http://jbcp.shahed.ac.ir 10.22070/JBCP.2021.14570.1144

Open Access Research Article

Journal of Basic and Clinical Pathophysiology
Print ISSN: 2322-1895, **Online ISSN:** 2345-4334
Vol. 9, No.1, June 2021

A Semiannual Publication of
Shahed University Press

Effect of simultaneous injection of l-arginine in dorsal hippocampus and laterodorsal periaqueductal gray matter on morphine-induced analgesia in rat's formalin test

Mahboobeh Hashemi[1], Manizheh Karami[1]*, Mohammadreza Zarrindast[2]

1. Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran
2. Department of Pharmacology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background and Objective: Injection of l-arginine, a precursor of nitric oxide, in the rat's hippocampus or periaqueductal gray matter reduces the analgesic effect of morphine on formalin-induced pain, but the effect of simultaneous injection of the substance in both areas have not been shown as our purpose of this research.

Materials and Methods: Wistar rats were used as control, morphine, and l-arginine groups. The rats were simultaneously cannulated in two areas of the dorsal hippocampus and laterodorsal PAG. One week later, the control animals received 50 µl of 2.5% formalin in the paw of the left foot under restrainer. The morphine group 10 min before formalin received the opioid (6 mg/kg, intraperitoneally). Other groups took l-arginine (0.25-2 µg/rat) in only one area (d hippocampus or ld PAG), prior to morphine administration. The effective dose of l-arginine (0.5 µg/rat) simultaneously was injected in both areas. The findings were analyzed by analysis of variance (ANOVA) under α=0.05.

Results: Morphine induced analgesic response. Injection of NO precursor both separately and simultaneously in the two nuclei reduced morphine-induced analgesia.

Conclusion: Increasing levels of NO due to exclusive or concurrent injection of l-arginine in the areas likely antagonize the morphine response.

Keywords: L-arginine, PAG, Hippocampus, Formalin test

1. Introduction

Nitric oxide is an endogenous gas that acts as a signal molecule in the human body (1). NO synthase (NOS) enzymes catalyze the oxidation of l-arginine to l-citrulline through a way dependent to NADPH and tetrahydrobiopterin (BH4) and NO is produced as one of the reaction products (2). Cytosolic guanylate cyclase (sGC) is the major intracellular receptor for NO (3). This molecule plays an important role in acute and chronic pain at the central and peripheral levels. Peripheral morphine analgesia occurs after activation of the l-arginine/NO-cGMP pathway (4).

The hippocampus is a part of the limbic system that has long been shown to play a role in memory and learning. There is evidence from human and animal studies that show that hippocampal formations can also play an important role in the pain process (5). An increase in neuronal NOS-positive cells in the hippocampus has repeatedly been observed with morphine injection in treated rats (6, 7).

The periaqueductal gray matter (PAG), the collection of bodies of neurons around the Sylvius duct, plays an important role in the analgesic process due to the presence of opioid-like peptides such as enkephalins and dinorphins (8). The role of NO in PAG in analgesic reactions and defense reflexes due to activation of NMDA glutamate receptor in the brain has previously been identified (8). Injecting NO inhibitors (such as L-NAME) into PAG significantly eliminates the analgesic effect of this area, and the researchers postulated that N2O (the oxide of nitrous or laughing gas) in PAG causes analgesia by an opioid

Revised: 09 May 2021, **Accepted:** 23 June 2021, **Available online:** 28 June 2021
Corresponding Author: Manizheh Karami **E-mail:** karami@shahed.ac.ir

receptor-dependent mechanism (9). The PAG consists of distinct nuclei that receive selective inputs from the frontal cortex, amygdala, hypothalamus, and pain pathways, and its lateral dorsal part contains neurons that express NADPH-positive neurons (10). As mentioned, this cofactor is involved in NO production, but despite the positive reaction of neurons in this area, the role of NO in this center of downstream pain path is not clear. Therefore, the aim of this study was to investigate the role of NO in the descending pain path. In one hand, we treated the animal model of inflammatory pain, which received effective anti-pain dose of morphine (i.p.), by l-arginine in one of two interested areas. On the other hand, aside to interact between the opioid and nitrergic systems on the d hippocampus and ld PAG levels, we assessed the modulatory involvement of NO on morphine-induced analgesic flow in the animal model of inflammatory pain by simultaneously injecting NO precursor in both nuclei.

2. Materials and Methods

2.1. Animals

Male Wistar rats weighing 250 g were used for experiments. The animals were kept in the Shahed University animal care center in light conditions for 12 h in the dark, 12 h in the light and at a temperature of 21 ± 3°C. Ethical considerations were made in accordance with the principles of working with animals and the research was approved by the local animal ethics commission (IR.SHAHED.REC.1399.107).

