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Agmatine modulates neuroadaptations of glutamate transmission in the nucleus accumbens of repeated morphine-treated rats

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ABSTRACT

It has been proved that agmatine inhibits opioid dependence, yet the neural mechanism remains unclear. In the present study, the effect of agmatine on the neuroadaptation of glutamate neurotransmission induced by morphine dependence, including changes of the extracellular glutamate level and glutamate receptors in the nucleus accumbens was investigated. We found that agmatine (2.5–20 mg/kg, s.c.) inhibited development of morphine dependence, which was consistent with our previous report. In rats repeatedly treated with morphine, the glutamate level in the nucleus accumbens dialysate was markedly increased after naloxone-precipitated withdrawal. When agmatine (20 mg/kg, s.c.) was co-pretreated with morphine or was applied before naloxone-precipitated withdrawal, this elevation of the extracellular glutamate level was inhibited. In the synaptosome model, repeated morphine treatment and naloxone precipitation induced an increase in glutamate release, while agmatine (20 mg/kg, s.c.) co-pretreated with morphine reversed the increase of glutamate release. However, neither morphine or agmatine treatment alone nor morphine and agmatine co-administration had any influence on [3H]-glutamate uptake. It indicated that the elevation of the glutamate level in the nucleus accumbens might be caused by the increase of glutamate release of synaptosome in the withdrawal conditions of morphine-dependent rat. Furthermore, agmatine concomitant treatment with morphine entirely abolished the up-regulation of the NR1 subunit of N-methyl-D-aspartate (NMDA) receptors in the nucleus accumbens in repeated morphine-treated rats. Taken together, the present study demonstrated that agmatine could modulate the neuroadaptations of glutamate transmission in the nucleus accumbens in the case of morphine dependence, including modulating extracellular glutamate concentration and NMDA receptor expression.

1. Introduction

Long-term administration of opioids leads to changes in the effects of these drugs, including tolerance, sensitization and dependence. Accumulating evidence has revealed that the neuronal adaptations (including molecular, cellular, synaptic and network levels) produced by long-term exposure to opioids, are responsible for the tolerance, sensitization and dependence (Williams et al., 2001; Nestler, 2004). The adaptations of glutamate transmission are an important event in neural plasticity, as well as in opioid dependence, which contain the alterations of glutamate transmitter and glutamate receptors, including ionotropic glutamate receptors (NMDA and AMPA receptor) and metabotropic glutamate receptors. It has been reported that opioid addiction leads to the changes in the basal glutamate level or presynaptic glutamate release and the expression of glutamate receptors, especially NMDA receptors in various brain regions. (Siggins et al., 2003; Glass et al., 2004; LaLumiere and Kalivas, 2008). On the other hand, the administration of agents to regulate the extracellular glutamate level or to block NMDA receptor inhibits the opioid analgesic tolerance and dependence (Trujillo, 2000).

The nucleus accumbens, a critical element of the mesocorticolimbic system, plays a key role in opioid reward, the motivation and signs of opioid withdrawal (Wise, 1989; Harris and Aston-Jones, 1994). This basal forebrain structure receives dopamine input from the ventral tegmental area, as well as the glutamate input from regions including the prefrontal cortex, amygdala and hippocampus. As such, it integrates inputs from limbic and cortical regions, linking motivation with action. Several studies showed that alterations of glutamate transmission in the nucleus accumbens affected opioid addiction (Kourrich et al., 2007; LaLumiere and Kalivas, 2008).

Agmatine is an endogenous biological substance. As a putative neurotransmitter and/or neuromodulator, agmatine is widely distributed in mammalian brains such as hypothalamus, hippocampus, cortex, locus ceruleus, raphe nucleus, et al. (Halaris and Plietz, 2007). It binds with high affinity to α2 adrenergic and imidazoline (I1) receptors (Reis and Regunathan, 2000). Besides, agmatine also antagonizes the NMDA

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2.1. Animals

Male Wistar rats (Beijing Animal Center, China) weighing 220–250 g were used in all experiments. The rats were grouped 6 per cage at an ambient temperature of 24–25 °C and a relative humidity of 50–60%. Animals were maintained on a 12 h light/dark cycle (lights on between 7:00 A.M. and 7:00 P.M.) and given ad libitum access to food and water. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). Formal approval to conduct the experiments described has been obtained from the Animal Subjects Review Board of Beijing Institute of Pharmacology and Toxicology. All efforts were made to minimize the number of animals used and their suffering.

