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Original Article:

The Comparison of Intraventricular Injection of Mu Receptor Agonist (Morphine) and Antagonist (Naloxone) on the Occurrence of Fear Behavior in Adult Male Rats

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Abstract

Introduction: Drugs are among the opioid-like compounds that lead to the development of emotional behaviors in humans and animals. One of the emotional behaviors is stress behavior caused by fear, which can be caused by opioid and quasi-opioid compounds. In this study, the effect of intraventricular injection of brain (I.C.V) agonist (morphine sulfate) and hair receptor antagonist (naloxone) on fear behavior in adult male Wistar rats was investigated.

Materials and Methods: In this study, pure harmalin was used as a hallucinogenic drug that causes hallucinations and fear in animals as a positive control and saline as a sham was used for comparative studies with groups treated with morphine sulfate and naloxone. In this study, stereotax machine was used for cannulation and injection of I.C.V and Elevated plus-maze machine was used for behavioral testing.

Results: The values used to treat the experimental groups for morphine sulfate (0.5, 1, 2.5, 5, 7µg/rat) and naloxone (0.5, 1, 2µg/rat) were selected. The results of intraventricular injection of (1, 2.5, 5µg/rat) morphine in the brains of rats in the experimental group showed a significant difference in the occurrence of fear behavior compared to the positive control group with $P < 0.05$.

While injection of values (0.5, 7µg/rat) did not show a significant difference with $p < 0.05$ compared to the positive control group. Also, the results of intraventricular injection (I.C.V) of naloxone (1µg/rat) showed a significant difference with $p < 0.05$ in the occurrence of fear behavior in comparison with the positive control group. While injection of values (0.5, 2.5µg/rat) did not show a significant difference with $P < 0.05$ compared to the positive control group. In this study, I.C.V (50 µg / rat) injection of pure harmalin, which is considered as a positive control group, shows the percentage of entry into the open arm and also the percentage of retention time in the open arm.

Conclusion: In conclusion, none of the data used in the present study in the area has a uniform performance and this diversity can be considered as a result of various mechanisms that require more detailed studies.

Keywords: Elevated plus-maze, Fear, Naloxone, Rat, Sulfate morphine

1. Introduction

Fear is a physiological defense response to acute danger by stimulating some special parts of brain. Areas involved in fear include the hypothalamus, limbic system, septum, and amygdala, in which the role of neurotransmitters such as noradrenaline, dopamine, GABA, and serotonin should not be overlooked. The link between stress and drugs and cocaine injection in humans triggers the release of adrenaline and corticosteroids. Prenatal drugs have been shown to cause long-term changes in the neurotransmitter system involved in stress responses and brain homeostatic balance in rats. Studies show that dinorphin is involved in the expression of stress-induced motor responses by activating kappa opioid receptors. Studies show that in addition to the mu receptor, kappa receptors are also involved in stress-induced behavioral responses [1] .Studies on codeine have shown that the opioid receptor agonists kappa, delta, and hair have distinct effects on the excitatory properties of cocaine in rats [2].

In relation to fear and opioids, studies have shown that opioid receptor antagonists such as naloxone cause jumping behavior in morphine-addicted rats [3]. Studies demonstrate that drug use affects the stress hormone cycle and the secretion of them in body [4]. On the other hand, research illustrate that drugs can reduce emotional responses by affecting the release of noradrenaline and the inhibitory effect on its secretion [5].

According the data of previous studies, dinorphine is involved in the expression of stress-induced motor responses by activating opioid K receptors. Studies show that in addition to the mu receptor, kappa receptors are also involved in stress-induced behavioral responses [6].

Opioids are drugs that have morphine-like properties. The word opium is derived from the scientific name of the poppy plant papaver sominiferum. Poppy juice contains many alkaloids, the most important of which is morphine [7].

Studies indicated that morphine use in the presence of conditioned fear can balance acute morphine dependence. Research showed that increased morphine analgesia occurs following conditioned fear [8]. Specifications of mu receptor agonist and antagonist:

Morphine is the largest component of opium alkaloids (Figure 1). The basis of the morphine building was proposed in 1925. The structure of morphine consists of 5 dense rings: phenolic ring (A), cyclohexane ring (B), silkohexanol ring (C), N-methyl-hyperidine ring (D) and eruption ring, part of which is saturated (E) [9].

