarajappa et al., 1997; Hungund et al., 2002) and cause an increase of level of endocannabinoids in the brain (Basavarajappa, 1999). Endocannabinoid system of the brain is one of the neurochemical systems, which is directly involved in physiological

* Corresponding author. E-mail: bondarenko_oleksandr@ukr.net

recovery after any stress effects. There are two types of cannabinoid receptors, CB1 and CB2 (Takayuki and Keizo, 2002). To date, there are two endogenous ligands of CB1 receptors, namely anandamide and 2-arachydonylglycerol (2-AG), which mimic the pharmacological action of Δ^9-tetrahydrocannabinol, the active compound of marijuana and other synthetic cannabinoids (Devane et al., 1992; Mechoulam and Fride, 1995; Hungund et al., 2002).

Considerable evidence suggests that cannabinoids are anxiolytics and modulate the behavioural and physiological response to stressful events (Viveros et al., 2007; Hill et al., 2010). In the searching of new molecular and neuronal targets it is important to create original anxiolytic drugs for the treatment of anxiety disorders (Millan, 2003). However, it was found that the administration of low doses of CB1 receptor agonists manifested mainly anxiolytic effect, while higher doses had mainly anxiogenic effect (Viveros et al., 2005). Although considerable evidence suggests that activation of CB1 receptors can induce learning and memory impairments (Sullivan, 2000; Robinson et al., 2003; O'Shea et al., 2004; Varvel et al., 2005), it is important to find substances with potential cannabimimetic properties that would not cause side effects. Moreover, according to the ever-increasing volume of alcohol consumption in the society and property of ethanol to disrupt the natural balance between endogenous cannabinoids (Basavarajappa and Hungund, 1999; Basavarajappa, 2000), it is reasonable to estimate the physiological properties of cannabinoids in conditions of chronic alcoholization.

Research of endocannabinoid system of the brain showed that along with the most studied anandamide there is synthesized a number of other N-acylethanolamines in the brain which functional properties are not fully elucidated (Gula et al., 2004). One of these compounds is N-stearoylethanolamine (NSE), the content of which in the brain of mice and humans is about 11–14% of total endocannabinoid whereas the content of anandamide does not exceed 7–8% (Maccarrone et al., 2002). This substance shows some cannabimimetic properties but it is not set to be bound with cannabinoid receptors. However, the introduction of this compound can cause a variety of physiological effects. In particular, it is shown that NSE possesses antioxodative, membranoprotective and adaptive properties (Goridko et al., 2007; Artamonov et al., 2005), has anti-inflammatory effect on experimental burn wound in rats (Gula et al., 2009) and inhibits the growth and metastasis of Lewis carcinoma (Gula et al., 2006). In our previous study we showed that administration of NSE in the dose of 5 mg/kg during 7 days led to reduction of vertical and locomotor activity in open field and had no significant effect on the parameters of the emotional activity (duration of grooming) after retesting (Bondarenko et al., 2013).

The objective of this study was to compare the anxiety-like behavior in elevated plus maze in rats in the case of chronic alcoholism and NSE administration.

MATERIALS AND METHODS

Subjects

In the investigation, 85 white male rats (weight: 150–200 g at the beginning of the experiment) were tested. All manipulations were carried out according to bioethics rules.

Separation on the groups

The rat behavior was evaluated using elevated plus maze, the light / dark box or the open-field tests (Belzung and Griebel, 2001; Bogdanov et al., 2013). The rat behavioral reactions in the open-field test and light / dark box were used to equable groups' formation. After testing rats were divided into 5 equable groups according to the recorded parameters:

I group – **"Control"** (n = 16) – intact animals;

II group – **"Control tube"** (n = 16) – animals which receive water through the tube during 7 days;

III group – **"NSE"** (n = 17) – animals which receive NSE (5 mg/kg) through the tube during 7 days;

IV group – **"Alcohol"** (n = 18) – animals with the model of 30-day chronic alcohol intoxication;

V group – **"Alc+NSE"** (n = 18) – animals which during the last 7 days of forced alcoholization were receiving NSE (5 mg/kg) water solution.

