

Positive Effect of Nitric Oxide on Morphine-Induced Place Conditioning in Wistar Rats Treated by Colchicine Intra-Hippocampal CA1

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Abstract

Colchicine is a potent alkaloid neurotoxin known as a selective neurotoxin of granular cells of hippocampal formation. This research aimed to assess the consequences of hippocampal reward circuitry damaged by colchicine. Male Wistar rats were examined for saline conditioning a week after receiving of colchicine (1-8 μ g/rat, intra-CA1). Control group was simply microinjected saline (1 μ l/rat, intra-CA1). In addition, two categories of animals pre-treated by colchicine (2,8 μ g/rat, intra-CA1) were evaluated for place preference induced of morphine (2.5-7.5 mg/kg, s.c.) using a three-day schedule of unbiased paradigm. Also sham operated legend "normal" group passed the conditioning by morphine. This group was pre-treated saline (1 μ l/rat, intra-CA1) instead of colchicine. According to the results, the injection of colchicine (1-8 μ g/rat, intra-CA1) induced a place aversion in saline conditioning at the higher doses (8 μ g/rat). Furthermore, the colchicine treated rats showed histologically a significant ($p < 0.001$) decrease in numbers of pyramidal cells of CA1. On the other hand, morphine induced a significant place preference in animals pre-treated by colchicine (2,8 μ g/rat, intra-CA1). Moreover, pre-microinjection of L-arginine (0.3-3 μ g/rat, intra-CA1), a precursor of nitric oxide (NO), pre-testing of morphine response caused a positive effect on expression of response to morphine. However, this effect was reversed when L-NAME (0.3-3 μ g/rat, intra-CA1) was injected into the CA1 prior to the administration of L-arginine (3 μ g/rat, intra-CA1). Based on data the neurotoxin, colchicine, may affect the place conditioning reward circuitry via decreasing of pyramidal cells in the CA1 area ($p < 0.01$), an effect which most likely can be improved by morphine administration peripherally. In conclusion the molecule NO into CA1 may have a positive force on morphine-induced place conditioning in Wistar rats received colchicine intra-CA1.

Keywords: Conditioned Place Preference; Morphine; Colchicine; CA1; Nitric Oxide

Introduction

Place conditioning is used to assess the rewarding properties of morphine [1-3]. Conditioning to place is mediated by mu-opioid [4,5] and other types of receptors [5]. Nitric oxide (NO) a main retrograde neurotransmitter [6] is implicated in opiates' actions [7]. Also, this molecule regulates the rewarding behavior [8] and involves in place conditioning-induced by morphine [9]. Morphine stimulates the release of NO in hippocampus through the routes sensitive for naloxone and N^G-Nitro-L-arginine Methyl Ester (L-NAME) [9], the fact demonstrating a key role of NO in morphine-induced place preference.

In recent years, a considerable interest has been generated by drugs that show a selective neurotoxic effect on cells. Colchicine, a plant derived alkaloid [10], is known as a potent inhibitor of behavioral processes because that this drug specifically binds with a high-affinity receptor site on tubulin [11]. The alkaloid, colchicine, is also categorized as the blocker of mitosis [12]. Moreover, it is introduced as a depolymerizer of cytoplasmic microtubules which consequently affects on cell's shape and developmental or neuronal functions such as axoplasmic transport [12,13]. The latter process is thought to be associated with neurological disorders, e.g. Alzheimer's disease [14]. This poison of spindles also is a candidate for leukemia and other cancers chemotherapeutically [11].

It has been demonstrated [15] that colchicine destroys the neuronal populations in the hippocampal formation and been introduced as a drug with a quality of being selective for cell lesion in the hippocampal formation [16]. Considering these and other findings showing that the mammalian brain contains a class of molecules that interact with the colchicine site on tubulin [11]. Also considering that the destruction of

CA1 cells may offer opportunities for studying of the behavioral effects of damaged hippocampal circuitry, we aimed to inject the toxin intra-CA1 in order to evaluate the consequences of hippocampal reward circuitry damaged by colchicine using the place conditioning task.

