**Administration of activated glial condition medium in the nucleus accumbens
extended extinction and intensified reinstatement of methamphetamine-induced
conditioned place preference**

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Abstract

Methamphetamine (METH) is a psychostimulant drug with significant abuse potential and neurotoxic effects. A high percentage of users relapse to use after detoxification and no effective medication has been developed for treatment of METH addiction. Developing evidences indicated the role of glial cells in drugs abused related phenomena. However, little is known about the role of these cells in the maintenance and reinstatement of METH-seeking behaviors. Therefore, the current study was conducted to clarify the role of glial cells in the maintenance and reinstatement of METH-induced conditioned place preference (CPP) in rats. Astrocyte condition medium (ACM) and neuroglia conditioned medium (NCM) are liquid mediums prepared from primary astrocyte and neuroglia cells. These mediums seem to contain many factors that release by glia cells. CPP was induced by systemic administration of METH (1 mg/kg for 5 days, s.c.). Following the establishment of CPP, the rats were given daily bilateral injections (0.5 μ l/side) of either vehicle, ACM or NCM into the nucleus accumbens (NAc) and then were tested for the maintenance and reinstatement. Intra-NAc administration of ACM treated with METH, could extend the extinction period and also, intensified the magnitude of METH reinstatement. Furthermore, intra-accumbal administration of NCM treated with METH notably delayed the extinction period by four days and significantly increased the magnitude of CPP score in the reinstatement phase compared to the post-test phase. Collectively, these findings suggested that activation of glial cells may be involved in the maintenance and reinstatement of METH-seeking behaviors. It provides new evidence that glia cells might be considered as a potential target for the treatment of METH addiction.

Keywords: Reward; Conditioned place preference; Reinstatement; Nucleus accumbens; Glial cell; Methamphetamine

1. Introduction

Methamphetamine (METH) is a neurotoxic psychostimulant with highly addictive potential (Panenka et al., 2013). Psychosis, overdose and dependence are the major symptoms and disorders related to chronic use of METH. In addition, a high percentage of users relapse to drug taking after detoxification (Panenka et al., 2013). Unfortunately, no effective medication has been developed for treatment of METH addiction and symptoms associated with that (Gonzales et al., 2010; Ling et al., 2006).

Long-term exposure to psychostimulants can induce neuronal plasticity (Hyman and Malenka, 2001; Nestler, 2001). Alterations in neuronal structure, biochemistry and function in brain areas that are related to reward processing including medial prefrontal cortex (mPFC), nucleus accumbens (NAc), limbic structures and ventral tegmental area (VTA) are considered the base for the initiation, maintenance and also physiopathological mechanisms of drug addiction (Miguel-Hidalgo, 2009). The NAc by receiving dopaminergic projections from the VTA and glutamatergic projection from the mPFC play a crucial role in drug reward related phenomena as well as maintenance and reinstatement (Hsieh et al., 2014; Rocha and Kalivas, 2010). In addition, it has been shown that serotonergic systems play a critical role in establishment of drug use-associated behaviors and transition from METH use to addiction (Müller and Homberg, 2015)

Astrocytes exert a number of important functions during development of central nerve system (CNS). In the mature brain astrocytes perform a range of activities including maintenance of the blood-brain barrier, regulation of cerebral blood flow and CNS metabolism, involvement in synaptic transmission, neurotransmitter metabolism

and turnover, glycogen storage and also regulation of extracellular pH and K⁺ (Jensen et al., 2013; Sofroniew and Vinters, 2010).

Over the past decade accumulating evidence has demonstrated new critical roles for glial cells in neural function and their involvement in many diseases and disorders (Heneka et al., 2009). In addition to their well-known roles, glial cells by release of chemokines and neurotrophic factors can affect neuronal function (Aloisi, 2001; Dong and Benveniste, 2001). Furthermore, glial cells including astrocytes and microglia undertake a process of activation, proliferation and morphological changes as a consequence of many stimuli and disorders such as drug abuse (Kovacs, 2012; Narita et al., 2006).

Developing evidence has indicated the role of glial cells in drug reward related phenomena. In a study by Johnson *et al.* they showed that chronic morphine treatment increased expression of glial fibrillary acidic protein (GFAP), a specific marker of astrocytes, in the VTA (Johnson et al., 1993). Also it has been shown that glial activation is involved in opioid analgesia, reward and dependence (Hutchinson et al., 2007). Repeated amphetamine treatment increases the level of GFAP in caudate putamen (Armstrong et al., 2004). In addition, in a clinical study it has been shown that astrocyte and microglia changed in brain of METH users (Kitamura et al., 2009).

