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# MORPHINE RELEASES GLUTAMATE THROUGH AMPA RECEPTORS IN THE VENTRAL TEGMENTAL AREA: A MICRODIALYSIS STUDY IN CONSCIOUS RATS

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## ABSTRACT

*In vivo* microdialysis was used to study the effects of morphine on glutamate release from the ventral tegmentum area (VTA) in freely moving rats. Intraperitoneal (i.p.) injection of acute and repeated morphine at increasing doses significantly enhanced glutamate release. Only a minor tolerance developed to this dosage of morphine. AP-5 (2-amino-5-phosphonovaleric acid, 0.5 mg/kg i.p.), a NMDA receptor antagonist, given 20 min before each repeated morphine injection, did not have a significant effect on the stimulated glutamate release. Conversely, injection of CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione, 0.5 mg/kg i.p.), an AMPA receptor antagonist, 20 min before each morphine dose was found to markedly inhibit morphine-induced glutamate release in the VTA. In all experiments, glutamate release reduced by time. These results show for the first time that acute and repeated injection of morphine stimulates glutamate release in the conscious rat VTA via AMPA receptors.

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**Keywords:** Morphine, Glutamate, VTA, Microdialysis.

## INTRODUCTION

Drug addiction has developed to a social illness. Psychostimulants, opiates, alcohol and nicotine abuse affects millions of people worldwide in virtually all classes of modern society. Intimate neurobiological mechanisms leading to craving and drug addiction still remain largely obscure and therefore any treatment gives still unsatisfactory results. Drug addiction is a chronic relapsing brain disorder characterized by neurobiological changes that lead to a compulsion to take a drug with loss of control over the drug intake. In the last decade, preclinical and clinical research in this field has made some progress to improve our understanding of the brain mechanisms of this illness. Much of this evidence has been obtained in identifying the midbrain-basal forebrain neural elements, notably dopaminergic rewared systems. Also acetylcholinergic, serotoninergic, glutaminergic and opioidergic systems are involved in the drug abuse.[1,2]

Changes in glutamate transmission have been recorded by the repeated administration of addictive drugs into the VTA.[3] Alterations of glutaminergic transmission induce a neuroadaptation that is underlying the development of behavioural sensitization to stimulant drugs. Ungless and co-workers (2001) have indicated that a single dose of cocaine increases transmission at synapses between glutaminergic and dopaminergic neurons in the VTA of the rat brain *in vivo* via the AMPA (alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate) receptors. This effect is blocked by an NMDA receptor antagonist.[4] Conversely, concentration and release

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of glutamate vanishes after an injection of amphetamine into the VTA.[5] Cocaine addiction has also been treated by glutamate antagonists.[6] Microinjections of the glutamate receptor agonists into VTA and nucleus accumbens enhances cocaine craving,[7] suggesting that injections of psychomotorically active drugs into this area are important in triggering drug-induced adaptations.[8]

Behavioural sensitization to psychomotor stimulants is accompanied by a number of super and sub-sensitive glutamate receptors in the VTA. NMDA receptor is the principal subtype of glutamate receptors, which mediates fast excitatory transmission at synapses in the central nervous system. Sensitivity of NMDA subunit receptor is altered by repeated amphetamine administration.[9] Li et al. (1999) have also reported that cocaine sensitization decreases by glutamate antagonists and DA receptors are also altered.[10] Others have suggested that AMPA-type glutamate receptors are involved in the drug addiction, and that GluR-A subunit itself is important for morphine to tolerance and dependence.[11] All these experiments suggest that the VTA might play a role in the neurochemical events triggered by morphine addiction.

To our knowledge, the release of excitatory amino acids into the VTA has not been studied in morphine-dependent animals. Many scientists have evaluated the effect of stimulant drugs (such as amphetamine, cocaine and nicotine) on dopamine and glutamate transmission in nucleus accumbens and VTA in anesthetized rats. In this project we studied the effect of acute and repeated, increasing doses of morphine on the release of glutamate into the VTA of freely moving, conscious rats. We also tried to characterize the glutamate receptor subtype by testing the effect of selective receptor antagonists on the action of repeated morphine administration.