2.2. Drugs

In this study, morphine (Temad, Iran), l-arginine (Sigma, Germany) and ketamine and xylazine (Veterinary Organization, Iran) were used.

2.3. Surgery and cannulation

The animals were placed into the stereotaxic system after injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and cannulated in the dorsal hippocampus with AP: -3.8 mm, L: 1.8 ± 2.2 mm, V: 3 mm (13) and the lateral dorsal part of PAG with these coordinates AP: 7.8 mm, L: 0.7 mm, V: 4.8 mm. One week after recovery, the animals were divided into control and experimental groups.

2.4. Prescribing drugs

Drugs (at a volume of 1 µl) were injected during 30 sec using a 5 µl Hamilton syringe attached to the polyethylene tubing equipped with an injection cannula (27 gauge) that was 1 mm longer than the guide cannula. To ensure injection of the substance in the target area, after additional 30 sec, the injection cannula was removed from the guide. Morphine at a dose of 6 mg/kg (13) was injected intraperitoneally (i.p.) to induce analgesia in the formalin test.

2.5. Formalin test

Formalin test was used to measure pain. In order to adapt, the rats were first left in the test chamber (a 30 × 30 × 30 cm glass chamber equipped with a mirror at a 45-degree angle) for about 15 min. L-arginine was prescribed intra-cerebrally prior to morphine, then intraperitoneal (i.p.) injection of the drug morphine, and then inoculation of 50 µl of formalin solution (2.5%) into the left foot paw of the rat was arranged. The treated animal was returned to the formalin test chamber, which during testing (1 h) all animal's behavioral signs was recorded and the results were quantitatively measured. Two time intervals (0-5 and 15-60 min) were considered as a measure of acute and chronic pain and throughout these time courses per 15 sec, the animal's behavioral observations were quantified according to the following criteria.

Zero: When the animal does not react and the injected foot is completely on the surface of the formalin mirror.

One: The animal exerts most or all of its weight pressure on the opposite leg and the injected leg rests (favoring).

Two: The injected foot is completely separated from the surface and hang up (elevating).

Three: The animal licks or bites the injected foot (licking and biting).

2.6. Euthanasia

At the end of the experiments, all animals were killed with carbon monoxide. In order to confirm the accuracy of the cannulation, 1 µl of methylene blue solution was injected with a Hamilton syringe into the desired areas. The brain samples were eventually fixed in formalin 10% and cut in slices.

2.7. Statistical calculations

All data were analyzed by analysis of variance (ANOVA) after normality test and $p < 0.05$ was considered significant. In case of rejecting the null hypothesis (equality of variances), further tests by Tukey's HSD were used to investigate the differences between the groups. Data were shown as mean ± standard error in the tables.

3. Result

3.1. The effect of l-arginine injection into the dorsal hippocampus on morphine-induced analgesia in formalin test

L-arginine in doses of 0.25, 0.5, 1 and 2 µg per rat was injected at a volume of 1 µl using a Hamilton syringe in the dorsal hippocampus; the same amount of saline (1 µl) was injected into the PAG. Then, morphine at a dose of 6 mg/kg was i.p. injected 10 min before the formalin test. Formalin was injected subcutaneously

(s.c) in the left foot paw of the animal and the animal was placed in the formalin mirror chamber and tested; the control group solely received saline (Table 1). As data show, administration of l-arginine in the d hippocampus (saline cross-section) prior to morphine injection independent of dose is effective on morphine response in formalin test in both acute and chronic phases: there is a statistically significant reduction in morphine-induced analgesia.

Table 1. The effect of l-arginine pre-injection in the d hippocampus on morphine-induced analgesia

Treatment	Chronic phase	Acute phase
Saline (1 µl)	377 ± 6.8	44 ± 3.8
Morphine (6 mg/kg)	*193 ± 19	*27 ± 7.2
L-Arginine 0.25 µg/rat	*249 ± 8	*36 ± 3
L-Arginine 0. 5 µg/rat	*299 ± 35	*46 ± 3
L-Arginine 1 µg/rat	*274 ± 25.6	*40 ± 3.5
L-Arginine 2 µg/rat	*278 ± 58	*39 ± 2.6

Data are given in the table as mean ± standard error. According to the statistical analyses, morphine-induced analgesia showed attenuation in the L-arginine treated rats compared to the control. Tukey's *post hoc* test provided the between group differences: *P<0.05

3.2. The effect of injection of l-arginine into the dorsolateral PAG on morphine-induced analgesia in the formalin test

L-arginine in doses of 0.25, 0.5, 1 and 2 micrograms per rat was injected using a Hamilton syringe at a volume of one µl in the dorsolateral region of the PAG and in the other region, the d hippocampus, 1 µl of saline was inoculated. Morphine (6 mg/kg, i.p.) was then injected and the formalin test was performed. After that, the animal was placed in the test chamber (for testing) and the results are shown in Table 2. As can be seen, l-arginine injection in this area as the precursor of NO is much more effective on the morphine response in the formalin test in both acute and chronic phases (reduction of morphine response more pronouncedly shown than Table 1).