Animals from I, II and III groups were maintained on a standard vivarium diet and free access to food and water. Animals from IV and V groups were limited in water access through the period of introduction of alcohol but without food deprivation during the whole experiment. After dividing into groups chronic alcoholization and administration of substance were carried out.

Alcoholization

The chronic alcoholic intoxication was made in 2 steps: at I stage we determined the predisposition of rats to ethanol by "two bottles" method. Animals that had not had this contact with ethanol before were placed into individual cages with two drinkers, one with water, the other with 15% solution of ethanol. During this stage during 14 days animals had free choice between ethanol solution and water. The value of individual volume of consumed alcohol per unit time (g/kg/day) and the percentage ratio of consumed alcohol and volume of whole fluid were calculated once a day. It was described (Kovalenko et al., 2010) that innate tendency to consume alcohol was linked to the level of behavioral responses, that is why this step allowed to balance rats in IV, V and VI groups based on behavioral characteristics and also by the level of spontaneous alcohol consumption before beginning of the 30-day forced alcoholization. At II stage forced alcoholization was performed by introducing ethanol as the sole source of fluid for 30 days. After that to assess the individual level of alcohol consumption during 4 days cages were equipped with two drinkers again (one with water, the other one with 15% solution of ethanol) (Parkhomenko et al., 2007).

Substance

NSE water solution was being entered per os using plastic tube during 7 days in the dose of 5 mg/kg. The substance was synthesized in the Department of Biochemistry of Lipids of Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Introduction of substance using thin and flexible plastic probe is not traumatic for rats and allows without extra tools for fixing in a stationary position of the animal quickly introduce exogenous substance directly into the stomach. After quick adaptation to such manipulation animals practically are not stressed which is important for the study of behavioral responses. One of the rat group received water with the help of plastic tube to check the influence of the procedure on the rat activity.

Behavioral studies

After termination of alcoholization and administration of NSE, elevated plus maze was conducted. Elevated plus maze was the main method for anxiety level evaluation in rats from different groups in the current research. In this setup the following measures were recorded during 5 minutes: spending time in closed and opened arms, spending time in the centre, the number of entries made into either the open or the closed arms, number of crossings of the central platform, number of looks down, the number of looks from the centre, quantity and duration of grooming. The experiment was recorded using a video camera; all required parameters were measured during records examination.

Data analysis

Data were analyzed by Statistica for Windows 7.0 (StatSoft). Since the distributions of the most parameters obtained in these studies

were different from normal (Shapiro-Wilk test) for comparing independent groups non-parametrical Mann-Whitney U-test was used. Differences were considered significant at $p \leq 0.05$. Median and inter quartile range used to describe the sampling distribution (Me [25%, 75%]).

RESULTS

After termination of alcoholization and administration of NSE in the elevated plus maze test it was shown that open arm time in rats of IV group was significantly lower compared with animals from I ($p \leq 0.01$) and II ($p \leq 0.05$) groups. In animals from III group this parameter was significantly lower compared with rats from I group ($p \leq 0.05$) and tended to decrease compared with animals from II group ($p = 0.08$). In animals from V group this parameter was significantly lower compared with rats from I group ($p \leq 0.05$) and tended to decrease compared with animals from II group ($p = 0.09$). There were no differences between the performance of animals between animals from I and II groups. In rats from V group the closed arm time was significantly higher compared with rats from I group ($p \leq 0.01$) and tended to increase compared to the animals from II group (Fig. 1). This result may indicate some increased anxiety-like reactions in rats from III, IV and V groups. There were no significant distinctions of spending time in the centre of the maze between animals of different groups, however, in rats from IV group this value was slightly higher compared with animals from I group.

The ratio between open arm time and closed arm time is an important indicator. This indicator rises with the growth of neophilia (a tendency to like anything new) of animals. In animals from IV ($p \leq 0.01$) and V ($p \leq 0.05$) groups this factor is significantly lower compared with animals from I group. In rats from III group it tended to decrease compared to the animals from I group ($p = 0.06$) (Fig. 2).