## MATERIAL AND METHODS

### Animals

In this experiment, 60 male Wistar (National Laboratory Animal Center, Kuopio, Finland) rats weighing 220-250 g were used. The rats were housed five per cage under standard conditions (temperature 22 ± 1°C) at a 12 h dark/light cycle (lights on at 7:00 a.m.). They had free and continuous access to fresh tap water and food pellets (Lactamin R36, Lactamin AB Söderalje, Sweden). The rats were divided to five different treatment groups as follows: 1) Control rats were given 0.9% NaCl instead of morphine or glutamate antagonists; 2) the second group was given a single dose of morphine hydrochloride (10 mg/kg, i.p. (Pharmacopoeia Nordica, Helsinki University Pharmacy, Helsinki, Finland); 3) the third group was given morphine repeatedly as follows: in the first 3 days 10 mg/kg, in the next 3 days 20 mg/kg, and in the last 3 days 40 mg/kg;[12] 4) in the fourth group, each morphine dose was preceded by 0.5 mg/kg of AP-5 (2-amino-5-phosphonovaleric acid, Sigma, St. Louis, MO, USA) i.p. 20 min earlier, and 5) in the fifth group by 0.5 mg/kg of CNQX, Sigma, St. Louis, MO, USA.

### Surgery

Rats were anesthetized with chloral hydrate (350 mg/kg, i.p., Merck, Darmstadt, Germany) and placed in a Kopf stereotaxic apparatus. A vertical guide cannula was implanted through a burr hole and secured with dental cement held on the skull with small screws. The final co-ordinates for the tip of the microdialysis probe (220 μm outer diameters and a 1 mm exposed membrane, AgnTho's, Lidingö, Sweden) in the right VTA relative to bregma were: anteroposterior (AP), 5.8; lateral (L), 0.5 and dorsoventral (DV), 9.0 (Paxinos & Watson,1994). Following recovery from surgery (one week) rats were housed singly.

### Microdialysis of freely moving rats

One week after surgery, the microdialysis probe was inserted into the guide cannula and perfused with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 145; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 1.2; and $Na_2HPO_4$, 2, pH 7.40, with a flow rate of 2μL/min. After a 60 min wash out period, dialysate samples were collected in 20 min period in vials and 20 μL was used for glutamate HPLC analysis.

### Determination of glutamate

Glutamate was analyzed from a fraction of the microdialysates as described previously.[14] The HPLC system consisted of a solvent delivery pump (Jasco PU-1580, connected to Jasco LG-1580-02 Low presure mixer, Jasco, Tokyo, Japan), a refrigerated microsampler (Model CMA/200, CMA/Microdialysis, Stockholm, Sweden), an analytical column (Micra NPS ODS-II, 100×4.6 mm, 3 μm particle size, Micra Scientific Inc., IL, USA) protected by a 0.5 um inlet filter and thermostated by a column heater (model CROCO CIL, Cluzeau Info-Labo, France), and a fluorescence detector (Jasco FP-1520, Jasco). Automated sample derivatization was carried out using a CMA/200 refrigerated autosampler at 4°C. The autoinjector was programmed to add 6 μL of the derivatizing reagent [OPA/mercaptoethanol; prepared daily by mixing 1 mL OPA borate buffer solution (OPA incomplete, Sigma, MO, USA) with 3 μL of 2-mercaptoethanol solution (Sigma, MO, USA)] to 15 μL of a microdialysis sample, to mix 2 times and to inject 20 μL onto the column after a reaction time of 1 min. The mobile phase A [0.05 M disodium hydrogen phosphate, pH 6.1 (adjusted with 85% phosphoric acid), acetonitrile 0.5% (v/v), tetrahydrofuran 1% (v/v)] was pumped at a flow rate of 1 mL/min, column temperature was maintained at 32 °C. The external low-pressure mixer was programmed to switch to the mobile phase C [acetonitrile 70% (v/v), tetrahydrofuran 1% (v/v), water 29%] after 3 min of the beginning of the run and to switch back to the mobile phase A



