ELSEVIER

Pathophysiology 12 (2005) 103–107

PATHOPHYSIOLOGY

www.elsevier.com/locate/pathophys

Ascorbic acid decreases morphine self-administration and withdrawal symptoms in rats

H. Alaei *, M. Esmaeili, A. Nasimi, A. Pourshanazari

National Research Center of Medical Sciences, Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Received 29 December 2004; received in revised form 1 March 2005; accepted 3 March 2005

Abstract

Recent studies have indicated that the glutamatergic system is involved in the motivational aspects during the initiation of drug self-administration. Ascorbic acid (AA), an antioxidant vitamin, is released from glutamatergic neurons, and it modulates the synaptic action of dopamine and glutamate. In this study the AA effects on the self-administration of morphine and on the morphine withdrawal syndrome have been investigated.

Wistar rats were allowed to self-administer morphine (1 mg/infusion) during 10 consecutive days for 2 h/session. The number of lever pressings was recorded. An intrapritoneal AA injection (500 mg/kg, i.p.), 30 min before morphine self-administration produced a significant decrease in the initiation of morphine self administration during all sessions. After the last test session morphine withdrawal symptom signs (MWS) were recorded after naloxone precipitation. Most of MWS (but not all) were decreased by AA application. In conclusion, AA may change the motivational processes underlying the morphine self-administration.

Keywords: Glutamatergic system; Ascorbic acid; Wistar rats

1. Introduction

Both acute and chronic administration of morphine affects the turnover of the classical brain monoamine neurotransmitters dopamine (DA) noradernaline and 5-hydroxytryptamine [1,2]. Recent studies have indicated that the glutamatergic system is also involved in the morphine tolerance and abstinence [3–6]. Administration of glutaminergic antagonist such as MK-801 and APV modulate the reinforcing effects of drug abuse in animals. Several studies have documented that MK-801 inhibits the cocaine self-administration [7] and reverses its facilitation after chronic exposure to amphetamine [8]. APV increases the lever pressings for cocaine when administered into the nucleus accumbens (NAC) in rats [9]. There is, however, little information about the effects of glutamate receptor antagonists on morphine self-administration.

Ascorbic acid (AA) is an antioxidant vitamin that is released from glutamatergic neurons as a part of the glutamate reuptake process, in which the high-affinity glutamate transporter exchanges AA for glutamate [10]. Although neurons are known to use this vitamin in many different chemical and enzymatic reactions, only recently some evidence has suggested a role for ascorbate in interneuronal communication. Experimental evidence suggests that AA plays a key role in modulating central glutamatergic [11] and dopaminergic [12] systems as well as behavior [13].

The perfusion of cerebral cortical neurons with high doses of AA (1–3 mM) rapidly and reversibly attenuates NMDA antagonist-induced inward currents [14]. Other experiments have shown that low doses of AA could increase the neuronal activity and induce widespread neuronal oxidative stress on the primary cortical neurons [15]. One possible explanation of the divergent effects of increasing AA concentration on neuronal activity is that AA may shift from a presynaptic facilitator of glutamate transmission at relatively low extracellular concentration to a blocker of postsynaptic glutamate

* Corresponding author. Tel.: +98 311 792 2433; fax: +98 311 668 7898.
E-mail address: alaei@med.mui.ac.ir (H. Alaei).
URL: http://www.mui.ac.ir.

0928-4680/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.pathophys.2005.03.004

receptors [16]. It has been reported that high doses of AA suppress the withdrawal symptoms in the opiate addicts [17] and prevent the development of tolerance and physical dependence on morphine in the mice [18,19]. Recently, it has been reported that exogenous AA markedly reduces the locomotor activity and the behavioral withdrawal responses in morphine-treated guinea pigs [20]. Since both glutamatergic and dopaminergic systems are involved in morphine tolerance and morphine withdrawal symptoms [21] and these behaviors are greatly affected by AA [16] it is interesting to evaluate the effects of AA on i.v. self-administration of morphine as well as on morphine withdrawal signs in rats.

2. Material and methods

2.1. Animals and housing conditions

Male wistar rats (250–300 g) were used. Before surgery for the infusion system on the basis of ethic committee permission of Isfahan University, the animals were group-housed and they received food and water ad libitum. They were maintained under a day–night cycle with lights on between 07:00 and 19:00 h. After surgery the animals were placed in individual home cages, and they were allowed to recover from the operation for 5 days before study itself was started. During the tests food and water were available ad libitum in the home cages. The day–night cycle was reversed for 3 days before tests, and the animals were recorded during the dark phase of the cycle.