Astrocyte condition medium (ACM) and neuroglia conditioned medium (NCM) are liquid mediums prepared from primary astrocyte and neuroglia cell culture respectively. Many stimuli can activate glia cells and induce the release of many factors including proinflammatory cytokines from them (John et al., 2003; Thomas et al., 2004a). It seems ACM and NCM, particularly their activated forms contain these factors.

Recently, we showed that intra-NAc administration of NCM can prolong the extinction

period and also increases the magnitude of morphine reinstatement (Arezoomandan et al., 2015). Another study by Narita *et al.* showed that chronic administration of METH significantly increased the GFAP expression in the NAc and cingulate cortex (CG). Furthermore, they showed that intra-NAc administration of ACM intensified the rewarding effects induced by METH and morphine (Narita et al., 2006). Many studies have mentioned that modulation of glia activation could attenuate behavioral effects induced by METH (Fujita et al., 2012; Narita et al., 2006; Zhang et al., 2006). In our previous studies we reported that intra-NAc administration of glia modulator, minocycline, attenuated the maintenance and blocked the drug-induced reinstatement of METH-CPP (Attarzadeh-Yazdi et al., 2014). Also, glial cell modulator, ibudilast, could attenuate drug- and cue-induced reinstatement of METH- CPP (Beardsley et al., 2010). Altogether, these data imply that glial cell activation may contribute to the synaptic plasticity and behavioral changes during the acquisition, extinction and reinstatement of METH. Therefore, in the present study the involvement of glial cells in the maintenance and reinstatement of METH was investigated using conditioned place preference (CPP) paradigm in rats.

2. Materials and Methods

2.1. Animals

The experiments were carried out on male adult Wistar rats weighing 250–300 g (Pasteur Institute, Tehran, Iran). Animals were acclimated to the vivarium (a climate-controlled environment on a 12h light/dark cycle), for at least one week prior to the onset of the experiments. The animals were randomly assigned to different experimental

groups, habituated to their new environment and handled for one week before the experimental procedure was started. Each experimental group consisted of 5–7 animals. The tests were done between 9:00 a.m. and 2:00 p.m. Animal care procedures were conducted in accordance to the guide for the care and use of laboratory animals (National Institutes of Health Publication No. 80-23, revised 1996) and were conducted with the approval of an institutional animal care and use committee at the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences. Additionally, all efforts were made to minimize animals' suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Tissue Processing

Primary cells were cultured using cerebral hemispheres of neonatal Wistar rat (1-2 days old). Briefly, the brains were removed and meninges and blood vessels were cleaned under a dissection microscope. After mechanical dissociation, tissues were treated with trypsin (0.025%, Gibco, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteinemonohydrate (Sigma-Aldrich, USA), 0.5% glucose, and 0.02% bovine serum albumin (Gibco, USA) at 37 °C for 15 min. Then, the cells were placed in a flask containing Dulbecco's Modified Eagle Medium (DMEM) at a concentration of 15×10^6 cells and incubated at 37°C in a moist 5% CO₂, 95% air atmosphere for 48-72 hours. DMEM is a medium that contains glucose, amino acids, vitamins and additional supplementary components. The medium was changed every other day until cells reached an appropriate confluency and could be taken for harvesting different cell populations (Narita et al., 2006; Skaper et al., 2012).

To prepare cortical neuroglia conditioned medium (NCM), the cultures were incubated for 5 days. After reaching to appropriate confluency, the cells moved to the other flasks and after reaching appropriate confluency, the cells were washed with Dulbecco's Modified Eagle Medium (DMEM) and covered with an equal volume of serum-free medium for 3 days at 37° C and 5% CO₂ in the presence of the 10 or 100 µM METH. After 3 consequence days' treatments with METH, supernatant was and centrifuged for 10 min at 1000 rpm. The final supernatant was used as NCM.

Purified cortical astrocyte was grown as follows: after seeding and reaching an appropriate confluency, the cells were washed and the medium was changed completely. To separate the microglia and oligodendrocytes, the flasks were shaken at 180 rpm for 12h. Contents of shaken flasks were aseptically poured into a new flask at density of 1×10^5 cells/cm³ in DMEM supplemented with 5% FBS, 5% horse serum (HS), 10 U/ml penicillin and 10 mg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Then, the cells were identified by immunofluorescence using mouse anti-GFAP antibody.

The astrocyte-conditioned medium (ACM) used in experimental procedure was prepared as follow; after reaching confluency, the cells were washed with DMEM and then covered with an equal volume of serum-free medium in the presence of the METH 10 or 100 µM treatment and were incubated for 3 days at 37° C and 5% CO₂. The supernatant was collected and centrifuged 10 min at 1000 rpm. The final supernatant was used as ACM.