2.2. Drugs

Morphine hydrochloride was purchased from Qinhai Pharmaceutical Factory (Xining, China). Agmatine sulfate, naloxone, pentobarbital sodium, glutamate, amino-oxyacetic acid, and o-phthalaldehyde were obtained from Sigma (St. Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Octanesulfonate sodium salt was obtained from Dickma (USA). All drugs for animal treatment were dissolved in 0.9% saline to final concentrations and injected in a volume of 1.0 ml/kg. Morphine and agmatine were given subcutaneously (s.c.), naloxone was given intraperitoneally (i.p.).

Goat polyclonal antibody for NMDA receptor NR1 subunit, anti-β-actin antibody, horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence detection kit were obtained from Santa Cruz Biotechnology (CA, USA). L-[3H]-Glutamic acid (45.0 Ci/mmol) was purchased from Amersham (USA).

2.3. Experimental procedure

2.3.1. Morphine dependence and withdrawal

Rats were given for 5 days twice daily ascending doses of morphine (s.c.) at 8:00 and 20:00 according to the following schedule: day 1, 10 mg/kg; day 2, 20 mg/kg; day 3, 30 mg/kg; day 4, 40 mg/kg; and day 5, 50 mg/kg. The control group received saline according to the same schedule. In order to study the effect of agmatine on the development of morphine dependence, agmatine (2.5, 7.5 and 20 mg/kg, s.c.) was injected 30 min before morphine administration according to the same schedule. For precipitation of morphine withdrawal, the rats were administrated with naloxone (5 mg/kg, i.p.) 5 h after the last morphine administration.

2.3.2. Behavior analysis

After morphine treatment and naloxone precipitation described as above, the rats were immediately placed into clear, plexiglass observation tanks (50 cm × 50 cm × 60 cm) and rated for withdrawal severity for 15 min by a rater blind to the treatment. Withdrawal intensity was evaluated by assessing withdrawal signs including jumping, wet-dog shaking, writhing posture, paw tremors, genital grooming, teeth-chattering, chewing, salivation, rearing, ptosis, diarrhea, and irritability. The characteristics of these behaviors were described by Fernandez-Espejo et al. (1995). The score for each group was calculated as the method described by Koob et al. (1992).

2.3.3. Microdialysis procedure

The surgical procedure and microdialysis sampling were performed using modifications of techniques described previously (Montiel et al., 2005). After anesthetized with pentobarbital sodium (50 mg/kg, i.p.), the rat’s skull was exposed and one small hole was drilled for implantation of a CMA/12 microdialysis guide cannula, the tip of which was located above the nucleus accumbens core. The coordinates of guide cannula were anteroposterior (AP) +1.7 mm from the bregma, mediolateral (ML) ±1.2 mm and dorsoventral (DV) −5.0 mm from the surface of the skull (Paxinos and Watson, 1997), and the efficient dialysis length of probe was 2.0 mm. When the rats recovered consciousness after operation 16 h, the drugs were administered according to the same schedule as repeated morphine treatment totally for 5 days.

After the last morphine treatment, rats were transferred to a free moving animal system (BAS/100). The microdialysis probes (CMA/12), which were perfused with Ringer’s solution (145 mM NaCl, 2.7 mM KCl, 2.2 mM CaCl2, and 1.1 mM Na2HPO4 pH 7.4) at a constant flow rate 2 μl/min, were inserted into the guide cannula. Following an equilibration period of 3 h, dialytic samples were collected every 30 min and the glutamate level in these samples was measured by HPLC/ECD assay. After the basal level of glutamate became stable (the difference between three sequential samples being lower than 10%), animals received naloxone injection (2 mg/kg, ip), and then dialytic samples were collected every 15 min in the first 30 min and every 30 min in 30–150 min. All samples were determined by HPLC/ECD. The subsequent values were expressed as a ratio to the pre-injection values. After the completion of microdialysis testing, each rat was deeply anesthetized with pentobarbital sodium. The brain was then removed and cut to observe the track of the microdialysis probe for verification of probe placement. Fig. 1 shows a photograph of a representative dialysis probe track in the core of the nucleus accumbens. The data from animals with incorrect probe placements were excluded after assessment of probe placements.

2.3.4. HPLC/electrochemical detection (ECD) assay

After precolumn derivatized by o-phthalaldehyde, the amount of glutamate in the dialytic samples (25 μl) was quantified by HPLC (Agilent 1100 series, USA) using an electrochemical detector, a Supelco Hypersil ODS column (150 mm × 4.6 mm, 5 μm) as previously described (Wen et al., 2004). The mobile phase consisted of 85 mM citric acid, 100 mM sodium acetate, 0.2 mM Na2EDTA, 0.9 mM 1-octanesulfonate sodium salt, and 10% methanol (v/v), pH 3.65. The flow rate was 1 ml/min. The vitreous carbon reference electrode was set at +0.7 V. External standard curves were used to quantify the amount of glutamate in each sample by the use of area under curve (AUC).