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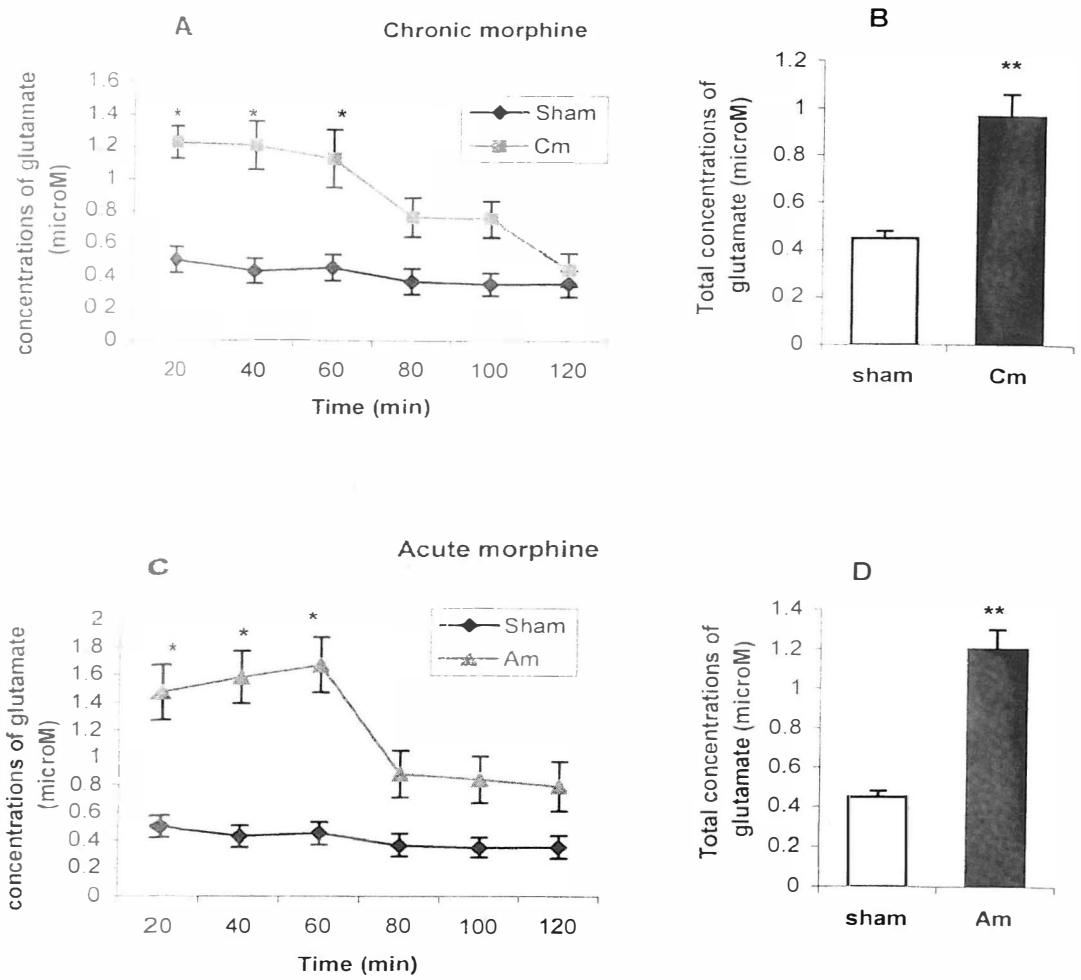


**Fig. 1.** Effect of chronic (Cm) and acute (Am) administration of morphine on glutamate release into VTA nuclei. After chronic morphine administration there was statistically significant difference in the concentration of glutamate between controls and morphine-dependent rats (A, B, *$p$<0.05). Glutamate concentration in the dialysates increased significantly 1 hour after injection of morphine in comparison with controls (*$p$<0.05,C). The average concentration of glutamate in 120 min significantly increased in morphine groups (D, ** $p$<0.01). Data are means ± standard error of the mean.

after 2 min (washout-step). The chromatograms were recorded on an integrator (model C-R5A Chromatopac, Shimadzu, Kyoto, Japan).