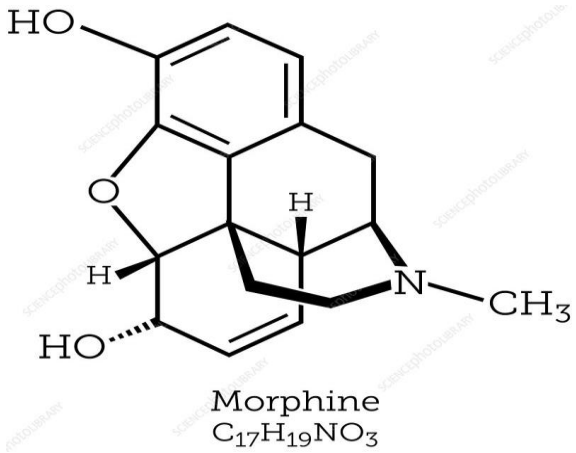

Figure 1. Chemical formula of morphine

Naloxone hydrochloride with formula C19H21NO4Hcl = 363.8 is a white powder or cream close to white. Soluble in water, strong and slightly alkaline, slightly soluble in alcohol, especially insoluble in chloroform and either (Figure 2).

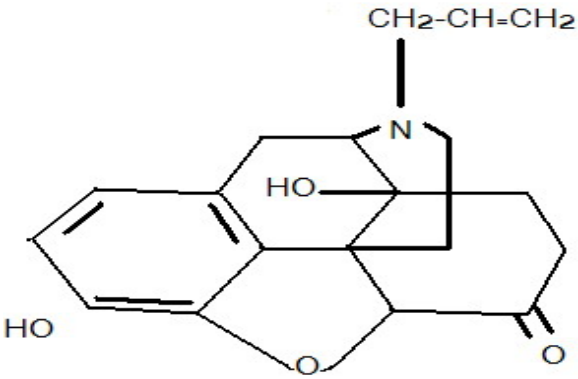

Figure 2. Chemical formula of Naloxone

Naloxone is the most important opioid antagonist that has an antagonistic effect on all three types of receptors [10]. The naloxonazine μ1 antagonist and the kappa (nor-binaltorphamine) antagonist reduce fear-like responses following electrical stimulation of the inferior colliculus [11]. In relation to fear and opioids, studies have shown that the mu opioid receptor antagonist, such as naloxone, induces jumping behavior in morphine-addicted rats [12].

The receptor pairs with G proteins that bind to cell membranes and travel seven times the width of the

membrane. Mu (hair) is known as the morphine receptor and is generally derived from morphine and its stimulation causes a decrease in temperature. Binding of morphine to the μ receptor regulates G protein and reduces adenylate cyclase activity, thereby reducing cAMP. In addition, activation of the μ receptor causes the release of potassium ions and cellular hydrogen. Both decrease in cAMP and increase in potassium ion output prevent calcium from entering the cell and decrease intracellular calcium free levels [13]. Studies have illustrated that harmalin and its derivatives cause visual impairment, loss of coordination, excitement, and confusion, and paralysis at high doses. Harmalin is a calcium channel antagonist that can inhibit Na + / H + pump in Escherichia coli at concentrations.

The most important chemical compounds of Esfand plant are its alkaloids. So far, 12 types of alkaloids have been reported in Esfand, the most important of which are harmalin, harmin, harmalol and peganin. Identified amino acids in Pecan seed include proline, glycine, alanine, valine, leucine and phenylalanine and histidine and small amounts of lysine, serine, treonine, tyrosine and aspartic acid and arginine [14]. In this study, the effect of intraventricular injection of brain (I.C.V) agonist (morphine sulfate) and hair receptor antagonist (naloxone) on fear behavior in adult male Wistar rats was investigated.

2. Materials and Methods

Animals

In this study, adult male Wistar rats with a mean weight of 180-200 g were used. These animals were prepared from the Pasteur Institute of Iran and transferred to the college's home.

Then, in special cages for keeping animals, they were divided into groups of 6-7 and ready water and food were available to them in sufficient quantities. Behavioral testing was performed on each animal only once on the day of the experiment.