2.3.5. Glutamate release and uptake assays

Animals were treated with drugs for 5 days according to the same schedule as repeated morphine treatment. The nucleus accumbens from the rat brain was rapidly dissected on the ice 15 min after the naloxone (5 mg/kg, i.p.) precipitation, and the synaptosomes were prepared according to the standard methods described previously (Silva Brum et al., 2001). Protein contents were determined by the Bradford method.
The synaptosomal fractions were stored on ice and used within 4 h for the glutamate release and uptake assays.

For release studies of basal conditions, synaptosomes (20 μg/300 μl) were incubated oscillatory in Krebs’–Ringer’s–HEPES buffer (NaCl 140 mM, KCl 5 mM NaHCO₃ 5 mM, MgCl₂·6H₂O 1 mM, Na₂HPO₄ 1.2 mM, glucose 10 mM, HEPES 20 mM, pH 7.4, equilibrated with 95% O₂/5%CO₂) containing CaCl₂ in a final concentration of 1.3 mM for 20 min at 37 °C. Incubations were stopped by centrifugation at 14,600 g for 10 min at 4 °C. The supernatants were stored at −70 °C until further HPLC/ECD assay.

As was the case (Ullensvang et al., 1997), the synaptosomes were incubated oscillatory in 400 μl of Krebs’–Ringer’s–HEPES buffer containing CaCl₂ 1.3 mM. After incubation at 37 °C for 15 min, the glutamate uptake was initiated by adding 100 μl [3H]-glutamic acid (10 nM for a final concentration, 45.0 Ci/mmol) to the reaction system at 37 °C for 4 min. Meanwhile, nonspecific uptake was determined by adding the non-radioactive labeling glutamate (1 μM) into the total 500 μl reaction systems. The uptake was terminated by filtration on GF/C glass-fiber filters (Whatman, UK) under vacuum. After being washed three times with 5 ml of ice-cold saline and stoving, the filters were added with scintillation fluid and the radioactivity was quantified by liquid scintillation counting.

2.3.6. Western blots for the NR1 subunit of NMDA receptors

Animals were treated with drugs for 5 days according to the same schedule as repeated morphine treatment. 5 h after the last drug exposure, animals were sacrificed and the nucleus accumbens from the brains was rapidly dissected on ice and stored at −70 °C. The stored tissues were homogenized in lysis buffer (w/v 1:15) (20 mM HEPES, 400 mM NaCl, 20% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP40, 5 mM dithiothreitol, containing the protease inhibitors leupeptin 1 mg/L, pepstatin A 1 mg/L, aprotin 1 mg/L, and phenylmethylsulfonyl fluoride 0.5 mM, pH 7.5) and centrifuged at 1000 g for 20 min at 4 °C. After measurement of protein concentration by the Bradford method, tissue lysates were denatured with 5× Laemmli buffer and frozen at −70 °C. An equal amount of proteins was separated with 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Membranes were blocked in 3% dry milk powder and 0.05% tween-20 in Tris-buffered saline for 2 h at the room temperature, and incubated with the primary antibodies (anti-NR1 antibody, 1:1000 diluted; anti-β-actin antibody, 1:5000 diluted) at 4 °C overnight. After incubation with corresponding secondary antibodies, bands were visualized using the enhanced chemiluminescence detection kit according to the manufacturer’s instructions. Western blots were scanned and analyzed by the NIH Image software. Results were expressed as a ratio of anti-NR1 blots to anti-β-actin blots to control for variation in protein loading.

2.3.7. Statistical analysis

Data were expressed as mean ± S.E.M. Student’s t-test was used to compare the difference of the other groups to control. One-way ANOVA followed by Dunnett’s t-test was used to compare the difference between agmatine treatment groups and morphine group. Differences were considered significant at *P<0.05.

3. Results

3.1. Preventive effects of agmatine on the development of morphine dependence

Rats with repeated morphine treatment displayed typical withdrawal syndromes following naloxone precipitation, such as teeth-chattering, jumping, erection, ptosis, chews, salivation, diarrhea, writhes, wet-dog shakes, rearing and irritability, which suggested that the morphine repeated dependence model has been established. Repeated agmatine (20 mg/kg) administration alone did not induce withdrawal syndromes after naloxone precipitation, while co-administration of agmatine (2.5, 7.5 and 20 mg/kg) with morphine attenuated the withdrawal syndrome in a dose-dependent manner (Fig. 2). No significant difference was detected between the high dose of agmatine (20 mg/kg) co-administrated with morphine group.
morphine group and saline control, so agmatine at dosage of 20 mg/kg was selected for the following experiments.

