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GLUTAMATE TRANSPORTER TYPE 3 PARTICIPATES IN MAINTAINING MORPHINE-INDUCED CONDITIONED PLACE PREFERENCE

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Abstract—Glutamate transporters (EAAT) have been implicated in the drug addiction behavior. We determined whether EAAT type 3 (EAAT3) played a role in morphine addiction. Six- to eight-week-old EAAT3 knockout (EAAT3$^{-/-}$) mice and their wild-type littermates received 3 intraperitoneal injections of 10 mg/kg morphine, each on an alternative day, to induce conditioned place preference (CPP). Two days after the place preference returned to baseline, mice received 2.5 mg/kg morphine to induce reinstatement. Some mice received intraperitoneal injection of 4 mg/kg riluzole, an EAAT activator, 30 min before morphine or saline injection. Hippocampus, medial prefrontal cortex, nucleus accumbens and ventral tegmental area were harvested for Western analysis 24 h after the last dose of morphine was injected. Morphine induced CPP in wild-type and EAAT3$^{-/-}$ mice. Gender is not a statistically significant factor to influence this behavior. This conditioned behavior extinguished after morphine administration was stopped for 8–9 days in wild-type mice, while this extinction occurred 6 days after discontinuation of morphine injection in EAAT3$^{-/-}$ mice. A small dose of morphine similarly reinstated the conditioned behavior in the wild-type and EAAT3$^{-/-}$ mice. Riluzole abolished morphine-induced CPP during the initial place preference. Morphine increased EAAT3 expression in the plasma membrane of medial prefrontal cortex, nucleus accumbens and ventral tegmental area but did not affect EAAT3 expression in the hippocampus. These results suggest that EAAT3 delays the extinction of morphine-induced CPP. EAAT activation may prevent the formation of morphine-induced CPP.

Key words: conditioned place preference, glutamate transporter, mice, morphine.

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Abbreviations: CPP, conditioned place preference; CS, conditioned stimuli; EAAT, excitatory amino acid transporters; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mPFC, medial prefrontal cortex; VTA, ventral tegmental area.

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INTRODUCTION

Opioids are a major class of analgesics used clinically. However, it was estimated that about 5.1 million Americans in 2010 abused opioids (topics in Brief, NIDA, 2011). Activation of various rewarding pathways, such as dopamine system in ventral tegmental area (VTA), contributes to the opioid addiction (Johnson and North, 1992). However, glutamatergic system may also be involved in opioid addiction (Del Pozo et al., 1996; Popik and Wrobel, 2002).

Glutamate, the major excitatory neurotransmitter, can be taken up by glutamate transporters (also called excitatory amino acid transporters, EAAT) into cells after it is released from presynaptic termini. In fact, glutamate uptake via EAAT is a major mechanism to regulate extracellular glutamate levels due to the lack of extracellular enzyme to metabolize glutamate (Danbolt, 2001). Consistent with this function, it has been shown that EAAT can regulate glutamate neurotransmission (Danbolt, 2001; Sepkuty et al., 2002).

Five types of EAAT have been known: EAAT1–5. EAAT1 and EAAT2 are mainly expressed in glia. EAAT3 and EAAT4 are mostly in neurons. EAAT5 exists in the retina. EAAT1–3 are expressed in many regions of the central nervous system; whereas EAAT4 is mainly in the cerebellum. Based on the amount of protein, EAAT2 and EAAT3 are considered the major glial and neuronal EAAT, respectively (Danbolt, 2001). Consistent with their functions of regulating glutamate neurotransmission, glial EAATs, especially EAAT2, have been indicated to play a role in drug addiction, such as cocaine addiction (Fujio et al., 2005; Fischer et al., 2013).

Morphine, a commonly used analgesic and addicted opioid, is known to regulate the expression of EAAT. For example, morphine can reduce EAAT1 and EAAT3 expression in the dorsal horn of spinal cord (Mao et al., 2002). Morphine withdrawal increases EAAT2 expression in the hippocampus (Xu et al., 2003). However, it is not known yet whether EAATs play a role in morphine addiction behavior.

Studies from our and other groups have shown that EAAT3, the major neuronal EAAT, participates in the learning and memory processes (Aoyama et al., 2006; Lee et al., 2012; Cao et al., 2014; Wang et al., 2014). Drug addiction is a pathological form of learning and memory related to rewards and cures that predict rewards (Hyman, 2005; Rosen et al., 2015). Thus, we hypothesize that EAAT3 plays a role in morphine addiction that can be considered as pathological learning and memory toward

morphine use. To test this hypothesis, we exposed wild-type and EAAT3 knockout (EAAT3$^{-/-}$) mice to morphine and measured their conditioned place preference (CPP). The conditioning, extinction and reinstatement behaviors of these mice were studied. In addition, we performed these tests in male and female mice to test whether there is a gender difference in these behaviors because inconsistent findings regarding gender difference of opioid addiction have been reported (Lee and Ho, 2013).