2.2. Experimental design

(A) Male rats ($n = 30$) were divided into three groups:
   (1) Control group, which received saline in the self-administration sessions.
   (2) Morphine group, which received morphine in saline solution (concentration 5 mg/ml) during the self-administration sessions.
   (3) Ascorbic acid–morphine group (AA + morphine), which received both AA (500 mg/kg, i.p.) 30 min before each session and morphine in the self-administration sessions.

(B) To determine the effects of the acute administration of AA on morphine withdrawal symptom signs 40 morphine treated male rats (250–300 g) were used. They were divided into four groups:
   (1) Control group, which had access to tap water for drinking during 21 days.
   (2) Morphine group, which received morphine (dissolved in water) for 21 days.
   (3) Ascorbic acid group, which received morphine for 21 days, and then divided to three sub groups which received AA with three doses (100, 500 or 1000 mg/kg in saline i.p.) at the end of experiment (21st day) 30 min before the naloxone injection was given to precipitate withdrawal symptoms.
   (4) Saline (vehicle) group which received only tap water for 21 days, at the end of experiment (21st day) 30 min before naloxone injection they received saline (0.5 ml, i.p.) as vehicle of AA.

(C) All of the animals at the end of experiment (21st day) received naloxone (2 mg/kg, s.c.) and withdrawal signs were recorded for 30 min.

2.3. Drugs

Morphine (HCl) was obtained from TEMAD Ltd., Teheran, Iran. Naloxone hydrochloride was purchased from Sigma Co., St Louis, USA. Ascorbic acid was purchased from Merck AG, Darmstadt, Germany. Morphine and AA were dissolved in saline.

2.4. Surgery

Animals were anaesthetized with ketamine (150 mg/kg) and rampon (0.1 mg/kg) and a cannula was inserted into the jugular vein. The cannula was guided subcutaneously up to the skull where it was fixed to acurred metal tube, which was secured on to the skull with small screws and fixed with dental acrylic cement.

2.5. Procedure

Details of the procedure have been reported previously [22]. Briefly, testing was done in standard conditioning cage (21 cm × 21 cm × 28 cm) placed in a sound attenuated room. The test cage was equipped with active and passive levers, 2 cm above the floor, and a red light located 4 cm above the active lever. The i.v. cannula of the animals tested was connected to the infusion pump. The pressing of the active lever, marked by the red light resulted in an i.v. infusion of 0.2 ml fluid (5 mg/ml morphine in saline solution or saline only) during 10 s. A depression of the active lever during this time (10 s) did not affect on the infusion of drug. The pressing of the passive lever had no programmed consequences.

The drug naïve animals were placed in the test cage and were allowed to i.v. self-administer a drug solution for 2 h a day (for 10 days). The number of the active and passive lever pressings is recorded by a oscillograph (Harvard).

2.6. Withdrawal syndrome signs

Withdrawal syndrome signs were precipitated with a s.c. naloxone injection (2 mg/kg). Withdrawal signs (body shakes, jumping, climbing, writhing, teeth chattering, irritability, ejaculation, ptosis and diarrhea) within 30 min after the injection of naloxone on the last day of experiment were recorded.

2.7. Statistical analysis

The data have been presented as means ± S.E.M. The data obtained during self-administration sessions were analyzed by using analysis of variance (ANOVA) with repeated measures (MANOVA). The number of active and passive lever pressings during every session were calculated. The differences in the total number of active and passive lever pressings (summed over ten sessions) between AA and morphine groups were analyzed by using Student's *t*-test. This method of analysis also was used in the analysis of withdrawal signs.

3. Results

3.1. Initiation of drug self administration

The self-infusion (SI) data during the initiation of morphine self-administration are presented in Fig. 1. Maximum values were obtained in a week. There was significant difference (ANOVA, $P < 0.05$) between numbers of self-infusions in three groups. Self-infusion of the morphine alone resulted in an addiction of the rats as shown by the several fold higher pressing number of the active lever providing the morphine solution. There was no significant difference between control (saline) and morphine + AA groups, indicating that AA (500 mg/kg, i.p.) resulted in a significant decrease in the number of self-infusions (Figs. 1 and 2). The total number of active