Anesthesia drug

To anesthetize the animals before surgery with a mixture of ketamine and glycine

- Physiological serum (saline)
- 10% formalin to stabilize brain tissue

Drugs injected I.C.V was dissolved in 1 ml of saline.
Medicines
The drugs used in this study are:
1 - Morphine sulfate (Morphine sulphate) prepared by Tamed Iran Company
2 - Naloxone
3- Harmalin
The instruments used in this research were:
1- Scales for weighing drugs with an accuracy of one tenth of a gram
2 - Scales for weighing animals
3 - Digital chronometer (timer) to measure the movement time of animals in the plus-maze
4 - A large number of 1 ml insulin syringes for injection of anesthetic
5- American-made Stereotax machine, Stoelting model
6 - Surgical instruments (scissors, pliers, scalpel ...)
7- Screw the glasses in large numbers
8- Screwdriver glasses
9 - A large number of dental Gage 21 needle heads to prepare the guide cannula
10 - A number of Gage 27 dental needles to prepare the injection cannula
11 - Polyethylene interface
12 - Hamilton syringe
13 - Stainless steel wire (to prevent clogging of the guide canal)
14- Dental acrylic and monomorphic for cement preparation
15 - Ruler
16 - Stone saw
17 - Penicillin powder and betadine solution for disinfection
18 - plus-maze device for behavioral testing

Animal surgery method

First, each rat was weighed and then a mixture of 2 ml of ketamine and glycine was used to anesthetize the animal. About 5 minutes after injection, the rat was completely anesthetized. After completing anesthesia and cutting the animal's hair, the animal was transferred to a stereotaxic device and to fix the animal's head, the ear rods were completely inserted into the incisor bar hole and the front teeth, then the skin on the skull from the middle. The two eyes make a vertical incision to the end of the occipital bone with a scalpel and remove the underlying muscles and clean the bone thoroughly with a cotton swab to determine the location of the bergama (where the frontal bones connect to the parietal) and the lambda (where the parietal bones connect to the occiput) (Figure 3) [15].

Then a hole with a diameter of one millimeter with anterior-posterior coordinates AP = 0.8 mm (Anterior-posterior)

Medium-lateral 1.6 = ML (Medial-lateral)

It was created in the skull by the head of a dental drill. 2 small screws of glasses were also screwed on the skull.

