Original Article

Striatum Hyperactivity Triggers Relapse to Morphine and Methamphetamine (Polydrug) Dependence in Mice

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Introduction: κ-opioid receptor (KOPr) system has been linked to relapse to many substances, especially opioids. Positive responses were recently reported in morphine and methamphetamine (polydrug)-dependent mice treated with buprenorphine and naltrexone, a functional κ antagonist. Objectives: This study aimed to determine the specific brain region that is responsive to KOPr treatment following polydrug dependence. Materials and Methods: The polydrug-dependent mice model was developed using conditioned place preference (CPP) method. Following successful withdrawal phase, the mice were treated with 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone. Four brain regions (hippocampus, prefrontal cortex, amygdala, and striatum) were investigated using immunohistochemistry technique. This is to quantify the changes in KOPr expression in each major brain region that was primarily involved in addiction neurocircuits of many substances. Unpaired Student’s t test was used to analyze all results, where $P < 0.05$ is considered significant. Results: The results showed that treatment with buprenorphine and naltrexone successfully attenuated relapse in 60% of mice ($n = 14$). A significant upregulation of KOPr was detected in striatum at the end of post-withdrawal phase ($P < 0.01, n = 12$). This treatment successfully suppressed KOPr in striatum ($P < 0.001, n = 12$), which supports the positive results seen in the CPP setting. No significant changes were observed in other brain regions studied. Conclusion: The hyperactivity of striatum suggests that the affected brain region following KOPr antagonist treatment is the region that primarily controls the drug rewarding activity, in which nucleus accumbens is located. This indicates that manipulation of KOPr system is one of the potential targets to treat morphine- or methamphetamine-dependence problem.
INTRODUCTION

Drug dependence is a chronic and relapsing brain disorder that causes uncontrolled compulsion to drug-seeking behavior despite its negative consequences such as negative emotional state and withdrawal syndrome.[1] Recent findings showed an increasing pattern of methamphetamine dependence among the methadone patients after enrolling into the methadone maintenance treatment (MMT) program.[2] This created another problem where the substance abusers start to abuse more than one class of drugs, therefore leading to polydrug dependence. The most commonly abused drugs by the polydrug users are morphine and methamphetamine.[3] To date, there is no Food and Drug Administration (FDA)-approved treatment for methamphetamine dependence.

The κ-opioid receptor (KOPr) activity was reported to be linked with drug relapse, including opioids and psychostimulants. The activation of KOPr that results in stress and dysphoria was believed to contribute to drug relapse.[4] Recent study showed that treatment combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone (partial NOPr agonist and mu opioid receptor [MOPr] or KOPr antagonists) was able to attenuate relapse to morphine and methamphetamine (polydrug) dependence in mice.[5] Thus, it strengthens the possibility that the opioid receptors can be manipulated to be a potential target to treat this dual dependency problem.

Therefore, the aim of this recent research work was to further investigate the involvement of KOPr system in mediating relapse to polydrug dependence, which involves morphine and methamphetamine. The ability of the buprenorphine and naltrexone treatment combination to interrupt the KOPr expression was also investigated during reinstatement related to this polydrug dependence at the brain level.

MATERIALS AND METHODS

Subjects

A total of 28 adult male Swiss albino mice were used, each weighed between 25 and 35 g (8–10 weeks old) for the conditioned place preference (CPP) test. The mice were placed in clear plastic cages with stainless steel cover lids and wood shaves as bedding. All mice were maintained under standard pelleted and water ad libitum until the time of experiment. The mice were housed in a room with a maintained temperature (21°C ± 0.5°C) and light on 12:12 h light–dark cycle (lights on at 7 AM, lights off at 7 PM). All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia (IIUM/IACUC Approval/2016/[11][64]).

Drugs

Morphine sulfate solution was obtained from Hameln Pharmaceuticals (Gloucester, UK), and methamphetamine hydrochloride was purchased from Lipomed (Arlesheim, Switzerland). Morphine sulfate and methamphetamine hydrochloride were dissolved in normal saline (0.9% sodium chloride) (Ain Medicare Sdn. Bhd., Kelantan, Malaysia). All drugs (and saline) were administered through intraperitoneal (ip) injection not more than 10 mL/kg of body weight. All drugs were prepared as a stock solution in normal saline and stored at −20°C until used.

Apparatus

The CPP box used was a three-chambered shuttle that comprised a small central compartment (10 cm × 10 cm) where the mice will be placed at the start of a test session, and two larger compartments (40 cm × 40 cm). One compartment was painted with horizontal black and white stripes, whereas the other one was with vertical black and white stripes. Both compartments were separated with removable partitions. The floors were made of stainless steel with different textures: punched-out holes and stripes.