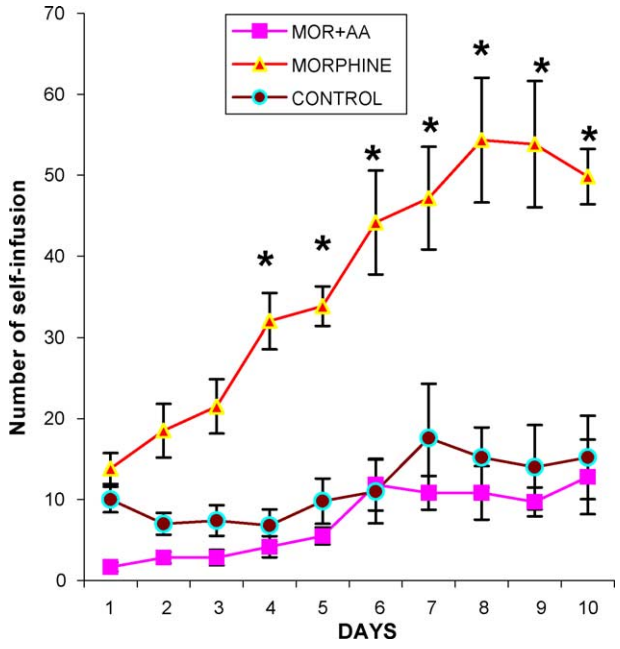

Fig. 1. The increase of self-infusions of morphine during the experiment. Please note that in the beginning morphine + ascorbic acid infusions were less than morphine only and that the increase was seen only after a few days, but it remained due to ascorbic acid at much lower level than morphine only (see also Fig. 2).

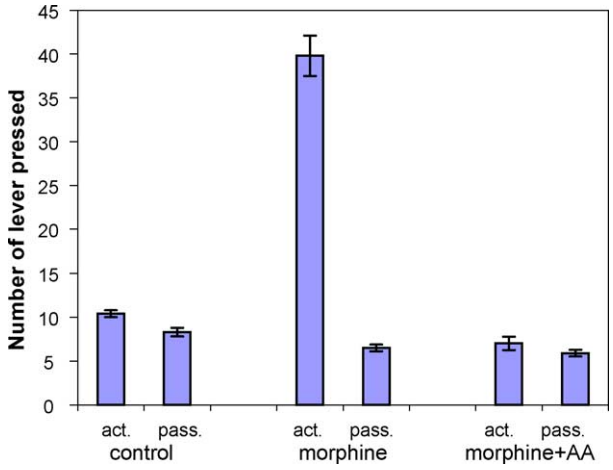

Fig. 2. The mean number of self-infusions indicated by the pressings of active and passive levers in the control, morphine and morphine + ascorbic acid rat groups during 10 days of self-administration. The passive lever provided saline as also the active lever (marked by a red lamp) in the control group while the active lever provided morphine saline solution in the two other groups. In the morphine group the number pressings of the active reinforcement lever giving morphine solution in the morphine alone group was several times higher than that of the passive, i.e., saline providing lever. There was a significant difference between the morphine alone ($P < 0.01$) and the control and morphine-AA groups, but there was no significant difference between control and morphine-AA groups. The red lamp above the active lever seemed to increase slightly the use of this lever compared to the passive lever as shown by the control and morphine-AA group data.

lever pressings in the AA receiving group was significantly less (*t*-test, $P < 0.01$) than in the group receiving morphine only (Fig. 2). There was no significant difference between the total active lever pressings of the control and AA groups. The total number of the passive lever pressings in the AA, vehicle and morphine groups did not reveal any significant differences (ANOVA, $P > 0.05$) (Fig. 3).

3.2. Withdrawal syndrome signs

Table 1 shows the effects of AA (500 mg/kg, i.p.) on the withdrawal syndrome signs. Controls did not show any of defined withdrawal signs. The i.p. administration of AA during the 10 days greatly attenuated most of the withdrawal signs specially ptosis, teeth chattering, wet dog shaking, jumping and climbing in comparison to these signs in the morphine group. In comparison with the morphine group, an acute AA administration 30 min before the naloxone injection greatly attenuated in morphine addicted rats the withdrawal signs like ptosis, teeth chattering and wet dog shaking, but it did not affect jumping, diarrhea, and it even increased ejaculations.

