Research Report

Methamphetamine modulates glutamatergic synaptic transmission in rat primary cultured hippocampal neurons

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A B S T R A C T

Methamphetamine (METH) is a psychostimulant drug. Abuse of METH produces long-term behavioral changes including behavioral, sensitization, tolerance, and dependence. It induces neurotoxic effects in several areas of the brain via enhancing dopamine (DA) level abnormally, which may cause a secondary release of glutamate (GLU). However, repeated administration of METH still increases release of GLU even when dopamine content in tissue is significantly depleted. It implies that some other mechanisms are likely to involve in METH-induced GLU release. The goal of this study was to observe METH affected glutamatergic synaptic transmission in rat primary cultured hippocampal neurons and to explore the mechanism of METH modulated GLU release. Using whole-cell patch-clamp recordings, we found that METH (0.1–50.0 μM) increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and miniature excitatory postsynaptic currents (mEPSCs). However, METH decreased the frequency of sEPSCs and mEPSCs at high concentration of 100 μM. The postsynaptic NMDA receptor currents and P/Q-type calcium channel were not affected by the use of METH (10,100 μM). METH did not present visible effect on N-type Ca2+ channel current at the concentration lower than 50.0 μM, but it was inhibited by use of METH at a 100 μM. The effect of METH on glutamatergic synaptic transmission was not reversed by pretreated with DA receptor antagonist SCH23390. These results suggest that METH directly modulated presynaptic GLU release at a different concentration, while dopaminergic system was not involved in METH modulated release of GLU in rat primary cultured hippocampal neurons.

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1. Introduction

Methamphetamine (METH) is a psychostimulant drug. METH abuse throughout the world has markedly increased during recent years (Lezcano and Bergson, 2002; Chirwa et al., 2005) and represents a significant public health concern (Reiner et al., 2009). Drug abuse, including METH, can cause long-lasting changes in neuronal systems (Cadet et al., 2005;
2. Results

2.1. METH affected the frequency and the amplitude of sEPSCs

After 10–14 days, the cultured hippocampal neurons were recorded. After the whole-cell recording configuration was established, the sEPSCs could be recorded in most neurons. The sEPSCs were blocked by a combined AMPA receptor antagonist CNQX (20 μM), NMDA receptor antagonist (L-AP5 50 μM), and KA receptor antagonist kynurenic acid (10 μM) (data not shown) in the presence of GABA A antagonist bicuculline, which revealed that the sEPSCs were mediated by neurotransmitter GLU. It was found that METH (10 μM) significantly increased the frequency of sEPSCs (K-S statistic, n = 10, p < 0.05), which was reversed by washing out for about 5 min (Fig. 1A and B). The frequency of sEPSCs was 1.43 ± 0.52 Hz for control, 2.73 ± 0.32 Hz in the presence of METH, 1.70 ± 0.31 Hz after washout (n = 10, p < 0.05). The amplitude of sEPSCs was enhanced by the use of METH (10 μM) (K-S statistic, n = 10, p < 0.05, Fig. 1C). The median amplitude of sEPSCs was 72.43 ± 8.78 pA for control, 89.45 ± 9.90 pA in the application of METH (10 μM), 77.93 ± 8.90 pA after washout (n = 10, p < 0.05). At concentrations 0.1, 1.0, 5.0, 10.0, and 50.0 μM, METH increased the frequency of sEPSCs for 223 ± 40%, 318 ± 46%, 246 ± 62%, 225 ± 39%, and 176 ± 41%, respectively (n = 10, p < 0.05). However, METH, at concentration of 100 μM, decreased the frequency of sEPSCs by 87 ± 13% (n = 10, p < 0.05, Fig. 1D). METH enhanced the amplitude of sEPSCs for 126.3 ± 9.80%, 145.6 ± 12.80%, 135.5 ± 7.15%, 132.8 ± 6.92%, 126 ± 4.70% at concentrations of 0.1, 1.0, 5.0, 10.0, and 50.0 μM, respectively (n = 10, p < 0.05, Fig. 1E). METH decreased the amplitude of sEPSCs by 59 ± 7.39% at concentration of 100 μM.