Rats from IV group had statistically lower number of open arm entries compared with animals from I group ($p \leq 0.05$) and tended to decrease compared with II group of rats ($p = 0.09$) that may indicate the strengthening of anxious behavior in these animals (rats who consumed only NSE). In addition, rats from III group had a reduction of number

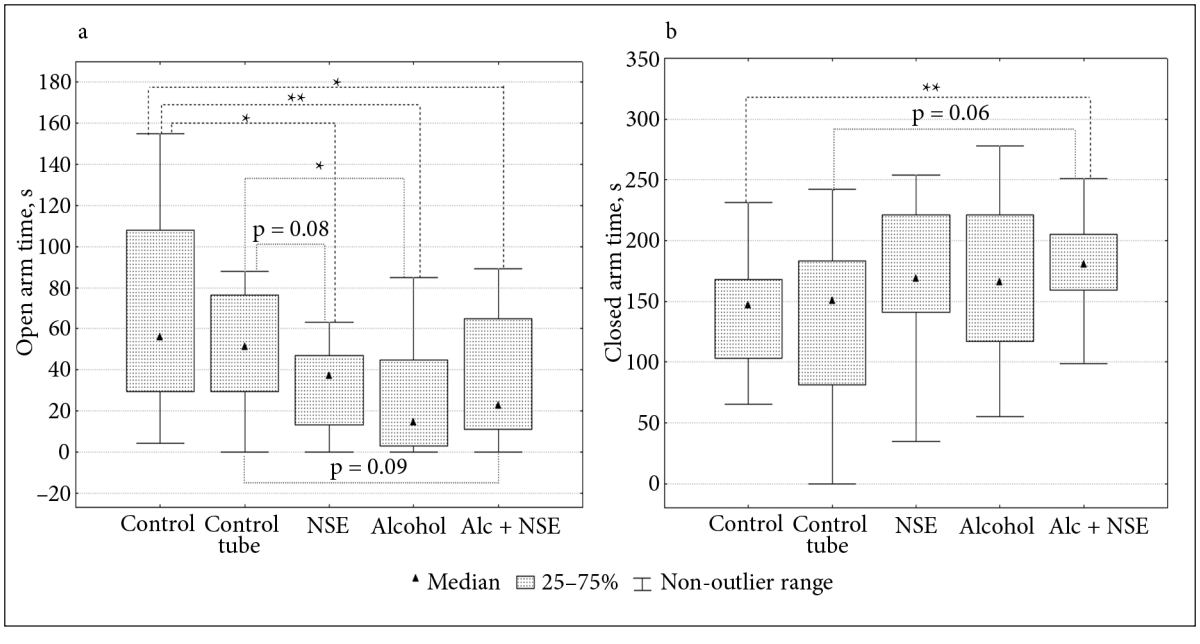

Fig. 1. Open arm (a) and closed arm (b) time in the elevated plus maze in rats from I–V groups. * $p \leq 0.05$; ** $p \leq 0.01$

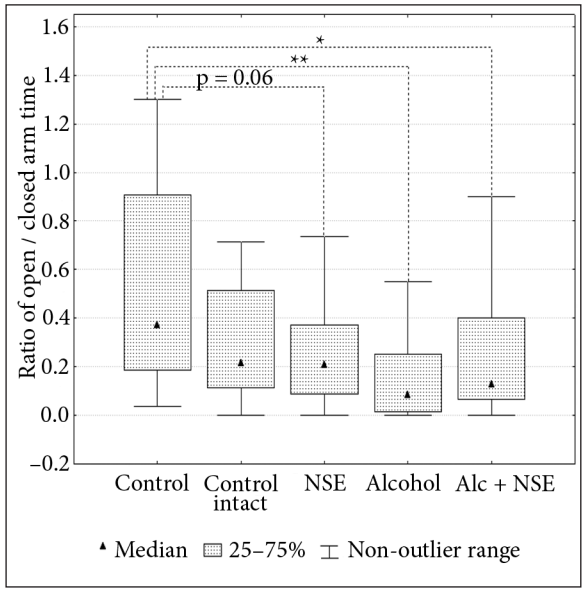

Fig. 2. The ratio factor of open arm time for closed arm time in elevated plus maze in rats from I–V groups. * $p \leq 0.05$; ** $p \leq 0.01$