Conditioned place preference test

All procedures used in the CPP test were adapted from an earlier study.[10] The CPP test was conducted between 8 AM and 4 PM at a consistent time for each mouse. The mice received a combination of morphine or methamphetamine (7.5 and 1.0 mg/kg, [polydrug]) during drug conditioning session. From Day 1 to Day 3, each mouse had a 15-min exploratory session. On Day 4, the mice underwent a 15-min baseline test. Any mouse that spent more than 70% of its time in either compartment was excluded from the subsequent conditioning session. The mice received a combination of morphine or methamphetamine (7.5 and 1.0 mg/kg, [polydrug]) during drug conditioning session. From Day 5 to Day 10. The mice were conditioned with the drug (morphine and methamphetamine [polydrug]) and alternated every other day with saline for at least 24-h interval for six consecutive days. Immediately after drug administration, the mice were confined in the drug-paired...
compartment for 60 min. During post-conditioning test, which was performed on Day 11, the mice were tested under a drug-free state for 15 min, and the percent of preference was calculated exactly as for the baseline preference. The mice that showed less than 30-s increment on the time spent at the drug-paired compartment (compared to its baseline) during post-conditioning test were excluded from the next procedure. Extinction training was performed for a maximum of 21 days, 15 min for each session. The mice that showed more than 10% preference at the drug-paired compartment over the baseline preference during extinction training were considered as failed to achieve drug abstinence. During reinstatement, the mice received priming dose of morphine and methamphetamine (polydrug) (2.5 and 1.0 mg/kg, ip, respectively) before 0.3 mg/kg buprenorphine/1.0 mg/kg naltrexone treatment.\(^5\)

**Immunohistochemistry**

The animals were killed by cervical dislocation immediately after the CPP experiment was completed. The brain tissues were dissected out, which was followed by the tissue processing.\(^6\) Only four parts of brain regions were used during immunohistochemistry (IHC), namely amygdala, hippocampus, prefrontal cortex, and striatum. Tissue sections were incubated with antigen retrieval solution (Dako, Santa Clara, CA, USA) using a commercial microwave for 10 min. Then, the tissues were incubated with ready-to-use endogenous peroxidase blocking reagent (Dako) for 10 min at room temperature. Next, the tissues were incubated with primary antibody (rabbit monoclonal antibody [EPR18881] to KOPr) with dilution factor of 1:500 for 30 min. The slides were then incubated with Dako Real EnVision HRP (horse radish peroxidase) polymer (ready-to-use secondary antibody) for 30 min. The tissues were then incubated with 3,3’-diaminobenzidine (DAB) substrate (1 mL DAB substrate + 20 μL DAB chromogen) for 10 min.

**Statistical analysis**

All data were expressed as mean ± standard error of mean (SEM) with 95% confidence intervals. The data from post-conditioning, extinction training, and reinstatement tests were compared with the baseline test data. The data were then statistically analyzed using paired samples \(t\) test. The positive cells refer to the presence of brown color on the cell membrane of the cells, which indicates the presence of KOPr. All data were expressed as mean ± SEM of the KOPr and analyzed using unpaired samples \(t\) test. The positive cells refer to the presence of brown color on the cell membrane of the cells, which indicates the presence of KOPr. All data were expressed as mean ± SEM of the KOPr and analyzed using unpaired samples \(t\) test.

**RESULTS**

**Establishment of morphine and methamphetamine (polydrug) relapse model**

Figure 1 shows the results for the effect of 7.5 mg/kg morphine and 1.0 mg/kg methamphetamine (polydrug) conditioning toward the establishment of a polydrug relapse model. A total number of 12 mice were used at the beginning of this experiment. Two mice were rejected during each baseline and post-conditioning tests due to failure of meeting the inclusion criteria for successful baseline test and drug conditioning. The results showed that a polydrug-dependence model was successfully established with an extremely significant difference compared to the baseline \((P < 0.001)\). Morphine and methamphetamine (polydrug) were also successfully reinstated \((73.54% ± 15.46%, n = 8)\), with a very significant difference compared to the baseline \((P < 0.01)\).