Experimental Procedure

Subjects

Subjects were male Wistar rats (Pasteur Institute of Iran, Tehran, Iran) weighing 200-220 gr. Animals were housed four per cage in a controlled colony room (temperature 21 \pm 3°C). They were maintained under a 12:12 h light/dark cycle with water and food provided *ad libitum*. 6-7 animals were used only once in each experiment. The experiments were approved by local ethics committee of Shahed University.

Drugs

Colchicine (Merck Co., Germany) was directly injected into the CA1. Morphine sulphate (TEMAD, Co., Tehran, Iran) was injected subcutaneously (s.c.). L-Arginine (Sigma Chemical Co., USA) and N^G-

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Nitro-L-arginine Methyl Ester (L-NAME; Research Biochemical Inc., USA) were microinjected into the area of interest (CA1). Ketamine (100 mg/kg) and xylazine (20 mg/kg) obtained from Veterinary organization of Iran were used i.p. Vehicle was 0.9% physiological saline. Cresyl violet (Merck Co., Germany) was used for staining of brain slices.

Surgery

Animals were anesthetized and placed in a stereotaxic apparatus, with the incisor bar set at 3.3 mm below horizontal zero to achieve a flat skull position. An incision was made to expose the rat skull. Two holes were drilled in the skull at stereotaxic coordinates: AP-3.8 mm posterior to bregma, and $L \pm 1.8$ to ± 2.2 mm according to the atlas of Paxinos and Watson [17]. Two guide cannulae (21-gauge) were inserted into the holes. For bilateral injections of colchicine into the animals' CA1 area, the guide cannulae were lowered 2.5 mm below bregma through the holes drilled at the above-mentioned coordinates. The guide cannulae were anchored with a jeweler's screw, and the incision was closed with dental cement. Under surgery, two injection cannulae attached with polyethylene tubing (0.6 mm internal diameter) to Hamilton syringes were inserted into the guide cannulae through which the alkaloid (1-8 μ g/rat) was gently (1-2 min) injected into the site. Tips of injection cannulae extended 0.5 mm beyond the guide cannulae. The injection cannulae were left in the site for another 60 sec to facilitate the diffusion of the drug into the area. All animals were allowed to recover for one week before behavioral testing began.

Histological verification

After completion of behavioral testing, animals were killed with an overdose of chloroform. The experimental animals' brains were removed and fixed in formalin solution 10% for 48 h before sectioning. Sections were taken through the brain areas of cannulae placements, and the placements were verified using the atlas of Paxinos and Watson [17]. Also the cell layer structure was studied under magnification; each brain sample was examined using the light microscope (OLYMPUS) at 100 μ m² defined as a unit of quantifying measurement. An Image Tool program [UTHSCSA Image Tool, version 2.03], the free image processing and analysis program for Microsoft Windows, was used for image analysis functions at the area (100 μ m²). Data from rats with injection sites located outside the hippocampal CA1 area were excluded from analyses.

Cresyl violet stain

The brain sections were placed in 50%, 75%, 95% (1 min) and in 100% ethanol (15 min). The sections were then rehydrated in 95%, 75%, and 50% ethanol and water (1 min) and stained with cresyl violet for 2 min. Subsequently, the sections were placed in 0.1% acetic acid in 75% ethanol for 30 sec, rinsed in water, dehydrated, and mounted in Entellan (Merck Co., Germany).

Statistical analysis

All data were calculated with analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. The histological results were evaluated by using the Image Tool program [UTHSCSA, version 2.03], to provide quantification data. P values less than 0.05 were considered as significant.

Place conditioning apparatus

A two compartment conditioned place preference apparatus (30 \times 60 \times 30 cm) was used in these experiments. Place conditioning was

conducted using an unbiased procedure with design been described previously [9,18].

Place conditioning paradigm

The experiment consisted of three following phases

Pre-conditioning (Familiarization): On day 1, animals received one habituation session. They were placed in the middle line of the apparatus and had free access to the entire apparatus for 15 min, while the removable wall was raised 12 cm above the floor. The time spent by rats in each compartment was recorded by an Ethovision system (model LVC-DV323ec of an Auto iris LG) located 120 cm above the apparatus. The records were then analyzed by an observer blind to the treatments. Groups displayed no significant preference for a compartment confirming that this procedure is unbiased.