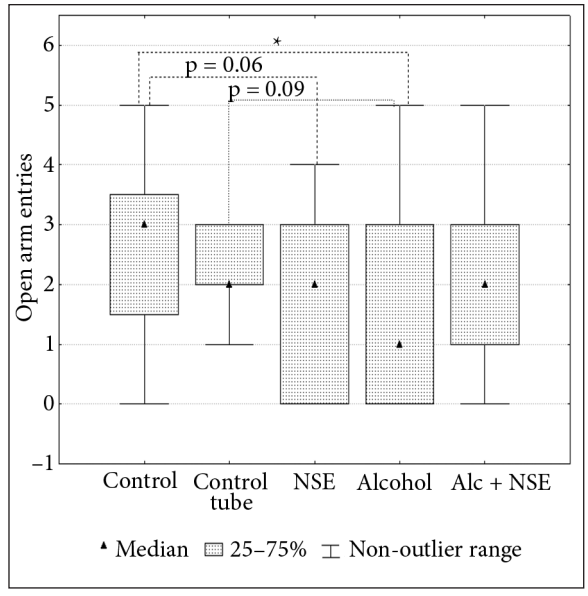

Fig. 3. Open arm entries in the elevated plus maze in rats from I–V groups. * $p \leq 0.05$

of open arm entries compared with animals from I group, but this decrease is only a trend ($p = 0.06$) (Fig. 3).

The analysis of grooming activity of animals from different groups showed that the number of grooming (Fig. 4a) in rats from V group was significantly lower ($p \leq 0.05$), and in rats from IV group tended to decrease ($p = 0.08$) compared with II group. Moreover, in rats from V group it tended to decrease compared to the animals from III group ($p = 0.07$) which may indicate some reduction of emotional activity in both groups of rats that were exposed to alcohol, although compared with the animals from I group significant difference was not observed. In rats from III group were no