**Expression of the \(\kappa\) opioid receptor at four different brain regions in buprenorphine- and naltrexone-treated mice**

Figure 2 shows the results for the expression of KOPr in morphine and methamphetamine (polydrug)-dependent mice in the striatum region. Different
samples of the brain tissues were used for each stage. The expression of KOPr during reinstatement was compared to its expression during the post-conditioning stage. It was found that there was a significant increment in the KOPr expression during reinstatement (33.390% ± 5.595%, n = 12) compared to the post-conditioning stage (16.730% ± 5.265%, n = 12, p < 0.01). In prefrontal cortex, a significant reduction of KOPr expression was observed during reinstatement (35.070% ± 3.505%, n = 12) when compared to KOPr expression during post-conditioning (44.090% ± 1.782%, n = 12, P < 0.01), whereas in hippocampus and striatum, no significant changes in KOPr expression were detected during reinstatement compared to the KOPr expression during post-conditioning (hippocampus, 65.250% ± 4.125%, n = 12 vs. 51.110% ± 6.870%, n = 12, P > 0.05; amygdala, 35.070% ± 3.484%, n = 12 vs. 38.160% ± 4.327%, n = 12, P > 0.05).

**Discussion**

On the basis of IHC results, a significant increase in the KOPr expression during reinstatement was only detected in the striatum compared to the KOPr expression during post-conditioning following polydrug priming of 2.5 mg/kg morphine and 1.0 mg/kg methamphetamine in mice. A previous study revealed that the prodynorphin (pDYN) expression in rats was increased in the striatum region following chronic cocaine dependence (50 mg/kg). The expression of pDYN was higher in the nucleus accumbens (NAc) as compared to the dorsal striatum.[6] This implies that an upregulation in the KOPr system activity in the striatum is related to drug seeking and rewarding effects of the addictive drugs. Similar finding was also reported in another study, which used quantitative in vitro autoradiography technique, where the KOPr expression was increased in the NAc of rats following chronic cocaine administration (45 mg/kg).[7] An earlier study by Pereira et al. showed that ip administration of 0.01 and 10 mg/kg buprenorphine significantly reduced the level of dopamine in striatum following the administration of 2.0 mg/kg methamphetamine in rats.[8] Therefore, it can be suggested that KOPr is activated in striatum, and this leads to drug-seeking behavior and relapse.

There was no significant difference in the KOPr expression during reinstatement compared to the KOPr expression during post-conditioning in amygdala and hippocampus. These results suggest that the KOPr system in these brain regions is not involved in mediating relapse to polydrug. Anker and Carroll[9] suggested that the activation of amygdala was linked to the aversive stimuli. Most of previous studies showed that KOPr in amygdala is associated with stress-induced reinstatement.[10-12] There were also reports on significant increase of c-Fos (gene marker of drug addiction and relapse) in amygdala after the animals were exposed to stress cue.[12] Therefore, it can be suggested that there is no difference in the KOPr expression at these two different stages of CPP in amygdala due to noninvolvement of stress exposure during the experimental procedure.

Similarly, no significant difference of the KOPr expression in the hippocampus was found during reinstatement compared to the KOPr expression in the hippocampus during post-conditioning. The increase of KOPr activity in the hippocampus has been linked to the exposure of stress cue, as seen in amygdala.[10,13] However, in the current experimental procedure, no stress cue was involved, which may explain the negative result observed in the hippocampal brain region. A study by Magnusson et al.[14] suggested that the elevated pDYN level in hippocampus may lead to impaired spatial memory. This view was supported by another study, which reported an increase in KOPr activity during addiction, which affects hippocampal function in memory regulation.[15] In this study, no significant difference in KOPr expression was observed in hippocampus following reinstatement. A long extinction training period has been associated with a reduction in memory toward the previous drug-taking habit.[14] This may contribute to the absence of difference seen in KOPr expression at the hippocampus during reinstatement phase compared to KOPr expression at the hippocampus during post-conditioning.

Contradictory to the findings in striatum, there was a significant reduction in the KOPr expression in the prefrontal cortex (PFC) during reinstatement compared to KOPr expression in the PFC during post-conditioning. It has been suggested that the KOPr system plays an important role in PFC by inhibiting the release of dopamine.[17] Many previous studies reported an increase in KOPr activity in PFC when the subjects were exposed to drug.[18] However, none of these studies relate the increase in KOPr activity in PFC with relapse following drug abstinence state. Therefore, the role of KOPr in PFC in mediating drug relapse remains unclear.

**Conclusion**

A priming dose of morphine and methamphetamine (polydrug) used has successfully induced relapse to polydrug dependence in mice. Surprisingly, the KOPr was not affected in amygdala, the brain regions that regulate emotion. However, KOPr upregulation was
observed at the striatum. Thus, it is suggested that this brain region might be triggered to oppose the stimulus-related reaction caused by polydrug abuse, which might be associated with the NAc activity.

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**Conflicts of interest**
There are no conflicts of interest.

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