2.2. METH regulated the frequency and amplitude of mEPSCs

We further examined the METH-mediated alteration of presynaptic GLU release by observing the effect of METH on mEPSCs, which were action potential-independent spontaneous GLU release from presynaptic terminals. The bath solution included...
bicuculline (100 μM) and TTX (1 μM) to block the GABA<sub>A</sub> receptor chloride channels and voltage-gated sodium channel, respectively. METH (10 μM and 100 μM) was applied in the following experiments. It was found that the frequency of mEPSCs was increased significantly in the presence of METH (10 μM) (K-S statistic, n = 8, p < 0.05, Fig. 2A and B), which suggested that METH enhanced presynaptic GLU release. K-S test showed that the amplitude of mEPSCs was not changed significantly by the application of METH (10 μM) (n = 8, p > 0.05, Fig. 2C). mEPSCs frequency was 1.01 ± 0.19 Hz for control, 1.86 ± 0.21 Hz in the presence of METH, 1.02 ± 0.21 Hz after 5 min of washout (n = 8, p < 0.05, Fig. 2D). mEPSCs amplitude was 21.78 ± 0.64 pA for control, 21.46 ± 0.53 pA in the presence of METH, 21.51 ± 0.61 pA after 5 min of washout (n = 8, p < 0.05, Fig. 2E). On the other hand, the frequency and the amplitude of mEPSCs were both decreased significantly by the application of METH (100 μM) (n = 8, each, p < 0.05, Fig. 2F-J).

2.3. METH had no significant effect on NMDA and AMPA receptor-mediated currents

To examine the possible involvement of GLU-mediated postsynaptic NMDA and AMPA receptors in METH-induced changes in glutamatergic synaptic transmission, AMPA and NMDA receptor-mediated currents were studied. To record NMDA receptor-mediated currents, exogenous NMDA (100 μM) and glycine (20 μM) were co-applied to hippocampal neurons at a holding potential of −50 mV. After 5 min pretreatment of METH (10 or 100 μM, in this and subsequent experiments, a total of METH superfusion time depended on when the agonist-induced current returned to original level, usually 6–7 min), the peak amplitudes of NMDA receptor-mediated currents before and after METH treatment were compared. On average, the peak NMDA receptor-mediated current was 441 ± 45 pA for the control group and 446 ± 56 pA and
443 ± 38 pA for 10 μM and 100 μM METH-treated groups, respectively (n=5, p > 0.05). This result showed that NMDA receptor-mediated currents were not affected directly by the application of METH (Fig. 3A–C). Exogenous application of AMPA (5 μM) induced an inward current at a holding potential of −70 mV. The mean amplitude of AMPA-mediated current was 251 ± 14 pA (Fig. 3D) for control and 260 ± 16 pA or 257 ± 15 pA in the presence of METH, 10 or 100 μM (n=5, p > 0.05, Fig. 3E–G), suggesting AMPA receptor was not directly affected by METH.

2.4. METH blocked N-type calcium channel currents, but not P/Q-type calcium channel currents

Both N-type and P/Q-type calcium channels are tightly coupled to GLU release (Inchauspe et al., 2007). Previous experiments
have shown that at least four distinct types of voltage-gated calcium channel (L-, N-, P/Q-, and R-type) are expressed in cultured hippocampal neurons and sensitive to different blockers (Takahashi and Momiyama, 1993). To observe the effect of METH on different kinds of voltage-gated calcium channels, three distinct blockers (verapamil for L-, MVIIA for N-, and ω-agatoxin IVA for P/Q-type calcium channels) were applied in the present experiment to define L-, N-, and P/Q-type currents, respectively (Wen et al., 2005). The voltage-gated P/Q-type Ca$^{2+}$ channel currents were elicited by 300 ms step depolarization to $+60$ mV from a holding potential of $-80$ mV in the presence of verapamil (10 μM) and MVIIA (3 μM). METH (10 μM, 100 μM) did not show any visible effect on the kinetic of P/Q-type Ca$^{2+}$ channel currents ($n=8$, $p>0.05$, Fig. 4A). The I-V curves showed that the amplitude was not affected by the application of METH either ($n=8$, $p>0.05$, Fig. 4B). This result indicated that METH had no significant effect on P/Q-type Ca$^{2+}$ channel currents. The voltage-gated N-type calcium channel currents were recorded by 300 ms step depolarization to $+60$ mV from a holding potential of $-80$ mV in the presence of verapamil (10 μM) and ω-agatoxin IVA (0.5 μM). METH, at 10 μM, did not show any visible effect on N-type Ca$^{2+}$ channel currents (Fig. 4C). However, N-type Ca$^{2+}$ channel currents were depressed when a high concentration of METH (100 μM) was applied to the neurons. The inhibitory rate reached 62±6% ($n=8$, $p<0.05$) at a holding potential of 0 mV (Fig. 4D).