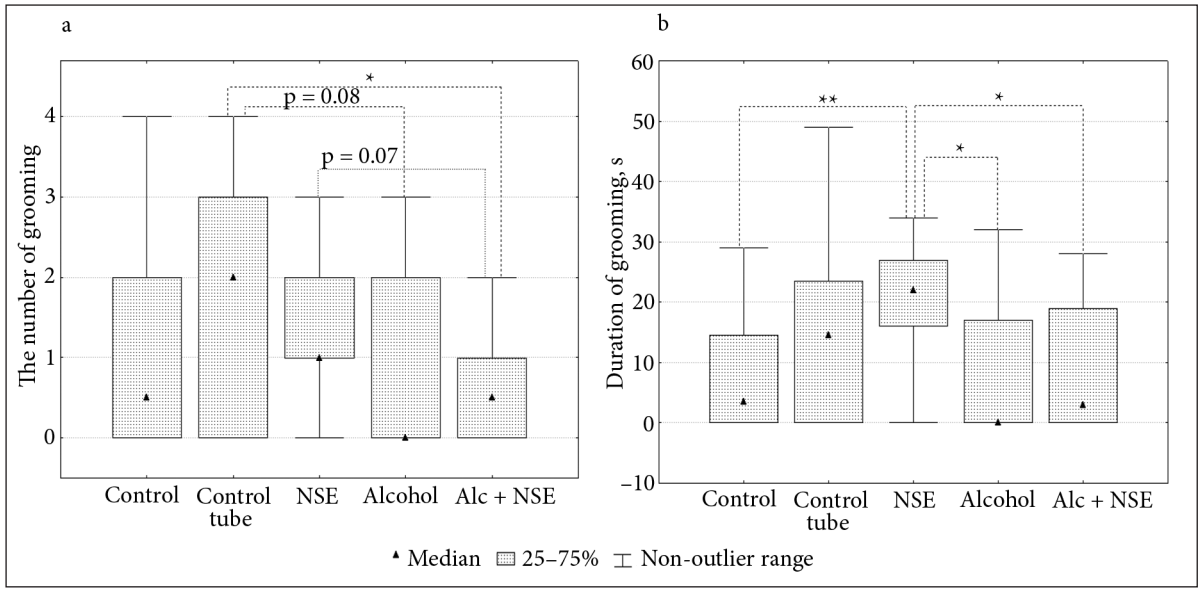

Fig. 4. The number (a) and duration (b) of grooming in the elevated plus maze in rats of I–V groups. * $p \leq 0.05$; ** $p \leq 0.01$

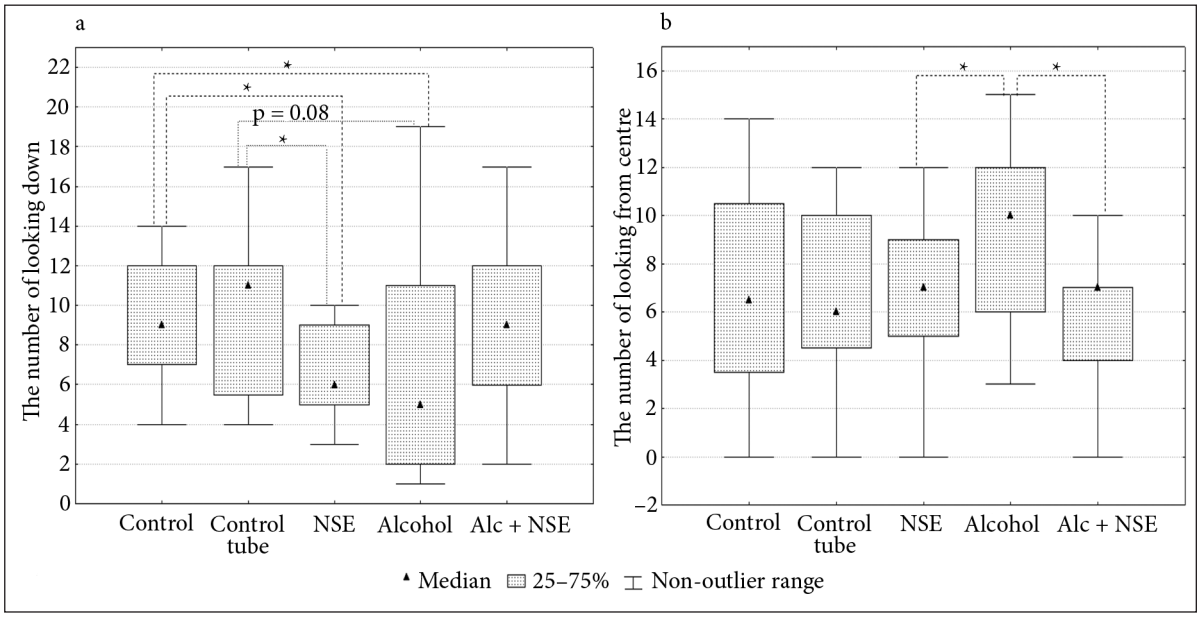

Fig. 5. The number of looking down (a) and looking from centre (b) in the elevated plus maze in the rats from I–V groups. * $p \leq 0.05$

significant differences compared with animals from I and II groups, but rats in this group had a slightly higher number of grooming compared with rats from I and IV group. The duration of grooming (Fig. 4b) was significantly higher in rats from III group compared with animals from I ($p \leq 0.01$), IV ($p \leq 0.05$) and V ($p \leq 0.05$) groups that may indicate some increase of emotional activity in the elevated plus maze in the animals from III group (in groups receiving only NSE).