Conditioning: This phase was started one day after familiarization and lasted three days. Conditioning to saline consisted of 6-saline pairings; each saline paired session lasted 45 min. Animals were injected saline (1 ml/kg, sc) twice daily with 6-h interval between two sessions. Control pre-exposed to saline group (1 μ l/rat, intra-CA1) also passed the same protocol. During the conditioning phase the removable wall was inserted along the seam separating the two compartments. In addition, two categories of animals' pre-treated colchicine (2,8 μ g/rat) were conditioned to morphine (2.5-7.5mg/kg, sc). For conditioning to morphine the conditioning phase consisted of 3-saline and 3- drug pairings; each session lasted 45 min. They were daily injected morphine or saline. After the injection of morphine (2.5-7.5mg/kg, sc) the animals were confined to one compartment of apparatus. Following administration of saline, they were confined to the other compartment. So, treatment compartment and order of presentation of morphine or saline were designed counterbalanced. Sham operated group named "normal" not exposed to colchicine passed the same protocol.

Testing (Post-Conditioning): Test sessions were carried out on day 5, 1 day after the last conditioning session. Each animal was tested only once. For testing, the removable wall was raised 12 cm above the floor and the animals were allowed free access to both compartments of apparatus for 15 min. Then the time spent in each compartment was calculated to subtract the time spent in one compartment throughout testing to that of spent in the same compartment on day of familiarization. The values representing the scores of changes in place preference (in sec), were expressed as mean \pm S.E.M.

Results

Effect of contact to colchicine (Intra-CA1) on saline conditioning

Injection of colchicine (1-8 μ g/rat, intra-CA1) one week before starting of saline conditioning program resulted in a place aversion response ($F_{3,28} = 2.357, p < 0.01$). The response was significant at higher doses of toxin (8 μ g/rat, intra-CA1) as post hoc analysis revealed (Figure 1).

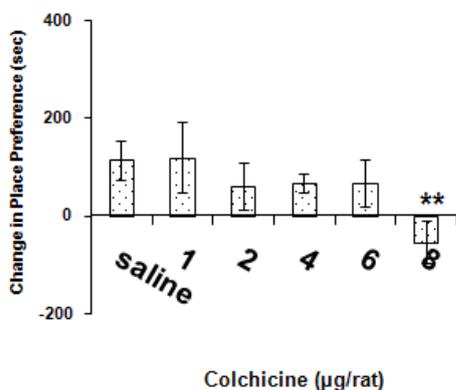
Effect of morphine injection in rats pre-exposed to colchicine (Intra-CA1) using place conditioning paradigm

Bilateral injection of colchicine a week before starting of place conditioning-induced of morphine, resulted in a significant place preference in the experimental groups according to one-way ANOVA [$F_{3,28} = 7.459, p < 0.001$ for category exposed to colchicine 8 μ g/rat, intra-CA1 or $F_{3,28} = 6.333, p < 0.001$ for other class received colchicine 2 μ g/rat, intra-CA1]. However, the conditioning response was differently

appeared in sham operated group legend "Normal". Moreover, the response was more pronounced at higher doses of opioid (5-7.5mg/kg) in this group in contrast to colchicine-treated categories (Figure 2).

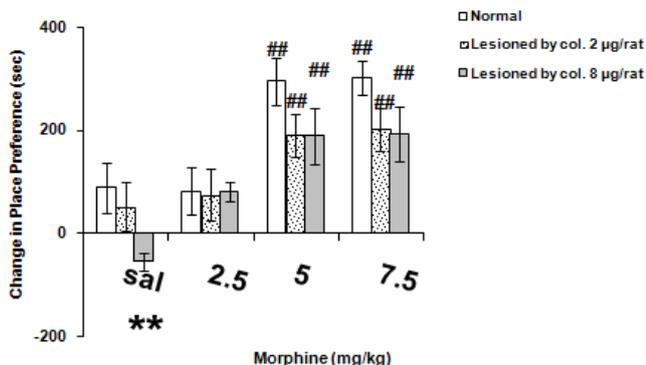
Effect of intra-ca1 injection of Nitric Oxide (NO) precursor, L-arginine, on place conditioning-induced by morphine in colchicine exposed rats