Table 2. Effect of l-arginine pre-injection in the ld PAG on morphine-induced analgesia

Treatment	Chronic phase	Acute phase
Saline (1 µl)	377 ± 6.8	44 ± 3.8
Morphine (6 mg/kg)	*193 ± 19	*26 ± 7.2
L-Arginine 0.25 µg/rat	*353 ± 5.3	*42 ± 5.6
L-Arginine 0. 5 µg/rat	*354 ± 38	*37 ± 5
L-Arginine 1 µg/rat	*302 ± 69	*38 ± 7
L-Arginine 2 µg/rat	*282 ± 6.7	*33.7 ± 1.5

Data are given in the table as mean ± standard error. The statistical analyses show that morphine-induced analgesia attenuated in the L-arginine treated rats compared to the control. The between group differences obtained by Tukey's *post hoc* test: *P<0.05.

3.3. Result of injection of effective dose of L-arginine simultaneously to both nuclei (dorsal hippocampus and dlPAG)

According to Tables 1 and 2, the effective doses of l-arginine (both at hippocampal area and PAG) was selected and injected simultaneously into both areas. The morphine at a dose of 6 mg/kg, ten min before the test was given. Formalin was injected and the animal was tested in a formalin mirror (Table 3). Injecting the l-arginine into both the gray matter and the hippocampus does not have a much more reducing effect on morphine-induced analgesia.

Table 3. Effect of injection of effective dose of l-arginine simultaneously to both target areas of rat's brain (d hippocampus and dl PAG)

Treatment	Chronic phase	Acute phase
Saline (1 µl)	377 ± 6.8	44 ± 3.8
Morphine (6 mg/kg)	*193 ± 19	*27 ± 7.2
L-Arginine 0.5 µg/rat	*324 ± 38	*37.5 ±

Data are given in the table as mean ± standard error. The analyses show that morphine-induced analgesia attenuated in the l-arginine treated rats compared to the control. between group differences were obtained by Tukey test: * P<0.05.

4. Discussion

The aim of this study was to investigate the effect of simultaneous injection of l-arginine on both the dorsal hippocampus and the laterodorsal periaqueductal gray matter on morphine-induced analgesia in the rat's formalin test. The control (saline) and morphine and l-arginine (a precursor of nitric oxide) groups were compared based on the ANOVA analyses. The precursor had a reducing effect on morphine analgesia either in one area or by simultaneous injection in the two areas.

With respect to specific role of the hippocampus in the pain memory (11), this finding is consistent with the previous results achieved by the precursor of NO (12), the l-arginine, in the rat formalin test (13). On the other hand, the PAG is known as an important area in the downward modulation pain path (14), and in this area, the NO precursor, l-arginine, reduced the analgesic effect of morphine, which is in line with the results of others (15, 16).

By comparing the result of injections of precursor individually in one of these two areas (the hippocampus or the periaqueductal gray matter), the material treatment in the PAG provided much more reduction of the analgesic effect of morphine, indicating that area as a key center in the path of pain. However, due to simultaneous injection of l-arginine into both regions, according to the data, not much difference was observed with the injection of this substance, in only one region, and this finding may show the NO mediation in the downstream pain path as been previously proposed (4).

Although pain as a whole is defined as a strong and unpleasant sensation, but chronic pain is associated with more complex emotional disorders such as anxiety-depressive symptoms and cognitive deficits than acute pain (17). The underlying mechanisms have not yet well understood, but the interactions between the frontal cortex and limbic structures is thought to play an important role in the matter.

As has recently been reviewed, evidence from studies on the rodent brain suggests that neuroplastic-related changes occur not only in the cerebral cortex but also below the cortex (amygdala and hippocampus) (17). Therefore, the present study can propose new interactions, but it is very highly complicated and can only be clearly interpreted by the help of additional studies.

The descending path of pain from the limbic area such as from d hippocampus to the gray matter around the Sylvius aqueduct shows interconnection. However, whether this association is related to prominent neurotransmitter and neuromodulatory systems, including glutamate and GABA and endogenous opioids, or whether NO directly mediates morphine analgesia, remain as vague that we suggest for future studies.