4. Discussion

In the present study AA injection (500 mg/kg, i.p.) 30 min before every session caused a decrease in the number of active

Table 1
Effects of i.p. administration of ascorbic acid (100, 500, 1000 mg/kg), 30 min before naloxone injection on naloxon-precipitated withdrawal syndrom signs in morphine dependent rats

Withdrawal signs	Morphine group only	AA and morphine (100 mg/kg)	AA and morphine (500 mg/kg)	AA and morphine (1000 mg/kg)
Wet-dog shakes	6.6	4.6	2.6*	1.6*
Teeth chattering	6.6	3.6*	1.6*	0.6*
Ptosis	6.6	1.6*	1.6*	0.6*
Jumping	6.6	5.6	6.6	5.6
Climbing	6.6	6.6	5.6	6.6
Diarrhea	6.6	5.6	5.6	4.6
Ejaculation	6.6	3.6*	5.6*	6.6*
Salivation	6.6	2.6*	0.6*	0.6*
Lacrimation	6.6	3.6*	0.6*	0.6*

Numbers denote the number of rats showing positive signs relative to the total number of rats tested.
* $P < 0.05$ compared with the morphine withdrawal group by the Fisher exact test.

lever pressings to obtain morphine in rats. The MWS signs were depressed in AA receiving rats in comparison with the morphine addicted animals. These findings are in agreement with the previous studies, which have shown that AA suppresses MWS in opiate addicts [23] and prevents the development of tolerance to and physical dependence on morphine in mice [24] and reduces the locomotor and behavioral withdrawal responses in morphine treated guinea pigs [20]. More recently Evangelou and Kalfakakou [23] have shown that a high orally administered AA dose decreased the withdrawal syndrome problems in heroin addicts.

We also found that, compared to the morphine group, the AA administration 30 min before each session significantly decreased, most of the withdrawal signs after naloxan injection. These results showing the inhibitory effects of chronic AA administration on opiate withdrawal signs confirm previous findings in humans [25] and mice [24].

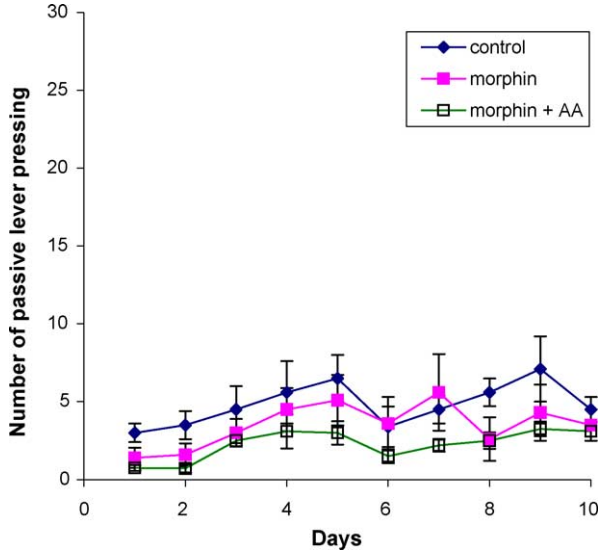

Fig. 3. The number of responses of the passive lever pressings plotted vs. the day of testing in the control (received normal saline), morphine (which received only morphine in self-administration sessions) and AA + morphine groups, which received ascorbic acid before sessions and morphine in self-administration sessions. There was no significant difference between three groups ($P > 0.05$).

On the other hand the injection of AA (500 mg/kg, i.p.) 30 min before thernaloxone injection in morphine addicted rats (acute effect) had no significant effects on some of morphine withdrawal signs including: jumping, climbing and diarrhea, and even it could increase ejaculation, although it could significantly reduce the head/body shaking and face washing and rearing (Table 1). These findings suggest that they are controlled by different mechanisms. It may also reflect a poor penetration of AA into the brain that has been reported following acute systemic AA administration [26]. Apart from these observations rats exhibited no other significant changes in the spontaneous behavior following acute or chronic administration of AA.

The mechanisms involved in the suppression of the tolerance development to and physical dependence on morphine and morphine withdrawal response by chronic AA treatment is unknown. That could include an inhibition of opioid receptor binding by AA, effects on second messengers or more subtle effects on transmitter release and neuronal function [27]. Although the inhibition of opioid receptor binding by AA has been observed in guinea pig brain homogenates, this effect is unlikely to have been involved in the present study, because AA has no effect on opioid receptor binding in intact tissue preparations [28]. Likewise, although AA has been shown to suppress the opiate induced compensatory increase of cAMP in NG 108-15 hybrid tumor cells [29], the significance of this change in cAMP with respect to development of opiate dependence in normal brain has been questioned [30].