### Location of microdialysis probes

At the end of each experiment the brain was removed, and the final position of the microdialysis probes was determined by inspection of Cresyl Violet stained brain section. Data were included in the final analysis only, if the final position of the microdialysis probe tip was found to be in the region of the VTA, as expected.

### Statistical analysis

All results have been expressed as means ± S.E.M. The statistical significance has been calculated by using the two-way analysis variances (ANOVA) test and unpaired Student's t-test. Differences with $p$< 0.05 were considered significant.

## RESULTS

### Effects of chronic and acute morphine on glutamate concentration released from VTA

The intraperitoneal injection of morphine during 9 days (chronic injection) significantly increased the release of glutamate up to 200% compared to control [f = 9.164, $p$<0.01]. The concentration of released glutamate was high in the first 20 min, and it decreased during 120 min (Fig.1A). The release of glutamate also was increased one-hour after the injection of acute morphine (Fig.1C, f = 6.480, $p$< 0.003). The average concentration of glutamate increased after the



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acute and chronic injection of morphine (Fig.1 B, D).

Fig. 2 indicates the comparative changes of glutamate in the VTA during the acute and chronic morphine injections. The concentration of glutamate release in acute morphine was insignificantly higher than chronic morphine groups.

**Effects of morphine administered with CNQX and AP-5 on glutamate concentration released from VTA**

Glutamate receptor antagonists were given in single doses 20 min before the injection of morphine during 9 days (chronic injection of morphine). The results of this treatment are shown in Fig. 3. CNQX (AMPA antagonists) significantly decreased glutamate release ($f = 10.72, p<0.001$) during 120 min (C1) and total concentration of glutamate after 120 min (D1). On the other hand, AP-5 (NMDA antagonists), given 20 min before the injection of morphine had insignificant effect on the average concentration of glutamate in comparison with the morphine group ($p<0.10$) (Fig.3 B1). In addition, the glutamate concentration decreased after injection of this drug measured at 80 min (Fig. 3 A1).

## DISCUSSION

VTA is known to be involved in the mediation of a variety of behavioral responses. Glutamate is one of the neurotransmitters in this area that plays an important role in addiction. Our results demonstrated that morphine addiction was associated with glutamatergic transmission in the VTA, especially with AMPA receptors, since repeated injections of morphine increased the glutamate release that was blocked by CNQX, an AMPA antagonist. Conversely, an NMDA antagonist AP-5 did not have a significant affect on release of glutamate in this area.

It is still unclear which area in the brain is mainly involved in the expression of opioid withdrawal signs precipitated by glutamate, but locus caeruleus (LC) has been most widely studied as an important anatomical site for mediating opioid withdrawal signs. Recently, it was also reported that extracellular glutamate in LC is increased during withdrawal from morphine.[15] Using *In vivo* microdialysis an elevation of extracellular glutamate level has been observed in LC during naloxane-precipitated morphine withdrawal.[16] Other regions in the brain are not ruled out, since it is well known that glutamate modulates dopamine release in the striatum, substantia nigra and VTA. Our results stress the role of the VTA in morphine dependence.

The cortico-striatal and cortico-accumbens projections utilize excitatory amino acids (EAA), such as glutamate/aspartate, as their neurotransmitters. These projections interact functionally and anatomically with the nigrostriatal and mesocortico-limbic dopaminergic pathways.[17] There is strong evidence that glutamate is as important as dopamine in the mammalian central nervous system in the expression of proper emotional and motivational responses. Also, glutamate receptors are currently regarded to play an important role in the formation of synaptic plasticity, which seems to be essential for the generation of compulsive behaviors in animals.[18] It is known that glutamate induces a variety of changes in the level of endogenous dopamine.[19] This phenomenon is possibly caused by the two types of glutamate-aspartate-dopamine interaction in the subcortical dopaminergic terminals.[20] The first type may be an action of EAA on the activity of dopaminergic neurons or dopamine release. One possibility could be that chronic morphine down-regulates dopamine receptors, decreasing dopamine inhibition of glutamate.