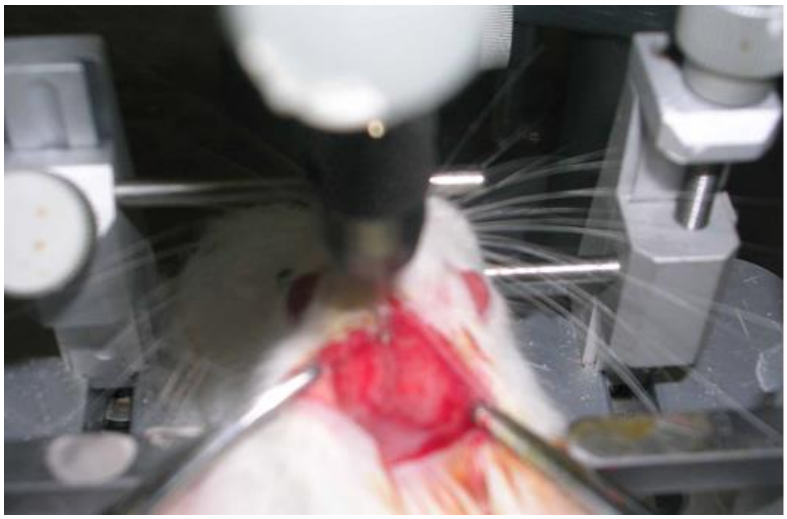

Figure 3. Animal skull and Bergma and Lambda spots

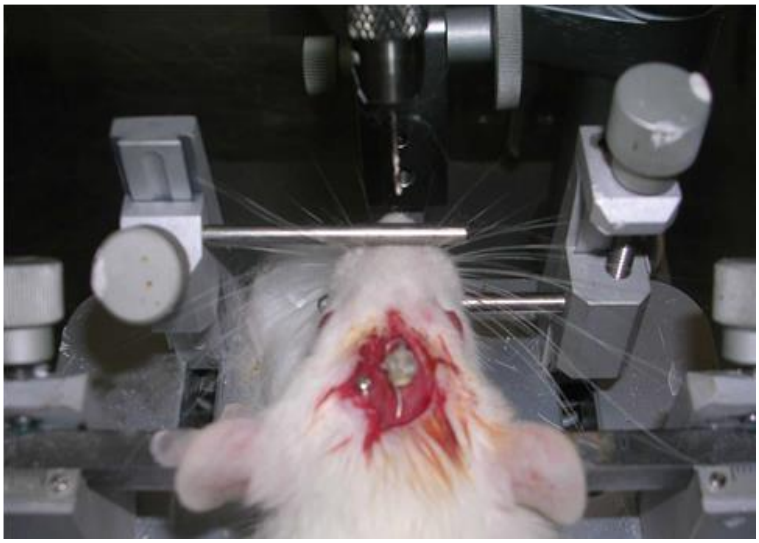

Figure 4. The guide cannula and screw are placed in the skull

How to place the guide cannula in the brain

After the animal underwent surgery and the location of the guide cannula was determined and the skull was pierced by the head of the dental drill, the mandible was punctured with a needle tip so as not to damage the mandible and arteries. Finally, the 3.5 mm guide cannula was fixed to the skull by dental cement and small spectacle screws (Figure 4). To prevent the cannula's duct from closing with blood, a stainless steel wire was inserted into the cannula's duct. Betadine and tetracycline powder were used for sterilization the wound. The operated mice were tested 5-7 days after surgery [16]. The Method of injection the drug into the lateral ventricle:

After a postoperative rest period, on the day of the experiment, the operated mice are transported to the laboratory. An injection cannula with a polyethylene interface and a Hamilton syringe was used for intraventricular injection. First, the injection cannula was connected to the polyethylene connector and then the connector was filled with the drug with an insulin syringe, then the Hamilton syringe was also filled with the drug and after connecting the polyethylene connector to the H syringe by driving some of the drug to the H syringe. The front and the appearance of a drop of medicine at the end of the injection cannula ensure that the route is open and full. The injection cannula is then inserted into the animal's brain through the guide cannula and one microliter of drug is slowly injected for one minute. After the injection, the injection cannula remains inside the cannula for about a minute and then exits.

It should be noted that in these experiments the needle head of the guide cannula is 3.5 mm and the needle head of the injection cannula was selected 3.8 - 3.7 mm to be 0.3 - 0.2 mm higher than the guide cannula. Follow the ICV injection completely into the ventricle on the day of injection after connecting the injection needle head to the guide cannula (Figure 5).

To ensure the correct injection site in surgical mice, 2 µl of methylene blue dye is injected into the ventricle through the guide canal after measuring the fear behavior.

The rat is then anesthetized with a high concentration of ether and the brain is removed from the skull and placed inside 10% formalin for a few days to stabilize the brain tissue.

Behavioral testing

Elevated plus-maze was used to measure fear. This tool is made of wood and has four arms in the figure of 4. The dimensions of the closed arm corridor were 50. 10 cm and the sides and end of the closed corridor had a wall 40 cm high and a 1 cm high edge made of glass was installed to prevent the rats from falling on both sides and the end of the open corridor. The four corridors lead to a central area measuring 10 x 10 cm. The maze is placed by bases at a height of 50 cm above the ground. Before being placed in the maze, each mouse was first placed in a box measuring 60 × 60 × 60 cm for 5 minutes. Previous studies have shown that this method increases the total number of animals entering different parts of the plus-maze. After the required time had elapsed, the animal was immediately transferred from the box to the maze, facing a closed corridor. Adequate lighting was provided by a 100-watt bulb 120 cm above the center of the maze. For 5 minutes the animal moved freely in different parts of the maze (Figure 6)[17].

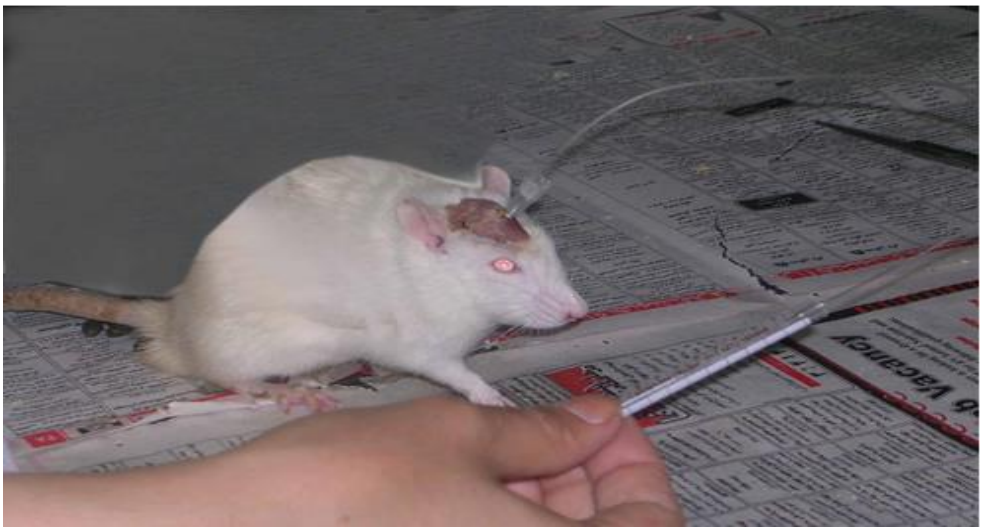

Figure 5. How to inject into the ventricle of the brain using a Hamilton syringe

Figure 6. Plus-maze device

The following parameters were measured by observation:
- The number of times the animal entered the open corridor.
- The number of times the animal entered the closed corridor.
How long the animal stays in the open corridor.
How long the animal stays in the closed corridor.