An intriguing possibility arises from the reported finding that AA and morphine have opposite effects on membrane Na^+, K^+-ATPase activity. AA has an inhibitory effect on this pump whereas morphine has a stimulatory effect [31,32]. Increased Na^+, K^+-ATPase activity has been suggested to be at least partly responsible for the two major opiate effects in the brain viz. membrane hyperpolarization and inhibition of neurotransmitter release [33]. Chronic AA administration may reduce the stimulatory effect of morphine on this pump activity and inhibit the development of dependence and thus the subsequent withdrawal response. As AA is a powerful hydrophilic antioxidant high doses may also have a role on the redox potentials in the brain, although rat is able to synthesize

its own AA. Some of the membrane proteins may be redox sensitive.

In conclusion the present study has confirmed that chronic administration of AA inhibits the development of morphine dependence, and it inhibits the morphine withdrawal responses. Rat model seems to be useful in further studies, which are needed in the further clarification of the molecular mechanisms.

References

[1] L.M.J. Anttila, L. Ahtee, Retardation of cerebral dopamine turnover after morphine withdrawal and its enhanced acceleration by acute morphine administration in rats, Naunyn Schmiedeberg's Arch. Pharmacol. 327 (1984) 201–207.

[2] R. Tao, S.B. Auerbach, Increased extracellular serotonin in rat brain after systemic or intraraphe administration of morphine, J. Neurochem. 63 (1994) 517–524.

[3] G.R. Siggins, G. Martin, M. Roberto, Z. Nie, S. Madamba, L. De Lecea, Glutamatergic transmission in opiate and alcohol dependence, Ann. NY Acad. Sci. 1003 (2003) 196–211.

[4] T. Ozawa, T. Nakagawa, Y. Sekiya, M. Minami, M. Satoh, Effect of gene transfer of GLT-1, a glutamate transporter, into the locus coeruleus by recombinant adenoviruses on morphine physical dependence in rats, Eur. J. Neurosci. 19 (1) (2004) 221–226.

[5] G.K. Aghajanian, J.H. Kogan, B. Moghaddam, Opiate withdrawal increases glutamate and aspartate efflux in the locus coeruleus: an in vivo microdialysis study, Brain Res. 636 (1994) 126–130.

[6] T. Zhang, T. Feng, R.W. Rockhold, Naloxone-precipitated morphine withdrawal increases pontine glutamate levels in the rat, Life Sci. 55 (1994) 25–31.

[7] S. Schenk, A. Valadez, C.M. Worley, C. Mcnamara, Blokade of the acquisition of cocaine self-administration by the NMDA antagonist MK-801, Behav. Pharmacol. (4) (1993) 652–659.

[8] S. Schenk, A. Valadez, C. McNamaraA, D.T. House, D. Higley, B.A. Honger, Development and expression of sensitization to cocaine's reinforcing properties: role of NMDA receptors, Psychopharmacology (11) (1993) 332–338.

[9] L. Pulvirenti, R. Maldonado-Lopez, G.F. Koob, NMDA receptors in the nucleus accumbens modulate intravenous cocaine but not heroin self-administration in the rat, Brain Res. 594 (1992) 327–330.

[10] S.F. Fan, S. Yazulla, Modulation of voltage-dependent K^+ currents (IK(V)) in retinal bipolar cells by ascorbate is mediated by dopamine D1 receptors, Vis. Neurosci. 16 (5) (1999) 923–931.

[11] M. Miele, M.G. Boutelle, The physiologically-induced release of ascorbate in rat brain is dependent on impulse traffic, calcium influx and glutamate uptake, Neuroscience 62 (1994) 87–91.

[12] L. Tolbert, Ascorbic acid modulate D1 and D2 antagonist binding in vitro, Soc. Neurosci. Abstr. (20) (1994) 521–522;
L. Tolbert, Ascorbic acid modulate D1 and D2 antagonist binding in vitro, Brain Res. 745 (1996) 173–182.

[13] L. De Angeli, Ascorbic acid and atypical anti psychotic drugs modulation of amino peptide-induced behavior in mice, Brain Res. 670 (1995) 303–307.

[14] A.R. White, K.J. Barnham, X. Huang, I. Voltakis, K. Beyreuther, C.L. Masters, R.A. Cherny, A.I. Bush, R. Cappai, Iron inhibits neurotoxicity induced by trace copper and biological reductants, J. Biol. Inorg. Chem. 9 (3) (2004) 269–280.

