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Prenatal morphine exposure induces molecular and structural alterations in the developing hippocampus of neonatal rats

Pooya Nadri [1], Zahra Daneshfar [2], Zahra Azarmehr [3], Samaneh Farrokhfar [4]*

[1] Student Research Committee, Ramsar Campus, Mazandaran University of Medical Sciences, Ramsar, Iran
[2] Ramsar Campus, Mazandaran University of Medical Sciences, Ramsar, Iran
[3] Department of Hematology, Tehran University of Medical Science, Tehran, Iran
[4] Department of Anatomical Sciences, Faculty of Medicine, Ramsar Campus, Mazandaran University of Medical Sciences, Ramsar, Iran

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ABSTRACT

Objective(s): Prenatal exposure to opioids such as morphine poses significant risks to fetal neurodevelopment, particularly in brain regions critical for cognition, such as the hippocampus. Despite the prescription and use of opioids during pregnancy, the molecular and histological consequences of such exposure remain insufficiently explored. To evaluate the effects of short-term prenatal morphine exposure on the expression of key neurodevelopmental genes and the structural integrity of the hippocampus in neonatal rats.

Materials and Methods: Pregnant Sprague Dawley rats were administered intraperitoneal injections of morphine sulfate (10 mg/kg) on gestational days 15 and 16. On postnatal day 12, offspring (n = 6 per group) were euthanized, and their hippocampal tissues were collected. Quantitative real-time PCR was performed to assess the expression levels of neurodevelopmental genes, including MDH2, Neurog1, and BDNF. Histological evaluations were conducted using hematoxylin and eosin and cresyl violet staining to assess cellular architecture and neuronal viability. Immunohistochemical staining for GFAP, S100, and synaptophysin was used to evaluate astrocytic integrity and synaptic density.

Results: The morphine-exposed group showed significant up-reglation of MDH2, Neurog1, and BDNF ($P<0.05$). Histological analyses revealed neuronal degeneration and inflammatory infiltration in the hippocampus. Immunohistochemistry demonstrated a marked reduction of GFAP, S100, and synaptophysin signals, indicating substantial glial loss and synaptic disruption.

Conclusion: Prenatal morphine exposure leads to marked molecular and histopathological changes in the developing hippocampus, suggesting long-term risks for neurocognitive dysfunction. These findings emphasize the importance of limiting opioid use during pregnancy and identifying molecular targets for future therapeutic interventions.

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Introduction

Morphine is a widely used opioid analgesic that acts on the central nervous system (CNS) to alleviate moderate to severe pain. Its mechanisms involve inhibition of cyclic AMP production, modulation of intracellular calcium dynamics, activation of potassium channels, neuronal hyperpolarization, and suppression of neurotransmitter release (1). In recent years, the prevalence of opioid use among pregnant women has increased substantially (2). Morphine is often prescribed during pregnancy for acute pain management in conditions such as renal colic, gallstones, and severe migraine (3). However, morphine readily crosses the placental barrier, exposing the developing fetus to its pharmacological effects. Prenatal opioid exposure has been associated with a range of adverse outcomes, including intrauterine growth restriction, preterm birth, low birth weight, and increased neonatal morbidity and mortality (4, 5). The perinatal period is a critical window for brain development, particularly in regions such as the hippocampus, which are essential for memory formation and cognitive processing. Disruption of early neurodevelopmental processes, including neurogenesis, gliogenesis, and synaptogenesis, by opioids may lead to long-term deficits in brain structure and function (6). The hippocampus is recognized not only for its essential functions in memory formation, learning, and anticipating future events, but also for its involvement in a wide range of neurological and psychological disorders (7-9). Moreover, recent studies have raised concerns regarding opioid-induced teratogenic effects and alterations in cortical organization, including reduced cortical thickness and ventricular abnormalities (2). Despite these findings, the specific molecular and histological effects of prenatal morphine exposure on hippocampal development remain poorly understood. Genes such as Malate dehydrogenase 2 (*MDH2*), Neurogenin 1 (*Neurog1*), and Brain-derived neurotrophic factor (*BDNF*) play critical roles

****Corresponding author:*** Samaneh Farrokhfar. Department of Anatomical Sciences, Faculty of Medicine, Ramsar Campus, Mazandaran University of Medical Sciences, Ramsar, Iran. Email: s.farrokhfar@mail.mazums.ac.ir

in neurodevelopment. *MDH2* plays a role in regulating behavioral and cognitive gene expression. Mutations in *MDH2* have been linked to developmental and epileptic encephalopathy 51, as well as hereditary paraganglioma-pheochromocytoma syndromes (10). *Neurog1,* expressed in neural progenitor cells, is crucial for neuronal differentiation, olfactory system development, and the regulation of cell proliferation, cell fate determination, and neuronal migration (11). *BDNF* is a key growth factor in the CNS, involved in neural plasticity and associated with psychiatric disorders, Parkinson's disease, and Alzheimer's disease (12). However, their regulation in the context of prenatal morphine exposure remains largely unexplored.