3.2. Effects of agmatine on the extracellular glutamate levels in the nucleus accumbens core of repeated morphine-treated rats

After repeated administration of morphine, no significant change in the extracellular glutamate level was observed in the nucleus accumbens dialysate. Compared with the saline-treated control group, naloxone precipitation induced a precipitous elevation of the glutamate level in the nucleus accumbens dialysates in the repeated morphine-treated group. The increase of extracellular glutamate reached the peak value of 221% at 15 min after naloxone precipitation and turned to the baseline after 60 min. Agmatine (20 mg/kg, s.c.) repeated co-pretreatment with morphine entirely inhibited the naloxone-induced elevation of extracellular glutamate in the nucleus accumbens (Fig. 3).

3.3. Effects of agmatine on the glutamate release and uptake in the nucleus accumbens of repeated morphine-treated rats

The extracellular glutamate level is regulated by its release and uptake. In order to explore the potential changes in the glutamate release and uptake during repeated morphine treatment and agmatine’s influence on it, a synaptosome model was used. Rats received ascending doses of morphine twice daily for 5 days, a procedure known to produce significant morphine tolerance and dependence, and the crude synaptosomal fractions from the nucleus accumbens were prepared 15 min after naloxone precipitation. The basal release of glutamate increased by 17% in morphine-dependent rats after naloxone precipitation, and agmatine (20 mg/kg) co-pretreatment with morphine completely abolished the increase of glutamate release. However, the increase in the basal release of glutamate in the nucleus accumbens was observed by repeated treatment of agmatine (20 mg/kg) alone (Fig. 4A).

In the [3H]-glutamate uptake experiment, no change in glutamate uptake in the nucleus accumbens synaptosomes was observed after repeated administration of agmatine, morphine or agmatine concomitantly with morphine (Fig. 4B). These results suggested that agmatine’s inhibition of the naloxone-induced elevation of the extracellular glutamate level in the nucleus accumbens in morphine-dependent rats was attributed to its decreasing glutamate release but not affected by the glutamate uptake procedure.

3.4. Effects of agmatine on NR1 subunit expression of NMDA receptors in the nucleus accumbens of repeated morphine-treated rats

The NMDA receptor is one of the important components of the glutamate transmission system and plays a crucial role in drug addiction. The NR1 subunit is the essential part of NMDA receptors. Repeated morphine treatment induced an increase in NR1 expression of NMDA receptors by 80% in the nucleus accumbens, while agmatine (20 mg/kg) co-pretreatment with morphine entirely abolished the up-regulation of the NR1 subunit. Agmatine (20 mg/kg) alone had no effect on NR1 subunit expression (Fig. 5).