2.3. Immunocytochemistry

After seeding in eight-well chamber slides, astrocyte cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then rinsed with PBS. Then, cells were permeabilized with 0.2% BSA/0.1% Triton X-100 solution for 30 min and then incubated overnight in a 1:100 dilution of GFAP antibody (Chemicon, International Inc.). After washing in PBC containing Tween 20 (PBST), the cover-slips were incubated for 1h with FITC-conjugated goat anti-rabbit IgG antibody (1: 4000) at room temperature. For counterstaining, the cells were incubated with propidium iodide (10µg/ml) for 30 min. Finally, cells were washed three times in PBST, and mounted to slides. The cells were then visualized (Fig. 1) under a fluorescence microscope (Olympus IX, Japan).

2.4. Stereotaxic surgery

Animals were anesthetized with intraperitoneal (i.p.) injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) placed into the stereotaxic apparatus (Stoelting, USA). Lidocaine with epinephrine (0.2 ml) was injected in several locations around the area that the incision was going to be made. The incision was made along the midline to expose the cranium, the area surrounding bregma was cleaned and dried. Then by using stereotaxic coordinates for the NAc (Paxinos and Watson, 2006) as follows: AP = 1.6 ± 0.15 mm anterior to the bregma; ML = ± 1.4 mm; DV = 6.7 mm ventral from the skull surface, two cannulae (11 mm in length, 23- gauge) were implanted 1 mm above this area on both sides. The guide cannulae were secured in place using two stainless steel screws anchored to the skull and dental acrylic cement. To prevent clogging and infection, stainless steel obturators were inserted into the guide cannulae. After the

cement completely dried, animals were individually housed and allowed to recover for 5-7 days before experiments.

2.5. Infusion procedures

Microinjections were performed by lowering a stainless steel injector cannula (30-gauge needle) with a length of 1-mm longer than the guide cannulae in the NAc. The injector cannula was connected to a 1- μ l Hamilton syringe by polyethylene tubing (PE-20). All drugs solution or vehicles were slowly administered over a period of 60s into the nuclei. Needles were left in place for an additional 60s to facilitate diffusion of the drugs and prevent drug backflow and then the stylets were reinserted into the guide cannulae. During the infusion procedure, the animals could freely move in a cage and all efforts were made to reduce stress of the animals.

2.6. Conditioning place preference apparatus and paradigm

The CPP procedure is used to evaluate the reinforcing effects of natural and pharmacological stimuli, including rewarding or aversive effects of drugs in animals (Huston et al., 2014) (Supplementary Fig. 1). This procedure has been described in our previous works in detail (Attarzadeh-Yazdi et al., 2014). Briefly, CPP studies were conducted using a three-compartment apparatus, two compartments were identical in size (30cm $\times$ 30cm $\times$ 40cm) but differed in shading and texture. The third and middle compartment (start box) was a red open alley (30cm $\times$ 15cm $\times$ 40cm) connecting the two preference compartments. This paradigm took place in seven continuous days and consisted of three phases: pre-conditioning, conditioning and post-conditioning phases.

Pre-conditioning phase: In Pre-conditioning test, each animal was placed in the start box with the guillotine door removed and the rats were allowed to move freely in all three chambers for 10 min. Animal movement and time spent in each compartment was recorded using a 3CCD camera (Panasonic Inc., Japan) and locomotion tracking was measured by Ethovision software (Version 7), a video tracking system for automation of behavioral experiments (Noldus Information Technology, the Netherlands). In the experimental setup used in this study, the animals should not show any preference for either of the compartments. The animals that spent $\geq 70\%$ of the total test time in each compartment were considered to have initial bias and were excluded from the study. From the fifty five rats used in this study, nine were excluded because of showing bias in pre-test day.

Conditioning phase: Conditioning phase started one day after pre-conditioning test and consist of a 5-day schedule. During the conditioning phase, the rats were injected with METH subcutaneously (1mg/kg) in the morning and immediately confined to the drug-paired compartment for 30 min; about 6 h later, the rats were injected with saline as a vehicle and immediately placed in the saline-paired compartment for 30 min. On the next day, the animals were injected with saline in the morning and METH in the afternoon. The injection schedule of the third day of conditioning was the same as the first day. This procedure was repeated until day six. Control animals received only saline instead of METH.

Post-conditioning phase: On the post-test day, in a METH free state, rats were placed into the CPP box and were tested for CPP with free access to all compartments. The time spent in each chamber during the 10-min period was recorded and analyzed using the Ethovision software. The time spent in the METH-paired compartment minus time

spent in the saline-paired compartment was considered as conditioning scores (CPP scores). Total distance traveled by each animal was also recorded in control and experimental groups.