[15] F. Fornai, S. Piaggi, M. Gesi, M. Saviozzi, P. Lenzi, A. Paparelli, A.F. Casini, Subcellular localization of a glutathione-dependent dehydroascorbate reductase within specific rat brain regions, Neuroscience 104 (1) (2001) 15–31.

[16] G.V. Rebec, C.P. Pierce, A vitamin as neuromodulator. Ascorbic acid release in to the extra cellular fluid of the brain regulate dopaminergic and glutaminergic transmission, Prog. Neurobiol. 43 (1994) 537–565.

[17] M.M. Garcia, A.T. Anderson, R. Edwards, R.E. Harlan, Morphine induction of c-fos expression in the rat forebrain through glutamatergic mechanisms: role of non-*n*-methyl-D-aspartate receptors, Neuroscience 119 (3) (2003) 787–794.

[18] J. Bockaert, O.J. Manzoni, D. Robbe, Metabotropic glutamate receptor 2/3-dependent long-term depression in the nucleus accumbens is blocked in morphine-withdrawn mice, Eur. J. Neurosci. 16 (11) (2002) 2231–2235.

[19] P. Popik, E. Kozela, M. Wrobel, K.M. Wozniak, B.S. Slusher, Morphine tolerance and reward but not expression of morphine dependence are inhibited by the selective glutamate carboxypeptidase II (GCP II, NAALADase) inhibitor, 2-PMPA, Neuropsychopharmacology 28 (3) (2003) 457–467.

[20] P.A. Johnston, L.A. Chahi, Chronic treatment with ascorbic acid inhibit the morphine withdrawal response in guina pigs, Neurosci. Lett. 135 (1) (1992) 23–27.

[21] N.J. Xu, L. Bao, H.P. Fan, G.B. Bao, L. Pu, Y.J. Lu, C.F. Wu, X. Zhang, G.J. Pei, Morphine withdrawal increases glutamate uptake and surface expression of glutamate transporter GLT1 at hippocampal synapses, Neurosci. 23 (11) (2003) 4775–4784.

[22] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1998.

[23] A. Evangelou, V. Kalfakakou, Ascorbic acid (Vitamine C) effects on withdrawal syndrome of heroin abusers, Invivo 14 (2) (2000) 363–366.

[24] N.C. Khanna, S.K. Sharma, Megadoses of Vitamin C prevent the development of tolerance and physical dependence on morphine in mice, Life Sci. 33 (1983) 401–404.

[25] M. Hong, B. Milne, Evidence for the involvement of excitatory amino acid pathway in the development of precipitated withdrawal from acute morphine, Brain Res. 623 (1993) 131–141.

[26] H. Koyuncuoglu, A. Nurten, N. Enginar, B. Ozerman, I. Kara, The effects of different 4-aminopyridine and morphine combinations on the intensity of morphine abstinence, Pharmacol Res. 43 (3) (2001) 245–250.

[27] R.C. Pierce, G.V. Rebec, Dopamine-, NMDA- and sigma-receptor antagonists exert differential effects on basal and amphetamine-induced changes in neostriatal ascorbate and DOPAC in awake behaving rats, Brain Res. 579 (1) (1992) 59–66.

[28] C.E. Dunlap, F.M. Leslie, Effect of ascorbate on the toxicity of morphine in mice, Neuropharmacology 24 (1985) 797–804.

[29] S.K. Sharma, N.C. Khanna, Ascorbate suppresses the opiate-induced compensatory increase in cyclic AMP in neuroblastomax glioma hybrid cells, Biochem. J. 208 (1982) 43–46.

[30] S.M. Johnson, W.W. Fleming, Mechanisms of cellular adaptive sensitivity changes: applications to opioid tolerance and dependence, Pharmacol. Rev. 41 (1989) 435–488.

[31] T. Nishikawa, T. Teramoto, Effect of morphine on Na, K-ATPase from homogenate of synaptosomes and of synaptic membrane of rat cerebral cortex, Brain Res. 510 (1990) 92–96.

[32] R. Goldshleger, G. Patchornik, M.B. Shimon, D.M. Tal, R.L. Post, S.J. Karlish, Structural organization and energy transduction mechanism of Na^+, K^+-ATPase studied with transition metal-catalyzed oxidative cleavage, J. Bioenerg. Biomembr. 33 (5) (2001) 387–399.

[33] V. Demidchik, A. Sokolik, V. Yurin, Characteristics of non-specific permeability and H^+-ATPase inhibition induced in the plasma membrane of Nitella flexilis by excessive Cu^{2+}, Planta 212 (4) (2001) 583–590.