The number of looking down in elevated plus maze is an important indicator of reduction of emotional stress in rats (Fig. 5a). In our studies we found a significant decrease of this parameter in rats from III group ($p \leq 0.05$) compared with the animals from I and II groups. In animals from IV group this parameter was significantly lower compared with rats from I group ($p \leq 0.05$) and tended to decrease compared with animals from II group ($p = 0.08$) that may indicate to an increase of anxiety in rats from III and IV groups. With regard to animals of V group (rats which received NSE on the background of alcohol) there is no difference against control. Opposite in meaning indicator is the number of rats looking from centre (Fig. 5b). Analyzing this factor we observed a significant increase of it in animals from IV group ($p \leq 0.05$) compared with those from II and V groups that may indicate an increase of emotional stress in animals from IV group, and its slight decrease in the rats from V group.

DISCUSSION

The elevated plus maze has been used extensively in rats and mice as a preclinical screen to predict therapeutic efficacy of putative anxiolytics (Carobrez and Bertoglio, 2005; Hogg, 1996; Jones et al., 2002; Pellow et al. 1985). This method allows determining the rate of expression of the emotional reactions of fear and anxiety, locomotor activity, the rate of orienting responses in animals that find themselves in a new situation. A drug induced increase in the proportion of time spent in the open arms is suggestive of an anxiolytic effect, whereas an increase in time spent in closed arms is suggestive of an anxiogenic effect.

In general it was found that the administration of water with the help of plastic tube did not alter any parameter of behavior reactions in elevated plus maze compared with control condition. After isolated administration of NSE at the dose of 5 mg/kg we did

not receive any data that could clearly indicate the anxious behavior in animals compared to the intact animals. On the one hand, there is a significant decrease of open arm time and number of looks down; on the other hand, we observed a significant increase of the duration of grooming in these rats, which is a measure of comfort behavior. The obtained results rather indicate to the more general suppression of locomotor activity than increased anxiety-like behavior as described that NSE has the same effects as anandamide on catalepsy and motility of mice (Maccarrone et al., 2002).

Instead the application of this model of chronic alcoholization increased anxious behavior in rats in the elevated plus maze that was reflected in significant decrease of the number of open arm entries and open arm time, reduction of the ratio factor of open / closed arm time, as well as reduction of the number of looks down compared to intact animals.

Analyzing the behavior of animals that received NSE at the dose of 5 mg/kg combined with alcohol, we also saw significant decrease of the open arm time and reduction of the ratio factor of opened / closed arm time. But, in contrast to animals that received only ethanol, there was no difference in the number of opened arm entries and the number of looks down compared with intact animals. In rats which received NSE at the dose of 5 mg/kg co-administrated with alcohol we observed a significant reduction of the number of looking from centre, compared with animal which received only NSE that in general may indicate the presence of a certain modulating influence of NSE on the behavior of animals after alcoholization. However, revealed significant increase in opened arm time compared to control animals could theoretically indicate that NSE at the dose of 5 mg/kg with high confidence can be threshold limit of the substance, and the continued usage of large doses may have a more pronounced anxiogenic effect on behavior of rats after alcoholization and control rats which is important for the future studies. Usage of smaller doses of NSE is relevant because in the conditions of chronic alcoholization alcohol-induced increase in brain levels of natural endocannabinoids (anandamide and 2-AG) in combination with NSE possibly undermine its anxiolytic effect.

CONCLUSIONS

It was revealed that NSE at the dose of 5 mg/kg did not increase anxiety-like behavior in the EPM. The administration of water with the help of plastic tube did not alter any parameter of behavior reactions in EPM compared with control condition. The chronic 30-day alcoholization increased anxious behavior of rats in the elevated plus maze. Introduction of NSE at the dose of 5 mg/kg changed locomotor and emotional behavior of animals after alcoholization but further usage of lower doses of the substance is promising. The data obtained are important for further evaluation of efficacy of NSE effect in terms of chronic alcohol intoxication on different types of learning and memory.