2.7. Maintenance (Extinction) experiment

Following establishment of CPP, the rats were given daily bilateral injection of either drugs or vehicle into the NAc (0.5µl) in their home cages. Every day 60 min after injection, rats were placed in the CPP box and tested for CPP. This procedure was repeated for each rat in the control and experimental groups until the calculated CPP score in two consecutive days in extinction period became similar to those in the pre-conditioning day (the CPP score reached its level on the pre-conditioning day for two consecutive days) (Attarzadeh-Yazdi et al., 2014).

2.8. Reinstatement

For reinstating the METH-seeking behavior, one day after the last extinction day, the rats received drugs or vehicle 0.5µl into the NAc and 60 min after microinjection; they received a priming injection of METH (0.5 mg/kg, s.c.). Then the animals were immediately placed in the start box with access to the entire apparatus for 10 min and the time spent and distance traveled for each compartment were measured.

2.9. Experimental design

2.9.1. Time course of the acquisition, maintenance and reinstatement of METH-induced conditioned place preference

Following the acquisition of METH CPP and during the extinction period, in sham-operated group without any injection and in control group with daily microinjection of DMEM (0.5µl/NAc), the animals were tested for CPP every day (Table 1). This procedure was repeated for each animal until complete extinction was acquired (the CPP score reached its level on the pre-conditioning day for two consecutive days). For reinstating the METH seeking behavior, one day after the last extinction trial, rats received a priming injection of METH (0.5 mg/kg; s.c.), and were immediately tested for CPP. The time spent in each compartment and the distance traveled were measured (Supplementary Fig. 1).

2.9.2. Effect of Intra-accumbal administration of ACM during the extinction period on the maintenance and reinstatement of METH-induced conditioned place preference

To investigate the role of astrocyte-related soluble factors on the maintenance and drug-induced reinstatement of METH, after the acquisition of CPP and during extinction period, animals were given daily bilateral intra-NAc administration of ACM, METH 10-ACM and METH 100-ACM (0.5 µl/ NAc) in their home cage. Sixty min after each injection, the rats were tested for CPP and the CPP scores were measured. After extinction was established, on reinstatement day, 60 min after the drug administration, the priming dose of METH (0.5 mg/kg; i.p.) was injected (Table 1) and

immediately the rats were placed in the CPP box and the time spent and distance traveled were recorded for 10-min period (Supplementary Fig. 1).

2.9.3. Effect of intra-accumbal administration of NCM during the extinction period on the maintenance and reinstatement of METH-induced conditioned place preference

To investigate the role of glia activation on the maintenance and reinstatement of METH, following the induction of CPP, rats received daily bilateral intra-NAc administration of NCM, METH 10-NCM and METH 100-NCM (0.5 µl/ NAc) in their home cage. One hour after each injection, the rats were tested for CPP. After the last extinction day, on the reinstatement day, 60 min after the drug injection, the priming dose of METH (0.5 mg/kg; s.c.) was injected (Table 1) and rats were placed in the CPP box and were tested for reinstatement (Supplementary Fig. 1).

2.10. Histology

At the end of the experiments, for confirmation of the appropriate placements of cannulae tips in the rat brains, the animals were deeply anesthetized with Ketamine and Xylazine. Then, they were transcardially perfused with 0.9% saline and 10% formalin solution. The brains were removed, fixed in 10% formalin solution for two days. Then, the brains were coronally cut in 50 µm sections and cannula placements were assessed by light microscopy. Each dot in Fig. 2 represents the approximate placements of the cannulae in the NAc. The neuroanatomical locations of cannula tips were confirmed using Paxinos and Watson rat brain atlas (Paxinos and Watson, 2006). Only the animals

with correct cannula placements in the NAc were included in the data analysis. Totally five rats were excluded from the study because of cannula misplacement (Fig. 2).

2.11. Statistical analysis

The CPP score represents the differences between the time spent in the drug-paired compartment and the time spent in the saline-paired compartment on the test day, and is expressed as mean $\pm$ SEM (standard error of mean). Data were processed by commercially available software GraphPad Prism® 5.0. In order to compare the CPP scores and distance traveled obtained in all groups (vehicle and experimental groups). A statistical analysis for place conditioning study was performed using one-way ANOVA followed by post-*hoc* analysis (Dunnett's or Newman-Keuls test) or Student's t-test (for two-paired comparison). *P*-values less than 0.05 ($P < 0.05$) were considered to be statistically significant.