The effects of glutamate receptor antagonists co-admin-

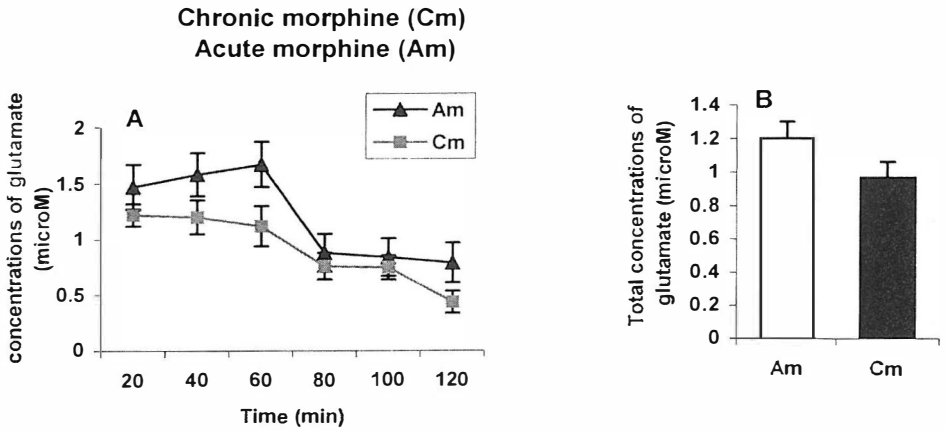


**Fig. 2.** Comparison of glutamate concentration in VTA perfusate after chronic (Cm) and acute (Am) morphine administration during 120 min (A).
The average glutamate concentrations in 120 min are shown in B.



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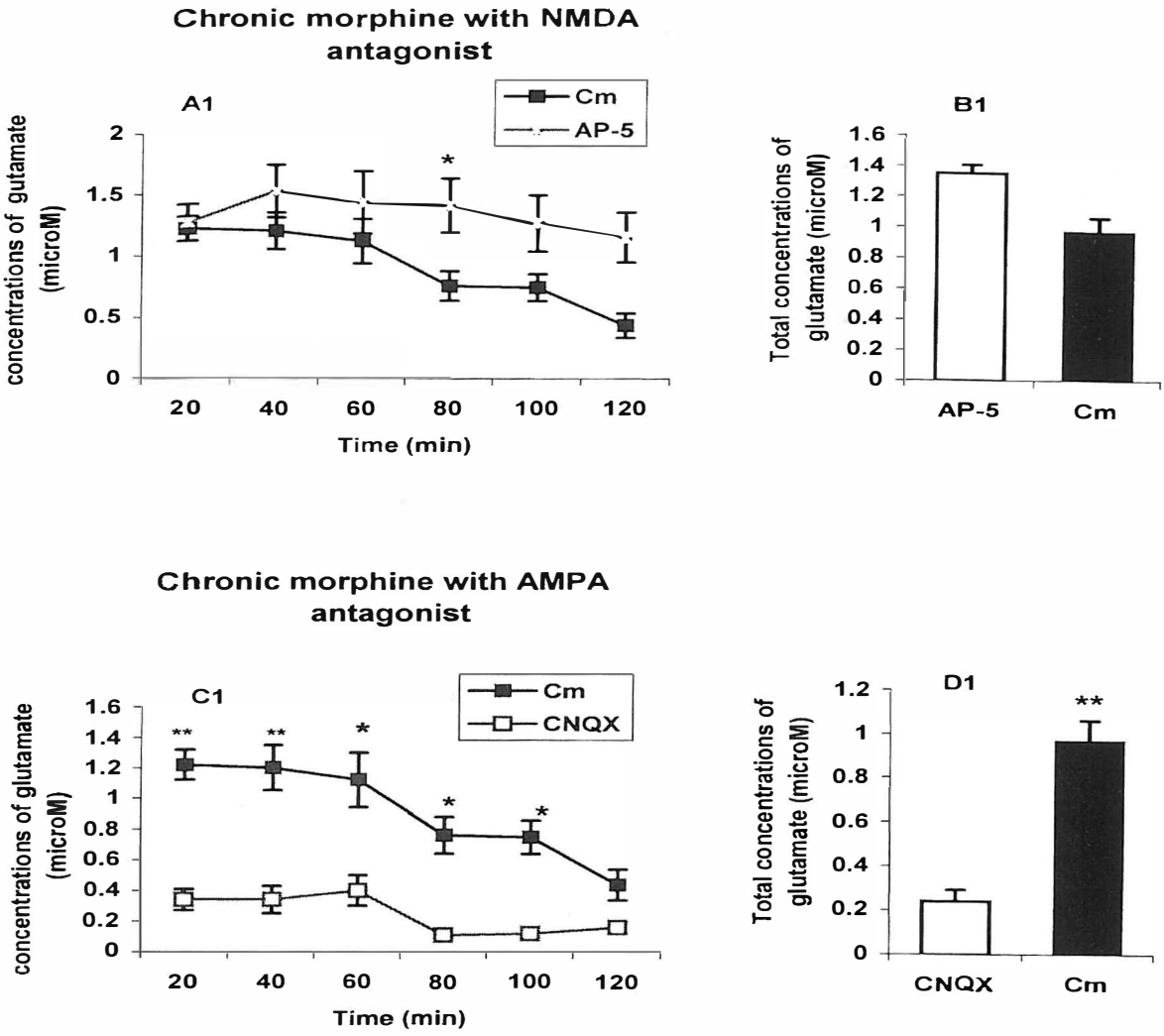