Received 21 December 2013
Accepted 05 January 2014

References

1. Artamonov M, Zhukov O, Shuba I, Storozhuk L, Khmel T, Klimashevsky V, Mikosha A, Gula N. Incorporation of labelled N-acylethanolamine (NAE) into rat brain regions *in vivo* and adaptive properties of saturated NAE under X-ray irradiation. Ukr Biokhim Zh 2005; 77(6): 51–62.
2. Basavarajappa BS, Hungund BL. Chronic ethanol increases the cannabinoid receptor agonist anandamide and its N-arachidonoylp hosphatidylethanolamine in SK-N-SH cells. J Neurochem 1999; 72(2): 522–528.
3. Basavarajappa BS, Saito M, Cooper TB, Hungund BL. Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. Biochim Biophysic Acta 2000; 1535: 78–86.

4. Basavarajappa BS, Saito M, Cooper TB, Hungund BL. Activation of arachidonic acid-specific phospholipase A2 in human neuroblastoma cells after chronic alcohol exposure: prevention by GM1 ganglioside. Alc Clin Exp Res 1997; 21: 1199–1203.

5. Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 2001; 125(1–2): 141–149.

6. Bogdanov VB, Bogdanova OV, Koulchitsky SV et al. Behavior in the open field predicts the number of KCl-induced cortical spreading depressions in rats. Behav Brain Res 2013; 236(1): 90–93.

7. Bondarenko O, Gulaya N, Makarchuk M, Goridko T, Baban V, Kovalenko O. Effects of n-stearoylethanolamine and chronic alcoholization on behavioral reactions of rats in "open-field". Visnyk of the Lviv University. Series Biology 2013; 62: 285–293.

8. Carobrez AP, Bertoglio LJ. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci Biobehav Rev 2005; 29: 1193–1205.

9. Devane WA, Hanus L, Breuer A et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992; 258: 1946–1949.

10. Goridko TM, Gula NM, Stogniy NA, Meged OF, Klimashevsky VM, Shovkun SA, Kindruk NL, Berdyshev AG. Influence of n-stearoylethanolamine on the lipid peroxidation process and lipid composition of the rat liver under acute morphine intoxication. Ukr Biokhim Zh 2007; 79(5): 175–185.

11. Gula NM, Khmel TO, Klimashevsky VM. The influence of n-stearoylethanolamine on lipid composition of the metastases and conditionally normal lung tissue in mice with Lewis carcinoma. Ukr Biokhim Zh 2006; 78(2): 97–105.

12. Gula NM, Chumak AA, Berdyshev AG, Megel EF, Goridko TM, Kindruk NL, Kosykova GV, Zhukov OD. Anti-inflammatory effect of N-stearoylethanolamine on experimental burn wound in rats. Ukr Biokhim Zh 2009; 81(2): 107–116.

13. Gula NM, Margitich VM, Artamonov MV, Zhukov OD, Goridko TM, Klimashevsky VM. Neuroprotective effect of n-acylethanolamines under chronic morphine dependence. i. rat brain phospholipids as a rational target of their action. Ukr Biokhim Zh 2004; 76(5): 123–131.

14. Hill MN, Patel S, Campolongo P, Tasker JG, Wotjak CT, Bains JS. Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. J Neurosci 2010; 10: 14980–14986.

15. Hogg S. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 1996; 54: 21–30.

16. Hungund BL, Basavarajappa BS, Vadasz C et al. Ethanol, endocannabinoids and cannabinoidergic signaling system. Alcohol Clin Exp Res 2002; 26: 565–574.

17. Jones N, King SM, Duxon MS. Further evidence for the predictive validity of the unstable elevated exposed plus-maze, a behavioural model of extreme anxiety in rats: differential effects of fluoxetine and chlordiazepoxide. Behav Pharmacol 2002; 13: 525–535.

18. Kovalenko OA, Ovcharyk EM, Bondarenko OV, Makarchuk MYu. Influence of behavioral reactions on ability to training of rats with different degree of alcoholic motivation. Visnyk of Luhansk National Taras Shevchenko University: Medical sciences 2010; 21(208): 54–59.

19. Kushner MG, Abrams K, Borchardt C. The relationship between anxiety disorders and alcohol use disorders: a review of major perspectives and findings. Clin Psychol Rev 2000; 20(2): 149–171.