3. Results

3.1. Time course of the acquisition, maintenance and reinstatement of METH-induced conditioned place preference

Subcutaneous injection of METH (1 mg/kg) during 5 conditioning days induced significant preference ($P < 0.001$) for the METH-paired chamber in comparison with saline-paired chamber. During the extinction period, without any injection, the CPP score was calculated every day. The CPP induction by METH gradually diminished over days and the time spent in METH-paired chamber did not differ from the saline one by tenth and eleventh extinction days. After the last extinction day, the animals

were tested for reinstatement. Subcutaneous injection of METH priming dose (0.5 mg/kg) could induced reinstatement and CPP score on reinstatement day significantly increased compare to pre-test phase (Supplementary Fig. 2)

In another experimental group, following induction of CPP, in the home cage DMEM, as a vehicle, was injected into the NAc and 60 min after each injection the animals were tested for CPP. As it can be seen in Fig. 3 similar to the sham group (Supplementary Fig. 2), extinction occurred on tenth and eleventh days. Also DMEM could not affect the reinstatement and there was no significant difference between reinstatement and post- test CPP score [$F(13, 83) = 12.73, P < 0.0001$].

3.2. Intra-accumbal administration of ACM prolongs the extinction period and increases the magnitude of METH reinstatement

To investigate the involvement of astrocyte activation in the maintenance and drug-induced reinstatement of METH, after acquisition of CPP and during extinction period, ACM was injected into the NAc. Administration of ACM and METH 10-ACM failed to change the extinction period and similar to control group, the preference for METH- paired compartment was extinguished on tenth and eleventh extinction days [$F(13, 97) = 11.92, P < 0.0001$], [$F(13, 69) = 13.01, P < 0.0001$]. Also, this treatment could not change the reinstatement CPP score in comparison with post-test (Fig. 4A and 4B).

Next, we examined the role of activated astrocyte-conditioned medium in the maintenance and reinstatement. Intra-NAc administration of METH 100-ACM prolonged the extinction period by three days and extinction occurred on the thirteenth and fourteenth days [$F(9, 69) = 8.28, P < 0.0001$] (Fig. 4C). In addition, the results showed this treatment could significantly intensify the reinstatement compare to the

control group and there was a significant increase in the percentage changes of CPP score in reinstatement phase compared to post-test phase [$t(12) = 5.045$, $P = 0.003$] (Fig. 4C and 6). These findings revealed that intra-NAc injection of activated astrocyte-related soluble factors could delay extinction period and also increase the magnitude of drug-induced reinstatement of METH, suggesting that activation of astrocytes may be implicated in the maintenance and reinstatement of METH.

3.3. Microinjection of NCM into NAc extends the extinction period and increases the magnitude of METH reinstatement

To examine the effect of NAc-neuroglia activation on the METH maintenance and drug-induced reinstatement, during extinction period, NCM was daily injected to the NAc. Administration of NCM failed to affect the maintenance and reinstatement compare to control group (Fig. 5A). Administration of METH 10-NCM and METH 100-NCM clearly prolonged the extinction period and intensified the reinstatement. In animals in which METH 10-NCM was injected into NAc, extinction occurred on eleventh and twelfth [$F(9, 69) = 8.28$, $P < 0.0001$, Fig. 5B], also this treatment could increase the magnitude of reinstatement in comparison to post-test day [$t(8) = 2.648$, $P = 0.028$, Fig. 6].

The ANOVA revealed, intra-NAc administration of METH 100-NCM dramatically affected the extinction period and reinstatement of METH [$F(11, 71) = 5.138$, $P < 0.0001$]. As it shows in Fig. 5C METH 100-NCM prolonged the extinction by four days and animals extinguished their preference on fourteenth and fifteenth days. On the other hand in these animals CPP score significantly increased on reinstatement phase compared to post-test day [$t(8) = 7.225$, $P < 0.001$] (Figs. 5C and 6). These results

suggest that neuroglia cells may be directly implicated in the maintenance and reinstatement of METH-seeking behavior.

3.4. Different treatments had no effect on locomotor activity

CPP score can be modified by locomotor effects of the drugs and treatments. To address that, total distance traveled of all rats in each experimental group were tested and then compared to each other. Results indicated that there were no significant differences in locomotor activity during acquisition, extinction and reinstatement phases in any of experimental group.

4. Discussion

In this study we investigated the possible roles of glial cells in the NAc in the maintenance and reinstatement of METH-induced CPP. We found that administration of astrocyte and neuroglia conditioned medium treated with METH into the NAc during the extinction period extended the maintenance of METH-induced CPP and also increased the magnitude of METH reinstatement.