2.5. The effect of METH on mEPSCs was not affected by pre-application of DA receptor antagonist SCH23390

To study whether DA was involved in METH-induced change of GLU synaptic transmission, D$_1$/D$_5$-selective DA receptor antagonist SCH23390 was used. The frequency of mEPSCs was increased by the application of METH (10 μM), which was not altered in the presence of SCH23390 (10 μM) ($n=8$, $p<0.05$, Fig. 5A and B). The frequency of mEPSCs was 0.93±0.34 Hz for control, 1.93±0.41 Hz in the presence of METH and SCH23390, and 0.89±0.32 Hz after washout ($n=8$, $p<0.05$).

The amplitude of mEPSCs was not affected in the presence of METH (10 μM) and SCH23390 (10 μM) ($n=8$, $p>0.05$, Fig. 5D–F).

2.6. Tyrosine hydroxylase (TH)-positive neuron was almost not found in rat primary cultured hippocampal neurons

To further study whether dopamine was involved in METH-induced increase of glutamate release, the TH was used as a selective marker to identify dopaminergic cells in our cultured hippocampal neurons (Fish et al., 2013). We found no TH-positive cells in most rat primary cultured hippocampal neurons (Fig. 6A and B). However, TH-positive cells were shown in rat cultured mesencephalic neurons (Fig. 6C and D), which was used as a TH staining positive control.

3. Discussion

Experiments in our manuscript provide evidence of significant effects of METH on excitatory synaptic transmission in primary cultured hippocampal neurons. METH (0.1–50 μM) increased the frequency and amplitude of sEPSCs, while the...
concentration of 100 μM decreased it. Furthermore, frequency of mEPSCs was enhanced by the application of METH and suggested that METH increases presynaptic membrane GLU release, which is not altered by use of D1/D5 receptor antagonist SCH23390, NMDA, AMPA, and P/Q-type calcium channel. Furthermore, N-type calcium channel is not involved in METH-mediated increase in GLU release. The current findings illustrate that the other unknown mechanism most likely involves METH-mediated increases in presynaptic GLU release in rat primary cultured hippocampal neurons.

According to prior reports, METH concentrations of 5–200 μM had no significant effects on cell toxicity, while 500 μM METH showed significant toxic effect to human neurons and astrocytes (about 50% cell death compared with control) following 24 h exposure (Abdul Muneer et al., 2011). Therefore, in our experiments we used METH concentrations between 0.1 and 100 μM. The clinical studies showed that a dose of METH 30 mg i.v. is estimated to result in a concentration of 14 μM in the human brain. METH abusers commonly inject dosages of tens to hundreds of milligrams (Fowler et al., 2008). The concentrations of METH used in these experiments (0.1–100 μM) fall within clinically relevant range.

Unlike METH-induced striatal DA release, which occurs directly via reverse transport (Panenka et al., 2013), METH-induced striatal GLU release is considered to be initiated indirectly by the activation of dopamine pathways (Sonsalla et al., 1989). However, the extracellular concentration of striatal GLU remains at elevated level for at least 24 h after METH, while METH-induced DA release is no longer present 4 h after METH consumption (Mark et al., 2007; Ricoy and Martinez, 2009). This study suggests that the other mechanism are likely involved in METH-mediated release of GLU.