20. Maccarrone M, Cartoni A, Parolaro D, Margonelli A, Massi P, Bari M, Battista N, Finazzi-Agrò. Cannabimimetic activity, binding, and degradation of stearoylethanolamide within the mouse central nervous system. Mol Cell Neurosci 2002; 21(1): 126–140.

21. Mechoulam R, Fride E. The unpaved road to the endogenous brain cannabinoid ligands, the anandamides. In: Pertwee RG (ed.). Cannabinoid Receptors. London: Academic Press. 2005: 233–258.

22. Millan MJ. The neurobiology and control of anxious states. Progr Neurobiol 2003; 70(2): 83–244.

23. O'Shea M, Singh ME, McGregor IS, Mallet PE. Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 2004; 18(4): 502–508.

24. Parkhomenko IuM, Donchenko GV, Pilipchuk SIu, Stepanenko SP, Chekhovskaia LI, Klimenko EP. Characteristic metabolic disturbances in the rat tissues caused by long-term use of alcohol. Ukr Biokhim Zh 2007; 79(3): 61–69.

25. Pellow S, Chopin P, File SE, Briley M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Meth 1985; 14: 149–167.

26. Piazza PV, Le Moal M. The role of stress in drug self-administration. Trends Pharmacol Sci 1998; 19: 67–74.

27. Roberts AJ, Heyser CJ, Cole M et al. Excessive ethanol drinking following a history of dependence: animal model of allostasis. Neuropsychopharmacol 2000; 22(6): 581–594.

28. Robinson L, Hinder L, Pertwee RG, Riedel G. Effects of delta-9-THC and WIN 55,212-2 on place preference in the water maze in rats. Psychopharmacol (Berl) 2003; 166: 40–50.

29. Sullivan JM. Cellular and molecular mechanisms underlying learning and memory impairments produced by cannabinoids. Learn Mem 2000; 7: 132–139.

30. Takayuki S, Keizo W. Cannabinoid receptors and their endogenous ligands. J Biochem 2002; 132(1): 7–12.

31. Varvel SA, Anum EA, Lichtman AH. Disruption of CB1 receptor signaling impairs extinction of spatial memory in mice. Psychopharmacol (Berl) 2005; 179: 863–872.

32. Viveros M, Marco E, Llorente R, Lopez-Gallardo M. Endocannabinoid system and synaptic plasticity: implications for emotional responses. Neural Plast 2007.

33. Viveros M, Marco EM, File SE. Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 2005; 81(2): 331–342.

34. Weiss F, Ciccocioppo R, Parsons LH et al. Compulsive drug-seeking behaviour and relapse. Neuroadaptation, stress, and conditioning factors. Ann NY Acad Sci 2001; 937: 1–26.

N-STEAROYLETHANOLAMINO POVEIKIS SU NERIMU SIEJAMOMS ŽIURKIŲ ELGSENOS REAKCIJOMS PO CHRONINĖS ALKOHOLIZACIJOS

Oleksandr V. Bondarenko, N. M. Hula, M. Yu. Makarchuk, T. M. Horid'ko

Santrauka

Tyrime nagrinėtas 5 mg/kg N-stearoylethanolamino (NSE) poveikis žiurkių elgsenos reakcijoms kontrolinėmis sąlygomis ir po 30 dienų alkoholizacijos. Tyrime naudotas elgsenos tyrimo labirintas (*Elevated plus maze*). Kontrolinėje grupėje NSE sumažino bendrą žiurkių lokomotorinį aktyvumą, bet nepadidino su nerimu siejamo aktyvumo. Lėtinė 30 dienų alkoholizacija padidino su nerimu siejamą žiurkių aktyvumą. Taikant alkoholizaciją kartu su NSE, žiurkių lokomotorinio bei su nerimu siejamo aktyvumo pokyčių nepastebėta. Naudota NSE dozė sumažino kontrolinės grupės lokomotorinį aktyvumą, todėl tikslinga atlikti tyrimus naudojant mažesnes NSE dozes.

Raktažodžiai: N-stearoylethanolaminas, elgsenos tyrimo labirintas, nerimas, elgsena