Astrocytes are the most numerous glial cells in the mature mammalian's brain (Miguel-Hidalgo, 2009). In addition to their well-established supportive roles in neural function, recently several lines of evidence have proved that astrocytes are involved in impulse conduction, neurotransmitters turnover, formation of synaptic connections and also exert a modulatory action on synaptic functions (Perea and Araque, 2002; Volterra and Meldolesi, 2005). Recently, accumulative line of evidence has mentioned that chronic administration of psychostimulants induces activation of astrocytes in humans,

rats and mice (Kitamura et al., 2010; Miguel-Hidalgo, 2009; Narita et al., 2008). Chronic exposure to psychostimulants including cocaine and METH activates astrocytes and increases the GFAP expression in brain areas that are related to reward such as VTA, NAc, PFC, caudate putamen, locus coeruleus and CG cortex (Armstrong et al., 2004; Fattore et al., 2002; Narita et al., 2006). GFAP is an intermediate-filament protein that is highly specific for astrocytes (Sofroniew et al., 2010). In cell culture, METH induced astrocytic activation in a dose- dependent manner that is in agreement with behavioral studies showing that sensitization to METH-induced hyperlocomotion lasted until two months after the cessation of METH treatment (Narita et al., 2005). Narita *et al.* showed that intra-NAc and -CG administration of ACM amplified the rewarding effects of METH and morphine (Narita et al., 2006). In addition, in our pervious study we indicated that intra-accumbal injection of ACM during the extinction period increased the magnitude of drug-induced reinstatement of morphine (Arezoomandan et al., 2015). In the current study, we showed that intra-NAc injection of METH 100-ACM, but not ACM, prolonged the extinction and reinstatement of METH. It seems that METH can activate and probably stimulate release of inflammatory factors from astrocytes. In this regard, METH 100-ACM contains the cocktail of factors that can affect reward phenomena. It's suggested that injection of these factors into the NAc after cessation of METH can mimic the effects of astrocyte activation in the brain and it may be involved in extending the maintenance and potentiation of METH reinstatement.

In addition, based on differences in cellular morphologies and anatomical locations, astrocytes can be divided into two main subtypes: protoplasmic or fibrous (Jensen et al., 2013). Although these two kinds of astrocytes exhibit many functions in common, there are distinct differences between them (Chaboub and Deneen, 2012).

While the involvement of astrocytes in reward related phenomena has been proved, this field is still growing and the different roles of protoplasmic or fibrous astrocytes in these phenomena has not been reported yet (Narita et al., 2008; Hutchinson et al., 2007; Miguel-Hidalgo, 2009; Narita et al., 2006) and future works will address that.

In another part of the study, we examined the effect of intra-NAc administration of glia-related soluble factors on the maintenance and reinstatement of METH-induced CPP. We found that microinjection of NCM into the NAc dramatically extended the extinction period and increased the magnitude of reinstatement induced by METH. It is clearly known that NAc is one of the most critical areas that are involved in rewarding behaviors induced by drug abuse. These findings further suggested that activation of glial cells in the NAc might be involved in the maintenance and reinstatement of METH-induced CPP. In response to various signals such as drug abuse, at least three functionally and morphologically distinct forms of microglia can be activated and various kinds of cytokines or neurotrophic factors may be released (Kovacs, 2012; Olah et al., 2011).

It has been shown that METH treatment caused microglia activation and increased the expression of their marker in purified cell culture (Narita et al., 2006). Treatment with METH for 3 days induced functional changes and astrocytic activation in cortical neuron/glia co-cultures in a dose-dependent manner (Narita et al., 2005). In addition, chronic METH administration activated microglia cells in the brain areas that are related to reward processing (Thomas et al., 2004a). Also, in a clinical study, Yoshimoto *et al.* showed that chronic administration of METH could cause microglia activation in the different brain areas of METH abusers (Sekine et al., 2008). In our previous study we demonstrated that intra-NAc injection of NCM prolonged the

extinction period and also significantly increased the magnitude of reinstatement induced by morphine (Arezoomandan et al., 2015). In addition, several lines of evidence have proved that modulation of glial cells by pharmacological agents could block or modulate the behavioral effects induced by repeated exposure to morphine, METH and cocaine (Beardsley et al., 2014; Beardsley et al., 2010; Chen et al., 2009; Narita et al., 2006; Snider et al., 2013). A recent study demonstrated that treatment with a glial cell modulator, AV411 (ibudilast), significantly reduced the rewarding behaviors induced by METH (Snider et al., 2013). Also, administration of AV411 could attenuate drug and stress-induced METH relapse (Beardsley et al., 2010). We have indicated in our recent studies that treatment with a glia cell modulators, minocycline, during the extinction period attenuated the maintenance and reinstatement of morphine and METH-seeking behavior in rat (Attarzadeh-Yazdi et al., 2014; Arezoomandan and Haghparast., 2015).

Glial cells can affect neuronal function by release of cytokines, chemokines and also involvement in neurotransmitters turnover. Under the physiological conditions, glial cells are not activated but many stimuli can activate them and induce the release of proinflammatory cytokines from these cells (John et al., 2003; Thomas et al., 2004a). In addition, many studies have shown that glia activation occurs in response to drug abuse (Guilarte et al., 2003; Hutchinson et al., 2007; Thomas et al., 2004b). As a consequence of activation, glial cells can release an array of factors known that they are implicated in reward related phenomena. In our study, glial cells were treated by METH and in this regard, glial cells conditioned medium contains a cocktail of cytokines and growth factors that, at least in part, are implicated in the drug related behaviors.