Statistical analysis

Statistical analysis: Statistical analysis of the results was performed using SPSS software. Unpaired T-test was used to compare the mean of the sham group with the mean of the positive control group and also to compare the means of the experimental groups with the means of the positive control group. To compare the means obtained from the experimental groups with the means obtained from the positive control group, we used one-way analysis of variance and, if necessary, two-way analysis. In all cases, $p < 0.05$ was considered as the limit of significant differences and all statistics were repeated in the text and figures as mean ± standard error (Mean) SEM).

3. Results

Testing groups include

-The effect of I.C.V injection of pure harmaline (50 µg/rat) against sham group (saline) on the occurrence of fear behavior.

- Evaluation of the effect of I.C.V morphine (0.5, 1, 2.5, 5 and 7 µg / rat) injection compared to sham group (saline) on the occurrence of fear behavior in male rats.

- The effect of I.C.V morphine sulfate (1, 2.5 and 5 µg/rat) injection on pure harmalin (50µg/rat) as positive control) in the occurrence of fear behavior in male rats.

- Evaluation of the effect of I.C.V naloxone (0.5, 1 and 2 µg/rat) injection compared to sham group (saline) on the occurrence of fear behavior in male rats

- Evaluation of the effect of I.C.V naloxone (1µg/rat) injection compared to the positive control group pure harmalin (50µg/rat) on the occurrence of fear behaviour in male rats.

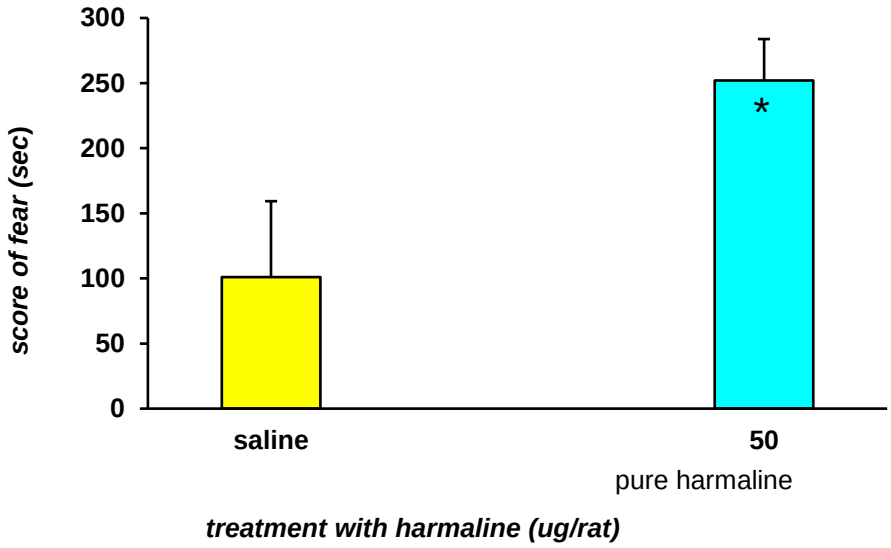

Chart 1. The effect of I.C.V injection of pure harmalin against sham group (saline) on the occurrence of fear behavior in male rats $P < 0.05$ $n = 6$.
According to the results, this dose of pure harmaline (50 µg / rat) was significantly ($p < 0.05$) effective in the occurrence of fear behavior. One way one-way analysis of variance - Anova and tukey test were performed, in all cases $P < 0.05$ indicates a significant level (n=6).