**Fig. 3.** Effects of co-administration of AP-5 (NMDA antagonist) and CNQX (AMPA antagonist) with morphine on the release of glutamate in VTA nuclei. (A1) AP-5 was injected 20 min before morphine during 9 days. Concentration of glutamate was reduced during 120 min in comparison with chronic morphine (Cm) administration alone. (B1) The average concentration of glutamate release in 120 min is shown in 6 samples. (C1) Concentration of glutamate was reduced in each 20 min in CNQX groups in comparison with (Cm) groups. (D1) The average glutamate is indicted in 120 min. Values are expressed as mean ± SEM, ** $p<0.01$ * $p<0.05$.

istered with morphine or given as a single dose before the induction of the abstinence behavior differ in several aspects. There is evidence that the mutual chronic treatment of mice with morphine and the non-competitive NMDA receptor antagonists reduce the intensity of tolerance to, and physical dependence on the opiate.[21] NMDA antagonists also decrease the intensity of the withdrawal syndrome when given in single doses a few min. before naloxone administration to morphine dependent mice.[22]

Our results showed that co-administration of an NMDA antagonist with morphine did not have any significant effect on the release of glutamate. This supports the studies of Bell and Beglan (1995) which indicated that the concomitant administration of morphine and non-competitive NMDA receptor antagonists does not modify the consequences of chronic opiate treatment.[23]

It has been shown that AMPA receptor antagonists attenuate the development of morphine tolerance and physical dependence without affecting the antinociceptive effect of morphine.[24] Some investigators have shown that



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intracerebroventricular injections of glutamate dose dependently induce withdrawal symptoms in morphine dependent rats.[25] Systemic injections of NMDA and non-NMDA glutamate receptor blockers prevent some features of the morphine withdrawal syndrome.[26] Therefore, it has been proposed that excitatory amino acids contribute to the morphine withdrawal syndrome.

*In vivo* microdialysis studies have demonstrated that the efflux of EAA (glutamate and aspartate) is dramatically increased in the LC during naloxone-precipitated morphine withdrawal.[15,16] We have shown an increase in glutamate release after chronic injection of morphine in the present study. AP-5 and CNQX have been shown to block morphine withdrawal responses of LC neurons but in our study AP-5 did not affect the efflux of glutamate in the VTA. Both glutamate receptor antagonists have been shown to inhibit the development of morphine tolerance and dependence.[27]

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