Brain derived neurotrophic factor (BDNF) which is mainly produced by astrocytes is implicated in neuronal survival and plasticity. In addition, several line of

evidence has suggested the role of BDNF in addictive behaviors. In METH users, the plasma BDNF concentration was significantly higher compared to the control group (Kim et al., 2005). The expression of BDNF mRNA in the mPFC increased followed by D-amphetamine-primed reinstatement in CPP paradigm (Shen et al., 2014). Furthermore, inhibition of BDNF activity or blockage of its TrkB receptor could modulate dopamine release induced by METH administration (Narita et al., 2005). Ghitza *et al.* showed that intra-NAc administration of BDNF increased cocaine self-administration, extinction and drug-induced reinstatement (Ghitza et al., 2010). Many studies have documented the direct role of astrocyte and microglia in release of IL-1, 6 and 10, IL- β , TNF- α (Kovacs, 2012; Narita et al., 2005). TNF- α and IL-1 β are produced by microglia and drug abuse such as morphine and METH can induce the generation of these factors in the NAc, striatum, hippocampus and amygdala (Niwa et al., 2007). It has been shown that IL-6 is involved in the rewarding effect induced by cocaine and conditioning with cocaine for three days increased IL-6 level in NAc and mPFC (Lin et al., 2011). Also, Goncalves *et al.* reported that the expression of TNF- α and IL-6 increased as a consequence of METH injection in mouse brain (Goncalves et al., 2008).

Under different conditions astrocytes and microglia can express both constitutive and inducible nitric oxide synthase (NOS), the enzyme that produces nitric oxide (NO) (Murphy et al., 1993). Furthermore, cytokines can upregulate iNOS expression (Nandi et al., 2010). Many studies have documented that NO synthesis is involved in the expression of behavioral effects of METH and also, can affect mesolimbic dopamine system. It has been demonstrated that pretreatment with 7-nitroindazole, a NO inhibitor, attenuated the reinstatement of METH (Li et al., 2002a). Furthermore, the administration of 7-nitroindazole before METH treatment could

significantly attenuate the acquisition and expression of METH-induced CPP (Li et al., 2002b). Also, administration of L-NAME, a nitric oxide synthesis inhibitor, before METH injection decreased the level of motor activity and locomotion stereotypy rating induced by METH (Abekawa et al., 1994).

Collectively, it seems that METH treatment causes activation of glia cells in brain areas that are related to reward phenomena (Fattore et al., 2002; Narita et al., 2006; Thomas et al., 2004a). As a consequence of glia activation, these cells can release an array of chemokines which, at least in part, have a critical role in neuron–glia interaction during the acquisition of drug abuse rewarding effects (Narita et al., 2006). On the other hand, it seems that activation of glial cells (Zhang et al., 2012) and perhaps release of chemokines factors remain elevated after the cessation of drug treatment. These findings suggest that glia activation and sustained elevation of these factors after cessation of METH treatment may be implicated in the extinction period and drug-induced reinstatement. In conclusion, our results showed that administration of activated glia-related soluble factors in the NAc can prolong the extinction period and increases the magnitude of drug-induced reinstatement. These findings are in agreement with our previous and other studies which indicated that activation of glial cells during development of rewarding effects, at least partially, is implicated in the maintenance and reinstatement of drug abused. Altogether, these findings suggest that glial cells might be considered as a potential target for therapy of METH addiction.

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Figure legends

Fig. 1. A representative image **A**: the neuroglia cell culture, **B**: the purified astrocyte cell culture stained with anti-GFAP antibody using immunofluorescence method.

Fig. 2. Representative images showing the microinjection sites in the Nucleus accumbens (○ Vehicle; ● Treatment; ▲ Misplacement). **aca**, anterior commissure, anterior part; **AcbC**, Accumbens nucleus, Core; **AcbSh**, Accumbens nucleus, Shell; **cc**, Corpus callosum; **CPu**, Caudate putamen (striatum); **DEn**, Dorsal endopiriform nucleus; **LV**, Lateral ventricle; **VP**, Ventral pallidum.