4. Discussion

In the present study, the modulation by agmatine to neuroadaptations of glutamate transmission in morphine dependence was investigated. It was found that the extracellular glutamate level in the nucleus accumbens was suddenly increased after naloxone precipitation in morphine-dependent rats, and agmatine abolished this elevation in extracellular glutamate levels. Further study showed that the inhibition of agmatine to extracellular glutamate elevation was due to the decrease in glutamate release in the nucleus accumbens. Meanwhile, agmatine reversed the up-regulation of NMDA receptors induced by repeated morphine treatment in the nucleus accumbens.
While agmatine's pharmacological effects are not only involved in transmodulator, is the endogenous ligand of imidazoline receptors. previous reports. Agmatine, acting as a novel neurotransmitter/accumbens of morphine-dependent rats, which was consistent with the present study, we found that extracellular glutamate concentration increased remarkably in locus ceruleus, the nucleus accumbens, and other brain structures (Sepulveda et al., 1998; Tokuyama et al., 2001). In our present study, we found that extracellular glutamate concentration was increased precipitously after naloxone precipitation in the nucleus accumbens of morphine-dependent rats, which was consistent with previous reports. Agmatine, acting as a novel neurotransmitter/transmodulator, is the endogenous ligand of imidazoline receptors. While agmatine's pharmacological effects are not only involved in imidazoline receptors (Reis and Regunathan, 2000). It had been improved that agmatine can generate a voltage- concentration-dependent block of the NMDA receptor channel in cultured hippocampal pyramidal cells of neonatal rat. This noncompetitive block indicates two binding sites with different affinity with agmatine in the NMDA receptor channel pore (Roberts et al., 2005). Pharmacologically, agmatine blocks some forms of hyperalgesia, morphine tolerance and withdrawal. Additionally, agmatine protects neurons from glutamate-induced neurotoxicity. All of its functions above may have very close relationship with its effect on the glutamate-NMDA receptor system. So agmatine possibly plays a part in the regulation of glutamate system adaptation in opioid dependence but it was rarely reported. The nucleus accumbens, as a cross point of the limbic system and motivational system in the brain, plays a crucial role in the pathological change of morphine dependence. It accepts inherent projection of glutamergic neurons from amygdala, hippocampus, dorsal medial nucleus of thalamus, etc. So the level of glutamate and NMDA receptor in the nucleus accumbens were detected in the present study. We found that agmatine (20 mg/kg) co-pretreatment with morphine abolished naloxone precipitation-induced increase in extracellular glutamate level in the nucleus accumbens of morphine-dependent rats. This kind of effect of agmatine was also observed during pentylenetetrazol-induced seizure (Feng et al., 2005). Since the sudden increase in the extracellular glutamate level was presumed as the key factor of the abstinence syndrome occurrence, it is conceivable that this might be one of the neurochemical mechanisms of agmatine inhibiting the development of morphine dependence as well as withdrawal syndromes. The microdialysis technique (Ungerstedt, 1991; Westerink and Cremers, 2007) is the most common method to get the sample and detect the level of neurotransmitters in the brain of living animals. During basal conditions, glutamate in microdialysates is mainly derived from non-synaptic sources. However, during conditions of chemical, electrical or behavioral stimulation, a significant part of glutamate might be derived from neurotransmission (Zeyden et al., 2008). As there is no enzyme to metabolize extracellular glutamate, the extracellular level of glutamate is mainly determined by two factors: the release from the pre-synaptic membrane and the uptake. The synaptosome is a suitable model for investigating neurotransmitter release and uptake. In this model, we found that the release of glutamate from the nucleus accumbens increased notably under a withdrawal condition in the morphine-dependent group, while the increase was reversed by agmatine (20 mg/kg) treatment accompanied by morphine. Unlike the release of glutamate, the uptake of glutamate in the nucleus accumbens had no change after naloxone precipitation either in repeated morphine alone or accompanied with agmatine. Thus, the decrease in the release of glutamate, rather than the uptake, might contribute to the inhibition of agmatine on naloxone precipitation-induced extracellular glutamate elevation in morphine-dependent rats. As is known, the neurotransmitter releases in a Ca2+-dependent manner from presynaptic membranes. Previous studies showed that agmatine could attenuate voltage-gated calcium channel currents in isolated neurohypophysial terminals and in cultured rat hippocampal neurons (Wang et al., 2002; Weng et al., 2003), which might result in the inhibition of agmatine to glutamate release. Neuroadaptations of glutamate transmission involve the change of glutamate concentration and glutamate receptors. The NMDA receptor is a tetrameric ligand-gated ion channel, which was assembled by NR1, NR2 (NR2A, NR2B, NR2C, NR2D) and NR3 (NR3A, NR3B) subunits. The common NR1 subunit is the essential part of NMDA receptors and the number of which is the amount signal of the NMDA receptor, while one or more of NR2 subunits regulate the properties of the channel (Cull-Candy, 2001). The differential expression of NR2 subunits in the various regions of the brain may account for the diversity of NMDA receptor subtypes (Watanabe et al., 1992). Interestingly, previous studies showed that chronic administration of morphine resulted in the up-regulation of the NR1 subunit of NMDA receptor in locus ceruleus, the paraventricular nucleus of hypothalamus and the ventral tegmental area (Tokuyama et al., 2001; Zhu et al., 1999). In our present study, agmatine (20 mg/kg) repeated treatment alone did not alter NR1 subunit expression in the nucleus accumbens, but it entirely reversed the up-regulation of the NR1 subunit in the nucleus accumbens in morphine dependence. This effect of agmatine was similar to NMDA receptor antagonist MK801. In fact, it has been found that agmatine could block the NMDA receptor current in rat hippocampal neurons (Boronat et al., 1998; Yang and Reis, 1999). It’s perhaps the mechanism of inducing the feedback up-regulation of the number of NMDA receptors. In conclusion, our present study demonstrated that agmatine modulated the neuroadaptations of glutamate transmission in the nucleus accumbens in morphine dependence, including modulating extracellular glutamate concentration and NMDA receptor expression. This might be one of the neural mechanisms of agmatine inhibiting opioid dependence.
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