Colchicine (2,8µg/rat, intra-CA1) was injected before starting of morphine place conditioning. L-arginine (0.3-3µg/rat) was microinjected intra-CA1 right pre-testing of morphine response. According to the results the effect of intra-CA1 injection of nitric oxide (NO) precursor, L-arginine, on place conditioning-induced by morphine in colchicine exposed rats was significant statistically [$F_{3,28} = 2.482, p < 0.01$ for one group which received colchicine 8µg/rat, intra-



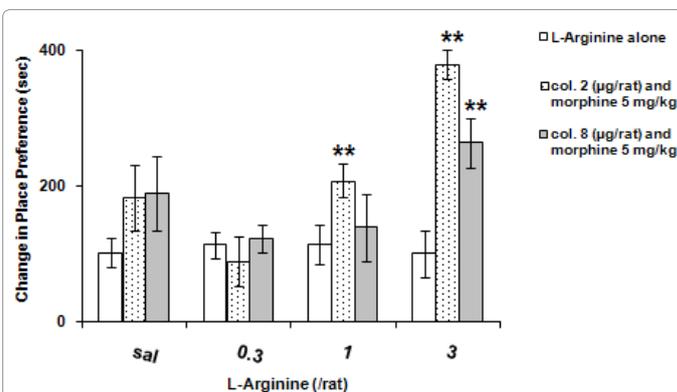
** $p < 0.01$ difference between colchicine-administered groups vs vehicle

Figure 1: Response to colchicine intra-CA1 in saline conditioning task. Each animal was microinjected colchicine (1-8µg/rat, intra-CA1) once. The animals after being recovered from surgery were assessed behaviorally by saline conditioning as detailed in experimental procedure. Control group avoided lesion-induced by colchicine but received saline (1 µl/rat, intra-CA1) and passed the same behavioral protocol.



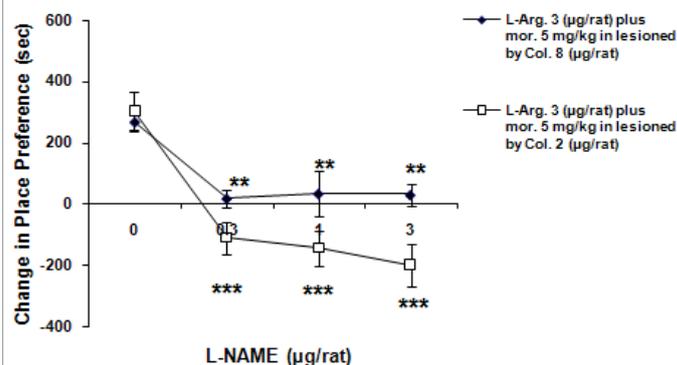
** $p < 0.01$ shows difference in saline groups (Legend sal)
$p < 0.01$ shows difference between dose groups vs respective saline group in each class

Figure 2: Response to morphine (2.5-7.5 mg/kg, sc) in colchicine treated animals using place conditioning task. Animals of lesioned classes were injected colchicine (2or8µg/rat, intra-CA1). The animals after being recovered were tested for place conditioning by morphine. A class legend "normal" was simply injected saline intra-CA1 and passed the same conditioning task. Control groups of all categories legend sal in Figure only received saline throughout the procedure (both intra-CA1 and peripherally).



** $p < 0.01$ shows difference between dose groups vs respective saline group in each class

Figure 3: Effect of L-arginine on expression of response to morphine in normal or colchicine lesioned categories using place conditioning task. Animals were injected colchicine (2or8µg/rat, intra-CA1), and after recovery they were tested for place conditioning induced of morphine (2.5-7.5 mg/kg, sc). The animals then were microinjected L-arginine (0.3-3µg/rat, intra-CA1) before testing of morphine response. A class legend "L-arginine alone" was simply injected saline intra-CA1 and passed the same conditioning task. Control groups of all classes legend sal in Figure only received saline throughout the procedure (both intra-CA1 and peripherally).