EXPERIMENTAL PROCEDURES

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications number 80–23) revised in 2011.

Animals

Six- to eight-week-old EAAT3$^{-/-}$ mice and their wild-type littermates were used in this study. The EAAT3 knockout mice were descendants of mice used in our previous studies (Lee et al., 2010; Li and Zuo, 2011). They were initially back-crossed with wild-type CD-1 mice for more than 10 generations to produce a strain of EAAT3 knock-out mice before they were used in our studies. These CD-1 wild-type mice were from Charles River Laboratories (Wilmington, MA, USA). It has been confirmed that the EAAT3 knock-out mice did not express EAAT3 in our previous study (Li and Zuo, 2011).

The animals were housed five animals per cage in standard plastic cages in room temperature (21 ± 1 °C) and humidity-controlled environment with an automatic 12-h light/dark cycle (light on 6:00 am, off 6:00 pm). Food and water were available *ad libitum*. Each experimental group consisted of 7–13 mice and each mouse was used only for one study.

Apparatus for testing CPP

The box for testing CPP (LE 893, automated place preference system, Panlab/HARVARD Apparatus, Barcelona, Spain) consisted of three Perspex compartments. The larger compartments on either side are the same size (19 cm width × 19 cm length × 25 cm height) with one in white color and the other in black color. These two compartments were connected by a central gray corridor (6.5 cm width × 9 cm length × 25 cm height). One guillotine door was at each end of the corridor. The door color was the same as that of the compartments the door led to. The compartments can be differentiated by both visual and tactile cues: the color of the walls and the texture of the floors (smooth or rough). These distinct cues served as conditioned stimuli (CS). The use of distinct colors and tactile floor cues allowed mice to be in direct contact with CS to experience its conditioned effect during preference experiment. Animal position is detected by transducers installed below the cage platform. Only the gray and black compartments had transducers underneath the floor. When the system did not detect the animal in the gray or black compartment, it was assumed that the animal was in the white compartment. The transducer system was connected to a PC-based software PPCWin V2.0, which provided a raw data table containing permanence time in each compartment. The behavioral test room had dim lighting with a 10 W bulb positioned about 1.5 m above the apparatus. The apparatus was kept clean.

Drugs

Morphine sulfate (10 mg/ml, Hospira Lake Forest, IL, USA) was diluted in normal saline to the concentration of 1 mg/ml. It was given intraperitoneally at 10 mg/kg. This dose was chosen based on previous studies (Nakagawa et al., 2001, 2005). Control group received normal saline at 10 ml/kg.

Riluzole hydrochloride (2-Amino-6-trifluoromethoxy benzothiazole hydrochloride; TOCRIS Bioscience, Bristol, UK), a glutamate transporter activator (Frizzo et al., 2004; Fumagalli et al., 2008), was first dissolved in DMSO (Fish Scientific, NJ, USA) to 100 mM (27 mg/ml), and then diluted in saline to 0.4 mg/ml with gentle warming. The dose of riluzole was 4 mg/kg injected intraperitoneally 30 min before each morphine or saline injection. The same concentration of DMSO (1.6%) was used as vehicle for injection in control group.

Experimental design

Total 114 male and female mice weighing 18–26 g were used in this experiment. The experiments were carried out between 9:00 am and 5:00 pm.

The experimental procedure consisted of four phases: (1) *Habituation* (preconditioning) (3 days, day -3 to day -1), (2) *Conditioning* with drugs or vehicle (6 days, day 0–day 5), (3) *Place preference and its extinction* (6–9 days), and (4) *Reinstatement* (1 day) (Fig. 1). Place preference was tested in the first, third and fourth phases.

During the preconditioning phase, mice were taken to the testing room, weighed and handled by the experimenter for at least 5 min including caressing the back, grasping them by the tail and turning them upside down to imitate the position needed for intraperitoneal injection to reduce their stress in response to experimental manipulation. Mice were carefully placed into the central gray compartment and the guillotine was removed to give mice free access to all three compartments for 15 min. The white compartment was paired with smooth floor and the black compartment was paired with rough floor.

Baseline placement preference of each mouse was assessed on the 3rd day. Mice showing strong unconditioned aversion (less than 33% of the session time) or preference (more than 67% session time) for the white or black compartments were discarded. Twelve of the initial 126 mice were excluded for this reason in these experiments. Although mice tended to stay in the black compartment in a dim room, there was