Therefore, we explored the mechanism whether dopamine system was involved in METH-mediated increase of GLU release in rat primary cultured hippocampal neurons. In the present experiment, TH-positive cells (dopaminergic neurons) less than 0.2% were found in rat primary cultured hippocampal neurons from postnatal rats 10–12 h. This finding was also supported by a previous report where the survival rate of dopaminergic neurons was 90% in primary cultured ventral mesencephalon of the E14 rat fetus, while the survival rate of dopaminergic neurons was 0.1–0.25% at cultured neurons of postnatal rats 10–12 h (Shimoda et al., 1992). This could represent the reason why the release of GLU...
was enhanced by the application of METH in rat primary cultured hippocampal neurons, which was not altered by the application of dopamine receptor antagonist SCH23390. We provide the evidence that METH facilitates GLU release in rat primary cultured hippocampal neurons and the effect is independent on dopaminergic systems. The D₁ and D₅ receptors are members of the D₁-like family of DA receptors and are expressed in rat hippocampal neurons (Kalueff et al., 2007). Both of them are involved in METH-induced psychostimulation and neurotoxicity (Friend and Keefe, 2013).

Preclinical and clinical evidence recently accumulated suggest that GLU plays a pivotal role in drug self-administration, reward-related processes, drug-induced conditioned associations, and relapse (Santos-Vera et al., 2013). Dissecting
the mechanism of how METH influences the GLU-mediated synaptic transmission in neurons will help us understand the complexity of its neurotoxicity. It is known that synaptic interactions between cultured hippocampal neurons can be very strong, especially in low density cultured neurons (Wang et al., 2009; Segal and Furshpan, 1990). To electrophysiological recording, density of cultured neuron was low in our preparation. In the region of the hippocampus, the two main neural populations, pyramidal neurons and interneurons establish intricate synaptic connections (Freund and Buzsaki, 1996). Due to superimposed polysynaptic circuitry, METH increases the glutamate release and enhances the excitability of pyramidal neuron or interneuron. Although the primary cultured hippocampal neurons do not keep the same neuron circuitry when compared to hippocampal slice, the use of cultured cells to study connectivity is controversial because the endogenous three-dimensional structure of hippocampus is completely dissolved and networks regrown in a two-dimensional sheet. On the other hand, major phenomena observed in culture, such as LTP and homeostatic plasticity, have also been observed in slice and in vivo, justifying the use of cultured cells as a model system for investigating the synapse transmission (Schultz, 2002; Huang and Kandel, 1995). In a local circuitry, it could be assumed that METH enhances presynaptic pyramidal neuron glutamate release, which mediates mEPSCs and sEPSCs and their reordering in pyramidal or interneuron in our preparation. Elevating GLU synaptic transmission in local circuitry and changes in the electrophysiological properties of neurons involved in the reward circuit seem to be of utmost importance in addiction (Ernst et al., 2000). The hippocampus is a particularly suitable structure for studying the role of psychostimulants in light of its exceptional monoaminergic innervation and its support of ICSS (Ursin et al., 1966; German and Bowden, 1974). The hippocampus has a significant site for drug reward/reinforcement, most likely due to its anatomical circuit to the VTA via the NAc and VP (Lisman and Grace, 2005). Thus, glutamate synaptic transmission enhanced by application of METH in hippocampus is most likely involved in reward circuitry in vivo.