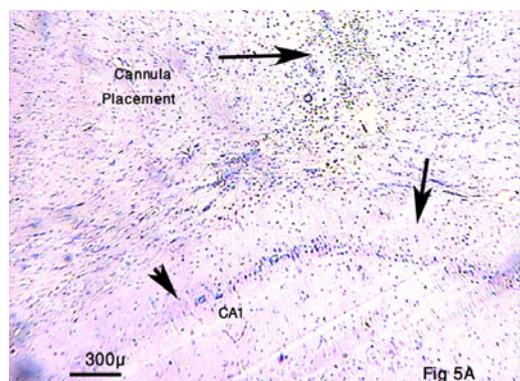
** $p < 0.01$ & *** $p < 0.001$ show differences between dose groups vs respective control group in each class

Figure 4: Effects of L-arginine (3µg/rat, intra-CA1) plus L-NAME (0.3-3µg/rat, intra-CA1) on expression of morphine response in lesioned categories using conditioning task. At first the animals were injected colchicine (2 or 8µg/rat, intra-CA1). They were then tested for place conditioning induced of morphine (after recovery). Before testing the animals were microinjected L-NAME (0.3-3µg/rat, intra-CA1) prior to L-arginine (3µg/rat, intra-CA1). Control groups of these classes legend 0 in Figure only received saline throughout the procedure (both intra-CA1 and peripherally).

CA1 or $F_{3,28} = 4.425, p < 0.01$ for colchicine 2µg/rat, intra-CA1, received group]. According to post hoc analysis the maximum response was produced at high dose (3µg/rat) of the precursor (Figure 3).

Effect of Nitric Oxide (NO) inhibitor, l-name, on response to l-arginine microinjection into ca1 in rats exposed to colchicine (intra-ca1) using place conditioning-induced by morphine

Colchicine (2,8µg/rat, intra-CA1) was injected before starting of place conditioning by morphine. L-NAME (0.3-3µg/rat) was microinjected prior to L-arginine (3µg/rat, intra-CA1) pre-testing of morphine response. This procedure showed a significant response in the experimental groups [$F_{3,28} = 2.876, p < 0.01$ for group of animals



Arrowheads show cannulae placements and pyramidal cells populations in CA1 layer under histological examination. Line is 300 μ

Figure 5: Verifying of lesion effect of colchicine (8 μ g/rat, intra-CA1) in rats' CA1 layer cells (Figure 5A). The rats were injected colchicine (8 μ g/rat, intra-CA1) once a week before of verification. Control slices (Figure 5B) were obtained from normal vehicle (0.9% physiological saline) injected group (1 μ l/rat, intra-CA1). As data shows the alkaloid (8 μ g/rat, intra-CA1) induced a significant lesion effect in CA1 layer cells of the experimental animals.

received colchicine 8 μ g/rat, intra-CA1 or $F_{3,28} = 9.972, p < 0.001$ for other group of rats exposed to colchicine 2 μ g/rat, intra-CA1]. Moreover, the inhibitor showed an attenuation on effect of precursor at all doses (Figure 4).

Effect of injection of colchicine (intra-ca1) on ca1 cell population of rats exposed to neurotoxin

Figure 5 shows the lesion effect of colchicine on pyramidal cells in CA1 area of rats' contacted neurotoxin. Administration of the alkaloid (1-8 μ g/rat, intra-CA1) a week before the place conditioning testing resulted in a significant decrease in the overall cell population compared with control ($p < 0.001$). In view of the results, the maximum lesion in the layer was induced by a higher dose of neurotoxin (8 μ g/rat, intra-CA1).

Discussion

Colchicine microinjection at higher doses (8 μ g/rat, intra-CA1) changed the behavioral measurement for saline conditioning in Wistar rats in respect to the control. The most striking result was that the microinjection of neurotoxin into CA1 reduced the pyramidal cell population in the layer (Figure 5). Exposure to colchicine at lower doses failed to show the result indicating dose-effect dependence.