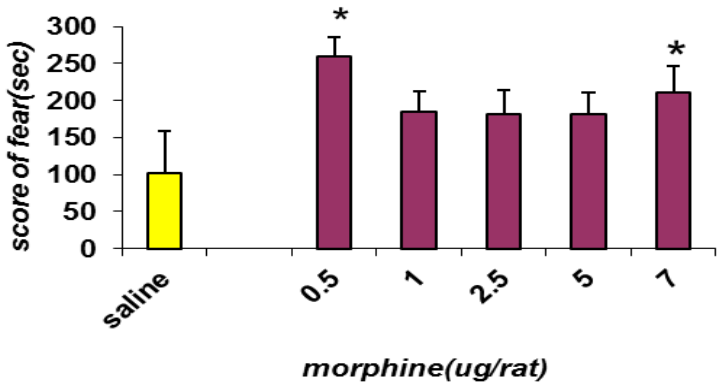

Chart 2. Evaluation of the effect of I.C.V morphine injection compared to sham group (saline) on the occurrence of fear behavior in male rats (P <0.05) n = 6.
In the above diagram, the effect of morphine sulfate injection at doses (0.5,1,2.5 , 5and 7 µg / rat) was compared with saline as sham group. According to the results, doses of (0.5 and ٧µ g/ rat) morphine sulfate were significantly effective in causing fear. In all cases (p <0.05) indicates a significant level (n =6).

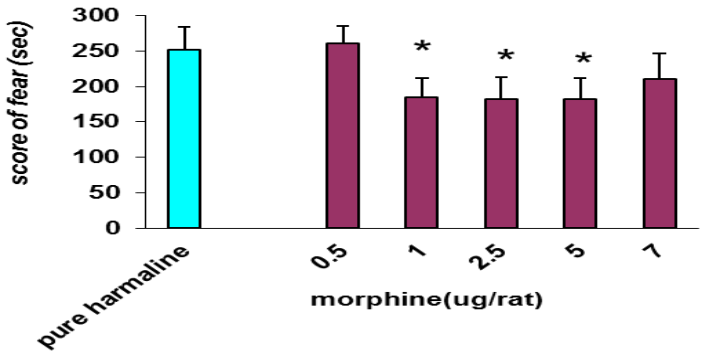

Chart 3. The effect of I.C.V morphine sulfate injection on pure harmalin (positive control) in the occurrence of fear behavior in male rats P <0.05 n = 6.
In the above diagram, the effect of morphine sulfate injection in doses (0.5,1,2.5, 5 and7 µg / rat) by I.C.V method and harmalin (50 µg/ rat) was compared as a positive control group. According to the results, doses (0.5, 1 and 2.5 µg / rat) of morphine sulfate are effective in comparison with pure harmalin and reduce fear behavior (P <0.05) in all cases .P <0.05 indicates a significant level. (n =6).

4. Discussion

In this study, after intraventricular administration (1 µl/rat) of saline in adult male rats (n =6 as sham group), harmalin (a component of Esfand alkaloids) with a dose (50 µg / rat) as Fear-inducing compounds were tested as a positive control group and different doses of morphine and naloxone were tested as an experimental group, and after comparing the results, the possible effect of the compound was confirmed (p <0.05).

Morphine was administered as a mu receptor agonist at doses of 5µg / rat (0.5, 1, 2.5, 5 and 7µg/rat) to study the fear behavior of the treated groups by I.C.V. (n =6).

The performance of the drug in the mentioned doses exposed that at the minimum (0.5 µg/rat) and maximum (7 µg/rat) morphine the time spent in the closed arm as well as the number of round trips in this arm in the plus device-increased the maze and was probably more effective than the control group treated with saline.

Studies indicated that morphine and morphine-like peptides such as methylvancephalin and endorphins can reduce noradrenaline release and reduce emotional responses. However, at intermediate doses (1,2.5 and 5 µg / rat), in comparison with the control group, no significant performance was observed in the occurrence of this process [18].