Fig. 3. Intra-NAc injection of DMEM failed to change the maintenance and reinstatement of METH in comparison with control group. It can be seen that extinction occurred on the tenth and eleventh days and there was no significant difference between CPP score in post- test and reinstatement phase. Each column represents the mean± SEM of 6 rats.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with pre-test day

⁺⁺⁺ $P < 0.001$ compared with last day of extinction period

Fig. 4. The effects of bilateral intra-NAc administration of astrocyte-conditioned medium (ACM) during extinction period on the maintenance and reinstatement of METH-induced CPP. **(A)** Injection of ACM failed to change the maintenance and reinstatement of METH-induced CPP. **(B)** Administration of METH (10 mM, 3 days)

treated conditioned medium (METH 10-ACM) could not change the maintenance and reinstatement of METH. (C) Intra-NAc injection of METH 100-ACM could prolong the extinction period by three days and extinction occurred on the thirteenth and fourteenth days. Also, METH 100-ACM significantly intensified the prim-induced reinstatement. It can be seen and that there was a significant increase in reinstatement CPP score in comparison with post- test day. Each column represents the mean $\pm$ SEM of 5-7 rats.

* $P<0.05$ ** $P<0.01$ and *** $P<0.001$ compared with pre-test day

+++ $P<0.01$ compared with the last day of extinction period

Fig. 5. The effects of administration of Neuroglia-conditioned medium (NCM) into the NAc on the maintenance and reinstatement of METH. (A) Intra- NAc administration of NCM failed to change maintenance and reinstatement of METH-induced CPP. (B) Injection of METH10-NCM prolonged the extinction period by one day. Also this treatment could intensify the METH reinstatement and there was a significant difference between CPP score in the reinstatement and post-test phase. (C) Administration of METH100-NCM prolonged the extinction period by four days in comparison with control group. Also, METH100-NCM could significantly intensify the reinstatement and there was a significant increase in CPP score compared with post- test day. Each column represents the mean $\pm$ SEM of 5 rats.

** $P<0.01$ and *** $P<0.001$ compared with pre-test day

+++ $P<0.001$ compared with last day of extinction period

Fig. 6. The effect of intra-NAc administration of ACM and NCM during the extinction period on the reinstatement induced by priming injection of METH. The figure indicates the percentage changes of post-test to pre- test and reinstatement to pre- test CPP score in different treatment groups. It can be seen that METH 100- ACM, METH 10- NCM and METH 100- NCM could significantly increase the magnitude of reinstatement CPP score.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to percentage changes of post-test to pre- test CPP score

Table 1. The different groups were used in this study. In all groups during conditioning period the animals were injected with METH (1 mg/kg, SC) for five days. After conditioning period and induction of condition place preference the rats were given either daily bilateral injection of treatments or vehicle into NAc (0.5µl) in the home cage. One day after the last extinction trial, the rats received drug or vehicle 0.5 µl/NAc. 60 min after microinjection, all rats received a priming injection of METH (0.5 mg/kg, SC).

Groups	Conditioning period (five days)	Extinction period	Reinstatement day
Sham group N= 7	SC injection of 1 mg/kg METH	-	SC injection of 0.5 mg/kg METH
Control group N=6	SC injection of 1 mg/kg METH	Daily intra- NAc injection of DMEM (0.5 µl/rat)	intra- NAc injection of DMEM; after 60 min SC injection of 0.5 mg/kg METH
ACM groups N=5-7	SC injection of 1 mg/kg METH	Daily intra- NAc injection of ACM (0.5 µl/rat)	intra- NAc injection of ACM; after 60 min SC injection of 0.5 mg/kg METH
	SC injection of 1 mg/kg METH	Daily intra- NAc injection of METH 10- ACM (0.5 µl/rat)	intra- NAc injection of METH 10- ACM; after 60 min SC injection of 0.5 mg/kg METH
	SC injection of 1 mg/kg METH	Daily intra- NAc injection of METH 100- ACM (0.5 µl/rat)	intra- NAc injection of METH 100- ACM; after 60 min SC injection of 0.5 mg/kg METH
NCM groups N=5	SC injection of 1 mg/kg METH	Daily intra- NAc injection of NCM (0.5 µl/rat)	intra- NAc injection of NCM; after 60 min SC injection of 0.5 mg/kg METH
	SC injection of 1 mg/kg METH	Daily intra- NAc injection of METH 10- NCM (0.5 µl/rat)	intra- NAc injection of METH 10- NCM; after 60 min SC injection of 0.5 mg/kg METH
	SC injection of 1 mg/kg METH	Daily intra- NAc injection of METH 100- NCM (0.5 µl/rat)	intra- NAc injection of METH 100- NCM; after 60 min SC injection of 0.5 mg/kg METH

ACM: Astrocyte-conditioned medium; **DMEM:** Dulbecco's modified Eagle's medium;
METH 10- ACM: ACM treated with METH (10 μ M); **METH 100- ACM:** ACM treated
with METH (100 μ M); **NCM:** Neuroglia conditioned medium; **METH 10- NCM:** NCM
treated with METH (10 μ M); **METH 100- NCM:** NCM treated with METH (100 μ M);
METH: Methamphetamine; **NAc:** Nucleus accumbens; **SC:** Subcutaneous