To observe the effects of METH on glutamatergic synaptic transmission in primary cultured hippocampal neurons, N-type calcium channel and P/Q-type calcium channel currents were recorded. It is established that synaptic transmission at most CNS synapses is mediated by joint actions of multiple subfamilies of the voltage-gated calcium channels, most prominently, P/Q- and N-type calcium channel (Takahashi, 2005; Takahashi and Momiyama, 1993). Presynaptic voltage-gated Ca\textsuperscript{2+} channels are activated on action potential (AP) invasion to allow for rapid influx of extracellular Ca\textsuperscript{2+}, which, in turn, triggers synaptic vesicle fusion and neurotransmitter release (Adams et al., 2003; Northrop et al., 2011). In the present study, METH had no significant effect on P/Q type Ca\textsuperscript{2+} channels. On the other hand, METH (10 μM) did not present visible effects on N-type Ca\textsuperscript{2+} channel currents, but had an inhibitory effect at a high concentration of 100 μM with an inhibitory rate of 62 ± 5.9%. Therefore, METH (100 μM) decreased the release of GLU, most likely through the inhibition of the frequency and amplitude of mEPSCs. In clinic, the use of METH at high doses produces the symptoms of hallucination, dizziness, and anxiety (Fowler et al., 2008). These symptoms are produced by blockade of N-type calcium...
channels, since N-type calcium channels inhibitors also produce the similar symptoms in clinic (Pope and Deer, 2013). Furthermore, in our study the high concentration (100 μM) of METH decreased the frequency of mEPSCs, which can likely be attributed to the effects on neuronal viability. This opinion was supported by a previous report where the high concentration of METH affected neuronal survival and direct toxicity of METH for neurons (Abdul Muneer et al., 2011). However, the detailed mechanisms are still being explored. As METH increases the frequency of mEPSCs, we believe that presynaptic mechanism involves the METH-induced phenomenon. Our findings indicate that N-type calcium and P/Q-type calcium channels are not involved in METH-enhanced release of glutamate. The previous report showed that external Ca$^{2+}$ triggers spontaneous glutamate release more weakly than evoked release in mouse neocortical neurons. Blockade of voltage-activated calcium channels has no effect on the spontaneous release rate or its dependence on [Ca$^{2+}$] (Vyleta and Smith, 2011). This is the most likely reason why N-type calcium channel currents and P/Q-type calcium channel are not involved in METH-induced release of glutamate.

sEPSCs and mEPSCs represent the GLU receptor postsynaptic response to presynaptic action potential dependent and independent release of the neurotransmitter vesicles at functional synapses, respectively. Both of them provide an index of synaptic efficacy and connectivity at a quantitative level (Liu et al., 2004). While the frequency of mEPSCs reflects presynaptic release quantitatively, the amplitude of sEPSCs and mEPSCs is affected by the amount of neurotransmitter release and sensitivity of postsynaptic receptors together. As METH enhances the amplitude of sEPSCs, we studied the direct effect of METH on NMDA and AMPA receptor-mediated currents. METH had no direct effect on NMDA and AMPA receptor-mediated currents in hippocampal neurons. Therefore, it was more likely that the increased amplitude of sEPSCs might be produced by an enhanced amount of GLU release, which induced a temporal and spatial summation of sEPSCs.

The study showed that serotonergic mechanism responsible for increase in baseline synaptic transmission is possible, since serotonin can reduce inhibition of excitatory synaptic transmission in the CA1 region of the hippocampus via activation of 5-HT1A receptors, while METH is a powerful releaser of SHT. (Steketee and Kalivas, 2011). METH is a substrate for the dopamine transporter and profoundly increases the concentration of extracellular monoamines dopamine (DA), serotonin (5-HT), and norepinephrine (NE) by redistributing these neurotransmitters from synaptic vesicles to the cytosol, in addition to inducing reverse transport and competing for transmitter uptake at their cognate transporters (Swant et al., 2010). However, the reported effects of 5-HT in the hippocampus are divergent, as some have documented inhibitory (Kimura et al., 2004; Pugliese et al., 1998; Jahnsen, 1980), while others have shown excitatory effects, or no effect on excitatory synaptic transmission (Jahnsen, 1980; Kimura et al., 2004). Therefore, the further exploration is needed in order to answer whether serotonergic changes are involved in the METH-induced effects on glutamatergic-mediated excitatory postsynaptic currents in our experiment.

Future studies will unequivocally discern whether serotonergic modulation is required for METH to increase glutamatergic synaptic transmission in rat primary cultured hippocampal neurons. Casually, the question of the exact mechanism on how METH induces GLU release remains unclear and required further investigation.