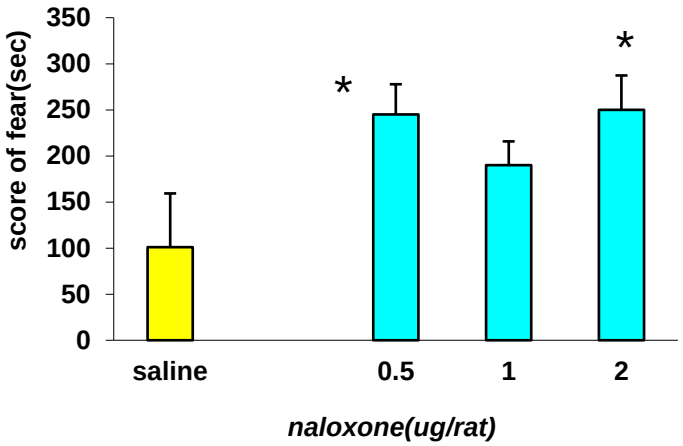

Chart 4. Evaluation of the effect of I.C.V naloxone injection compared to sham group (saline) on the occurrence of fear behavior in male rats P <0.05 n = 6.
In the above diagram, the effect of naloxone injection in doses (0.5, 1 and 2 µg / rat) was compared with i.c.v and saline (sham) methods. According to the results, doses of naloxone (0.5&2 µg / rat) in comparison with saline (sham) were effective in causing fear behavior. (P <0.05 in all cases (p <0.05 indicates a significant level. (n=6).In this star diagram at doses of (0.5&2µg / rat) indicates (p<0.05).

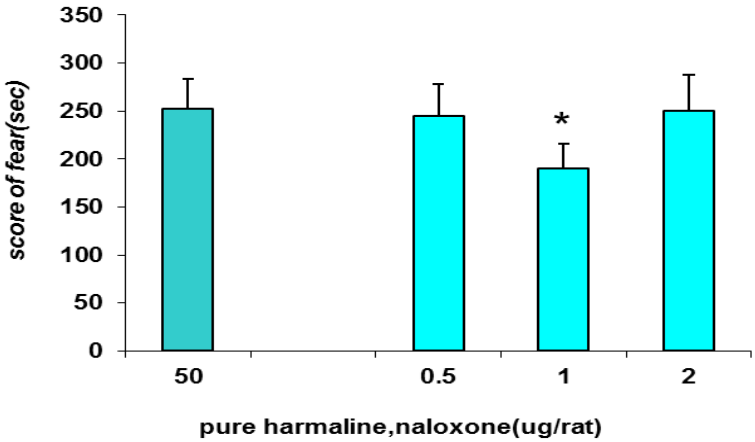

Chart 5. Evaluation of the effect of I.C.V naloxone injection compared to the positive control group (pure harmalin) on the occurrence of fear behaviour in male rats P <0.05 n = 6. In the above diagram, the effect of naloxone injection at doses (0.5, 1 and 2µg/rat) by I.C.V method and harmalin 50 / rat as a positive control group was compared. According to the results, 1 µg / rat dose of naloxone was more effective than harmalin. In this diagram, the dose of µg / rat (1) indicates (p <0.05).

However, Kapp and Galgher (1978) showed that opioids reduced the incidence of fear, so that injection of the mu receptor agonist into the amygdala reduced fear behavior. Research shows that stimulation of opioid receptors such as mu stimulates kappa receptors. It is possible that the doses of morphine (1,2.5 and 5 µg/rat) and naloxone (1 µg/rat) ineffective on fear behavior due to the role of kappa receptors [19].

In the study of moderate doses of morphine sulfate (1, 2.5 and 5 µg / rat), we found that the number of trips in the closed arm was higher but the time spent in the closed arm was reduced because in these doses,

comparison with control group did not have significant differences.

In 1996, Smart and colleagues demonstrated that opioids increase intracytoplasmic calcium. Administration of morphine releases calcium ions from brain cells and prevents them from being reabsorbed into the cell. Calcium prevents morphine-induced analgesia. Following these studies, high doses of morphine and naloxone may be effective due to the depletion of calcium ions, which stimulates the animal [20]. It should be noted that the mu and delta receptor agonists reduce cAMP synthesis by inhibiting the enzyme adenylyl cyclase from the G pathway of inhibitory proteins [21].

Thus, mechanisms associated with emotional responses such as fear may be blocked following cAMP inhibition and morphine injection. The ineffectiveness of moderate doses of morphine (1, 2.5 and 5 µg / rat) can be generalized to this problem. The scientists showed that there was an increase in post-synaptic dopamine receptor sensitivity following repeated morphine use. It is possible that local changes in the activity of dopamine pathways may play a role in causing fear behavior [22].

At doses of (1, 2.5& 5 µg/rat) morphine and (1 µg/rat) naloxone, there is a decrease in the excitability of the glutamatergic system. Blocking glutamate stimulation can have anti-fear effects. In fact, the application of NMDA and non-NMDA receptor antagonists into the lateral-basal part of the amygdala reduces fear in the animal.

The researchers pointed to the role of mu receptors in causing such behavior and noted that the effects of morphine-injected hatred may be explained by involvement of kappa receptors. Morphine injection at doses of 20 and 4 nmol / ml has been shown to induce fear behavior [23] .