In summary, increasing levels of NO due to exclusive or concurrent injection of l-arginine in the studied areas likely antagonize the morphine response.

Acknowledgment

Shahed University is appreciated for its support of post-graduate research program.

References

1. Tenopoulou M, Doulias PT. Endothelial nitric oxide synthase-derived nitric oxide in the regulation of metabolism. F1000 Research 2020; 9:F1000 Faculty Rev-1190. doi: 10.12688/f1000research.19998.1. eCollection 2020.
2. Ghimire K, Altmann HM, Straub AC, Isenberg JS. Nitric oxide: what's new to NO?. American Journal of Physiology 2017; 312(3):C254-C262. doi: 10.1152/ajpcell.00315.2016.
3. Zhang N, Beuve A, Townes-Anderson E. The nitric oxide-cGMP signaling pathway differentially regulates presynaptic structural plasticity in cone and rod cells. The Journal

of Neuroscience 2005 ; 25(10):2761-70. doi: 10.1523/JNEUROSCI.3195-04.2005.
4. Cury Y, Picolo G, Gutierrez V. Pain and analgesia: The dual effect of nitric oxide in the nociceptive system. Nitric Oxide 2011; 25(3):243-54. doi: 10.1016/j.niox.2011.06.004.
5. LIU M-G, Chen J. Roles of the hippocampal formation in pain information processing. Neuroscience Bulletin 2009; 25(5):237-66. doi: 10.1007/s12264-009-0905-4.
6. Toda N, Kishioka S, Hatano Y, Toda H. Modulation of opioid actions by nitric oxide signaling. Anesthesiology 2009; 110(1):166-81. doi: 10.1097/ALN.0b013e31819146a9.
7. Nazeri M, Razavinasab M, Abareghi F, Shabani M. Role of nitric oxide in altered nociception and memory following chronic stress. Physiology Behavior 2014; 129:214-20. doi: 10.1016/j.physbeh.2014.02.054.
8. TadeuMiguela T, LuizNunes-de-Souza R. Defensive-like behaviors and antinociception induced by NMDA injection into the periaqueductal gray of mice depend on nitric oxide synthesis. Brain Research 2006; 1076(1):42-8. doi: 10.1016/j.brainres.2005.12.095. Epub 2006 Feb 13.
9. Emmanouil DE. Nitrous oxide-antinociception is mediated by opioid receptors and nitric oxide in the periaqueductal gray region of the midbrain. European Neuropsychopharmacology 2008; 18(3):194-9. doi: 10.1016/j.euroneuro.2007.06.008.
10. Benarroch EE. Periaqueductal gray; An interface for behavioral control. Neurology 2012; 78(3):210-7. doi: 10.1212/WNL.0b013e31823fcdee.
11. Tyrtyshnaia A, Manzhulo I. Neuropathic Pain Causes Memory Deficits and Dendrite Tree Morphology Changes in Mouse Hippocampus. Journal of Pain Researcg 2020; 13:345-354. doi: 10.2147/JPR.S238458.
12. Hafeshjani ZK, Karami M, Biglarnia M. Nitric oxide in the hippocampal cortical area interacts with naloxone in inducing pain. Indian Journal of Pharmacology 2012; 44(4):443-7. doi: 10.4103/0253-7613.99299.
13. Hashemi M, Karami M, Zarrindast MR, Sahebgharani M. Role of nitric oxide in the rat hippocampal CA1 in morphine antinociception. Brain Research 2010; 1313:79-88. doi: 10.1016/j.brainres.2009.11.020.
14. Huang J, Gadotti VM, Chen L, Souza IA, Huang S, Wang D, Ramakrishnan C, Deisseroth K, Zhang Z, Zamponi GW. A neuronal circuit for activating descending modulation of neuropathic pain. Nature Neuroscience 2019; 22(10):1659-1668. doi: 10.1038/s41593-019-0481-5.
15. Lin J, Zhang X, Li C, Zhang Y, Lu H, Chen J, Li Z, Yang X, Wu Z. Evodiamine via targeting nNOS and AMPA receptor GluA1 inhibits nitroglycerin-induced migraine-like response. Journal of Ethnopharmacology 2020; 254:112727. doi: 10.1016/j.jep.2020.112727.
16. Ally A, Powell I, Ally MM, Chaitoff K, Nauli SM. Role of neuronal nitric oxide synthase on cardiovascular functions in physiological and pathophysiological states. Nitric Oxide 2020; 102:52-73. doi: 10.1016/j.niox.2020.06.004.
17. Thompson JM, Neugebauer V. Cortico-limbic pain mechanisms. Neuroscience Letter 2019; 702:15-23. doi: 10.1016/j.neulet.2018.11.037.