Previous research has primarily focused on behavioral outcomes, with limited molecular and histological analysis. Studies in animal models show that prenatal morphine exposure reduces hippocampal pyramidal neurons (13), spatial learning, and long-term potentiation (14) and leads to altered memory performance (15), inhibitory control, and learning tasks, as well as attention deficits in adulthood (16). Another study shows that μ-opioid receptors in brain structures controlling seizures are sex-specifically altered by prenatal morphine exposure in adult offspring (17). In MRI studies, infants exposed to opioids during pregnancy had smaller volumes in several brain regions—including the cerebellum, deep gray matter, bilateral ventrolateral thalamic nuclei, bilateral insular white matter, subthalamic nuclei, and brainstem—while the right cingulate gyrus and left occipital white matter were larger compared with controls (18, 19). Studies show significant differences in fiber count in connections between the right superior frontal gyrus and the right paracentral lobule and between the right superior occipital gyrus and the right fusiform gyrus (20). In the present study, we aimed to investigate the impact of short-term maternal morphine administration during pregnancy on the expression of key neurodevelopmental genes and the histological integrity of the hippocampus in newborn rats. Particular attention was given to changes in astrocytic and synaptic markers, as well as evidence of neuronal damage and inflammation, to better understand the potential mechanisms of morphine-induced neurotoxicity during the perinatal period.

Materials and Methods

Animal treatment

Six adult female Sprague Dawley rats were housed under standard laboratory conditions (12-hr light/dark cycle, controlled temperature and humidity, and ad libitum access to food and water) and acclimatized for 10 days. After mating and confirmation of pregnancy via the presence of a vaginal plug, the animals were randomly divided into two groups: control and morphine-treated. On gestational days 15 and 16, the control group received intraperitoneal injections of 1 ml of normal saline, while the treatment group received intraperitoneal injections of morphine sulfate (10 mg/kg; Temad Co., Iran) (21, 22) once daily for 2 consecutive days.

Experimental procedures

On postnatal day 12, a critical time point for hippocampal development (23), two offspring from each dam (n = 6 pups per group) were randomly selected and euthanized in accordance with institutional ethical guidelines. The hippocampi were rapidly extracted for molecular, histological, and immunohistochemical analyses.

Molecular assessment (qRT-PCR)

Total RNA was extracted from hippocampal tissue using a standard TRIzol-based method. RNA concentration and purity were determined spectrophotometrically, and cDNA was synthesized using a reverse transcription kit (SMOBIO, Korea). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Ampliqon, Denmark) to assess the expression of *MDH2*, *Neurog1*, and *BDNF*, with Actb as the housekeeping gene. Primers were designed using the NCBI Primer-BLAST and OligoAnalyzer tools and synthesized in lyophilized form (Table 1).

The study was conducted in accordance with the ethical standards established by the Laboratory Animal Center of the National Institutes of Health Research.

Histological and immunohistochemical analysis

Extracted hippocampal tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm. Sections were stained with Hematoxylin and Eosin (H&E) for general morphology and Cresyl Violet for Nissl body visualization. Immunohistochemical staining was performed using primary antibodies against glial fibrillary acidic protein (GFAP), S100, and synaptophysin (Syn) using chromogen-based detection kits. Sections were examined under a light microscope.

Statistical analysis

Data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, USA). Normality was assessed using the Shapiro-Wilk test. Differences between groups were evaluated using an independent samples t-test. A

Table 1. Sequence of primers used for real-time PCR

Gene	Primer sequences Forward/Reverse	Length	Melting temperature (° C)
MDH2	F: GATCTCTCAGTGTACCCCCAA	21	58.53
	R: CTTCAGTGCCAGCCTCCT	18	58.92
Neurog1	F: AAGCCCATTCCCTCCCTGA	19	60.23
	R: CACTTACTGTCCGTATGACCCG	22	60.48
BDNF	F: GCCTCCTCTGCTCTTTCTG	19	57.54
	R: TTATCTGCCGCTGTGACC	18	57.07

P-value<0.05 was considered statistically significant.

Ethical approval

All experimental procedures were conducted in compliance with the ethical standards of the Mazandaran University of Medical Sciences (Ramsar Campus) and were approved by the Institutional Animal Care and Use Committee (Ethics code: IR.MAZUMS.RIB.REC.1401.038), in accordance with the Helsinki Declaration.

Results

Gene expression analysis by qRT-PCR

Quantitative real-time PCR analysis revealed that the expression levels of MDH2, Neurog1, and BDNF were significantly increased in the morphine-treated pregnant mice (M group) compared to the control group (C) (*P*<0.05). The relative fold changes in gene expression are illustrated in Figure 1a. The differences were statistically significant, as indicated by asterisks (*). The presence of specific amplification bands for all three genes in both groups was confirmed by agarose gel electrophoresis (Figure 1b), supporting the reliability of the qRT-PCR results.