4. Conclusion

The effect of METH on glutamatergic synaptic transmission was not reversed by the pretreatment of DA receptor antagonist SCH23390. These results suggest that METH modulates presynaptic GLU release without the involvement of the dopaminergic system in rat primary cultured hippocampal neurons.

5. Materials and methods

5.1. Cultures

Wistar rats at postnatal younger than 12 h were anesthetized with ether and decapitated, in accordance with the guideline of the Beijing Institute of Biological Science Animal Research Advisory Committee (SCXK-JUN-2007-004). The experimental protocol was approved by Beijing Institute of Biological Science Animal Research Advisory Committee. Primary culture of hippocampal cells was established using a previously reported protocol (Zhang et al., 2008).

5.2. Electrophysiology

Hippocampal cultures at 10–14 days were transferred to a recording chamber placed on the stage of an inverted Olympus IX70 microscope. In most neurons, the excitatory postsynaptic currents could be recorded in whole-cell voltage clamp mode with a clamping membrane potential of −70 mV. The recording electrodes (borosilicate glass; 4–5 MΩ) were filled with an internal solution containing (in mM): Cs gluconate 130, MgCl$_2$ 2, HEPES 10, and Na$_2$ATP 2 (pH was adjusted to 7.35 with CsOH, osmolality was approximately 280 mOsm). For the mEPSCs experiment, TTX (1 μM) and bicuculline (100 μM) were added to the recording medium. For Ca$^{2+}$ current recording, 1 μM TTX, 5 mM TEA-Cl, and 2 mM 4-AP were added to the extracellular solution to block Na$^+$ and K$^+$ channels, respectively. The intracellular pipette solution contained (in mM): CsCl 140, HEPES 10, EGTA 10, TEA-Cl 5, and Na$_2$ATP 2 (pH was adjusted to 7.35 with CsOH, osmolality was about 280 mOsm) (Xia and Arai, 2011; Finnegan et al., 2005). Signals were amplified with Axopatch-1D (Axon Instruments, USA) and recorded with pClamp8.2 software (Axon Instruments, USA). Data were analyzed with the software Clampfit10.2 (Axon Instruments, USA) and Origin7.5 (OriginLab Corporation, USA).

5.3. Immunohistochemistry

The rat primary hippocampal neurons cultured on top of coverslips were washed with phosphate buffered saline (PBS) buffer and fixed with iced methanol. Cells were stained with
rabbit anti-TH antibody 1:1000 (ab41528, Abcam) overnight. After that, they were washed with PBS buffer three times for total of 30 min. The cells were then immersed in PBS buffer containing Cy3-conjugated donkey anti-rabbit antibody (1:300; Jackson ImmunoResearch Laboratories Inc.) for 60 min. Thereafter, cells were washed three times with PBS for total of 60 min. Coverslips were then mounted on slides and pictures were captured with fluorescence microscope (Olympus, Japan) and analyzed using Adobe Photoshop version 7.

5.4. Drugs

TTX, bicuculline, CNQX, verapamil, MVIIA, and ω-agatoxin IVA were purchased from Sigma (St Louis, MO, USA). TTX was dissolved in aqueous sodium acetate buffer (pH 4.5). Bicuculline and CNQX were dissolved in DMSO. Verapamil, MVIIA, and ω-agatoxin IVA were dissolved in dH2O. All drugs were stocked at a high concentration and stored at –20°C. They were dissolved in the extracellular solution for appropriate concentration just before administration.

5.5. Statistical analysis

Original current traces were analyzed using the Clampfit 10.2 software (Molecular Devices, UK). All curve fittings were carried out using Origin 7.5 (OriginLab Corporation, USA). All data were reported as mean ± SEM. The nonparametric Kolmogorov–Smirnov test was used to compare amplitudes and frequencies of mEPSCs and sEPSCs between two experimental groups. The level of significance was set at p < 0.05.

Disclosure

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Conflict of Interest

The authors declare no conflict of interest.

REFERENCE