Colchicine is known as an alkaloid extracted from *Colchicum autumnale* L. This neurotoxin selectively binds to tubulin dimers in vitro that result in tubulin-colchicine complex acting primarily to prevent microtubule assembly [19]. In animal cells colchicine is considered as a lethal agent even at the lowest concentrations ordered to block mitosis (10⁻⁷ M) [20,21].

As present data showed the neurotoxin irreversibly damages the pyramidal cell population in the rat CA1 area although other researchers have reported only reversible effects after using similar doses of colchicine [22]. Colchicine irreversibly damages dendrites by disrupting their microtubular supporting network [23], a mechanism which explains the toxic effect of colchicine in dentate granule cells [24]. The concentration of colchicine used in this study though sufficiently produced a high level damage of CA1 pyramidal cells, however, the exact mechanisms remain unclear. Although, we assessed the possible mechanism involving hippocampal reward circuitry by using a simple learning program (place conditioning) in accordance to a previously described task [9,18].

After conditioning testing of animals exposed to colchicine (1-8 μ g/rat, in the CA1 area), the animals dose-dependently exhibited a place aversion compared to the control group. This alkaloid been proposed to inhibit the rapid axonal transport by binding to tubulin subunits of microtubules [25]. Other researchers have suggested long-lasting morphological changes in neurons and glia [25-27]. Investigators have also suggested the axonal membrane [28] and dendrites [29] as the places enriched of microtubules and specific receptors [30] as the possible sites of action of colchicine. Several authors also have suggested the specific uptake systems in neurons for colchicine [31]. Colchicine, the microtubule-depolymerizing agent, blocks the axonal transport [32,33]. This plant alkaloid impairs the functional properties of nerve cells since, for instance, it inhibits the transport of amine storage vesicles in sympathetic nerves [34,35]. The toxin affects protein kinase C-induced modulation of synaptic transmission in cultured hippocampal pyramidal cells [35]. Thus, uncoupling the nerve terminals from its axon may affect the responsiveness of a neuronal cell to agents acting at a presynaptic site. Another explanation is that the alkaloid induces disruption of microtubule-network [36], thereby, causes the blockade of the axonal transport of vesicles [37,38]. The present effects thus may be the consequences of the similar changes in CA1 that necessarily are linked with synaptic transmission of the place conditioning task learning. Finally, colchicine has been introduced as an allosteric modulator of the GABA-aminobutyric acid (GABA) A type receptor [10]. The GABAergic interneurons are found abundantly in the CA1 of the dorsal hippocampus. Changes in firing rate of majority of the field CA1 putative GABAergic interneurons may induce a long lasting depression of synaptic excitability of CA1 pyramidal cell [39].

We microinjected NO producer (L-arginine) intra-CA1 to survey on the role of CA1 NO system in rats' morphine rewarding treated by colchicine. In a view to the present work morphine dose-dependently induced place conditioning in colchicine treated rats. NO precursor showed a significant effect on the expression of morphine response.

In support, it is well known that NO participates in morphine-induced place preference [40,41]. These data may reflect that the free-radical NO in the CA1 participated in the place conditioning induced of morphine at the area of research. Further event was that L-NAME (0.3-3µg/rat, intra-CA1), a highly selective neuronal NOS (nNOS) inhibitor, blocked this effect when was microinjected in combination with L-arginine, reflecting clearly that NO a highly active neurotransmitter, in the CA1 may mediate the pharmacological effects of morphine in colchicine exposed rats.

The potency of morphine was much more promoted in the groups pre-treated by lower doses of neurotoxin, colchicine, than the other category that may indicate a link between the level of neuronal destruction and the dose effect of colchicine. This part of the finding, based on the behavioral measurement may confirm the present histological evidence verifying the cell population decrease in CA1 of colchicine contacted rats.

In conclusion the present work offers opportunities for studying of behavioral effects of damaged hippocampal circuitry by colchicine usage. It proposes sensitivity to colchicine's neurotoxicity for other cell population in hippocampal formation. This achievement conflict with other researches pointing out that the poison, colchicine, only damages dentate granule cells [42].

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