In the present study, it was shown that increasing the dose of morphine (2.5 mg/kg) reduces anxiety activity and at low dose (0.5 µg/rat) this behavior occurs.

Morris (1978) showed that increasing the dose of morphine had anti-emotional and anti-fear effects. In this experiment, a low dose of (0.75 mg/kg) was effective [24].

Anseloni in 1999 showed that low-dose injection of morphine sulfate (7.6 µg/rat) had more pronounced effects on fear than higher doses (23 µg/rat) [25].

Similar to the evidence from morphine I.C.V injection, naloxone I.C.V injection is not dose-dependent in the development of fear behavior (p <0.05). As we know, naloxone reverses all the effects of morphine.

They showed that naloxone did not induce ethanol-induced conditioning preference place. The role of naloxone in increasing stress following elevated noradrenaline has been demonstrated in rats [5].

Studies show that injection of (µg/rat 2) naloxone into the midbrain of the mice causes fear behavior [26].

Therefore, it can be concluded that naloxone is probably involved in the development of emotional responses. Another study conducted in 1999 by researchers at the University of Medical Sciences found that naloxone induced such responses in addicted mice.

Researchers in 2002 showed that naloxone causes jumping behavior in addicted mice [27].

In this study, I.C.V (50 µg/rat) injection of pure harmalin, which is considered as a positive control group, shows the percentage of entry into the open arm and also the percentage of retention time in the open arm. Harmalin has been reported to tend to bind to 5-HT2A receptors due to its similar structure to serotonin.

Studies show that the more restorative 5-HT2A is occupied by harmaline, the more likely it is that fear behavior will occur [28].

According to the results of this study, pure harmalin compared to doses of 1, 2.5 and5 µg / rat) morphine sulfate and µg/rat) 1) naloxone occupy more 5-HT2A receptor and on the other hand compare Harmalin at doses of (0.5 and 7 µg/rat) morphine sulfate and (0.5 and 2µg/rat) naloxone indicate the effectiveness of these doses and their involvement with the 5-HT2A receptor. Therefore, it can be concluded that these doses have similar effects to harmalin.

On the other hand, studies have shown that the similarity of the molecular structure of alkaloids such as morphine sulfate and harmalin to the combination of serotonin can explain the high tendency of these compounds to bind to serotonergic receptors, which can potentially compete with serotonin as a competitive compound for occupying sites [28].

According to research, two types of receptors called NMDA (N-Methyl-D-aspartate) and AMPA may be involved in the occurrence of fear behavior. Increases and possibly causes fear behavior [29]. Therefore, it can be concluded that in the present study, the NMDA receptor at doses of µg/rat (1.2.5 and 5 µg/rat) morphine sulfate and (1 µg/rat) naloxone and also

(50µg / rat) pure harmalin in the behavior Fear does not play a significant role. On the other hand, doses of (0.5 and 7µg/rat) morphine sulfate and naloxane at the doses of (0.5 and 2µg/rat) showed similar effects in comparison with harmaline, which may be due to the multiplicity of NMDA receptors in these doses.

It should be noted that comparing the results of different doses of morphine sulfate and naloxone in the experimental groups shows that the dose of these drugs is not dose dependent in the occurrence of fear behavior. Perhaps the phenomenon of neuronal adaptation can be discussed here. Thus, when these drugs have an effect on the areas affected by the behavior of opioid receptors, they act. Mastuzawa's 1999 study showed that the kappa receptor has a modulatory role in the development of fear behavior. Therefore, this receptor has probably played a prominent role in doses of (1,2.5 and 5µg/rat) morphine sulfate and naloxone(1µg/rat) and has moderated this behavior [26].

5. Conclusion

In conclusion, the comparative effect of harmalin as a frightening substance with different doses of morphine and naloxone indicates that these compounds are involved in the occurrence of fear behavior in relation to harmalin. As the results show, none of the data used in the present study in the area has a uniform performance and this diversity can be considered as a result of various mechanisms that require more detailed studies.

Ethical Considerations

Compliance with ethical guidelines

As the format of article is review and we have not used any animal sample for getting data so the ethical considerations are according principles of this concept. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them. A written consent has been obtained from the subjects.

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Author's contributions

Farzaneh Najar is a main writer and corresponding author from department of basic science, Medicine Branch, Islamic Azad University, Tehran, Iran and Azam Afagh is my colleague from department of Biology, Sofian Branch, Islamic Azad University, Sofian, Iran. She has helped me during selecting best references for article.

Conflict of interest

The Authors declare that there is no conflict of interest.

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