Histological evaluation of the hippocampus

Histological analysis of hippocampal tissue revealed clear signs of neuronal damage in the morphine-treated group. Cresyl Violet staining demonstrated numerous pyknotic nuclei, appearing as white and shrunken regions, indicative of neuronal degeneration (Figure 2a). In addition, Hematoxylin and Eosin (H&E) staining showed dense infiltration of inflammatory cells along the inner boundary of the hippocampus (Figure 2b), suggesting the presence of a localized inflammatory response.

Immunohistochemical analysis

Immunohistochemical staining for GFAP, S100, and synaptophysin showed complete absence of immunoreactivity in the hippocampal sections of morphine-treated mice. GFAP and S100 are established markers for astrocytes, and their absence suggests severe astrocytic loss. Similarly, the lack of synaptophysin staining—a presynaptic vesicle protein—indicates significant disruption of synaptic integrity. These findings are indicative of extensive cellular and structural damage in the hippocampal region (Figure 3).

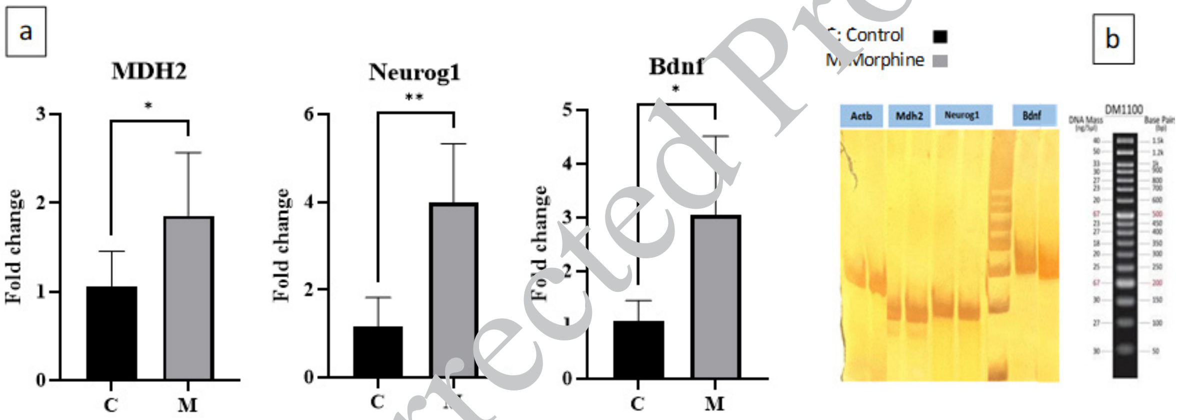

Figure 1. Comparison of gene expression alterations between the control group and the morphine-treated rat group
a. The study results demonstrate a significant increase (*P*<0.05) in the expression of MDH2, Neurog1, and BDNF genes in pregnant mice treated with morphine compared to the control group. This difference is marked by an asterisk (*). b. Photo of agarose gel electrophoresis to confirm the bands of the genes under investigation

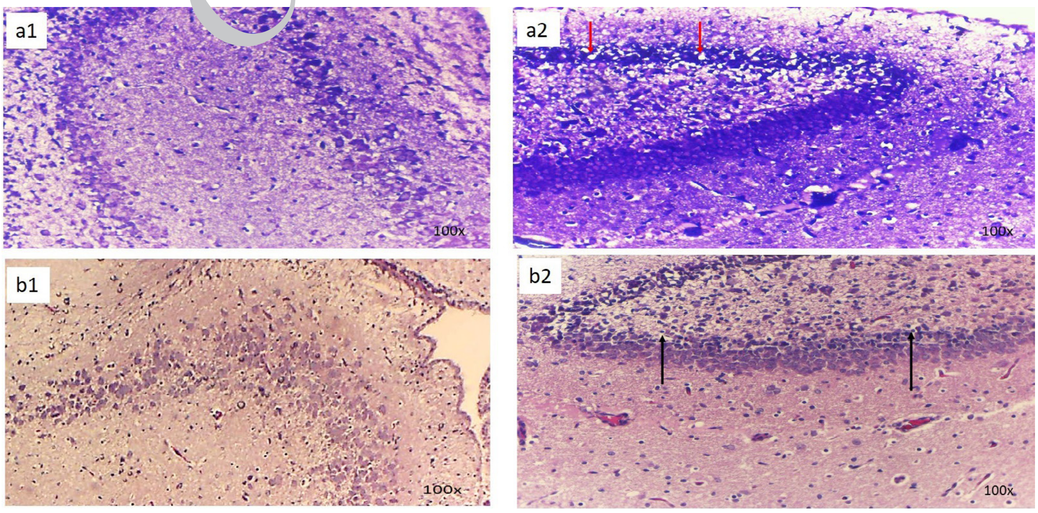

Figure 2. a) Cresyl violet is used for staining to assess cellular architecture and neuronal viability. a) Image a is a Cresyl Violet stain showing white pyknotic spots in the hippocampus region indicating damage and indicated by arrows. b) In H&E staining, the accumulation of inflammatory cells at the inner periphery of the hippocampus is indicated by arrows (Groups a1 and b1 are the control groups).

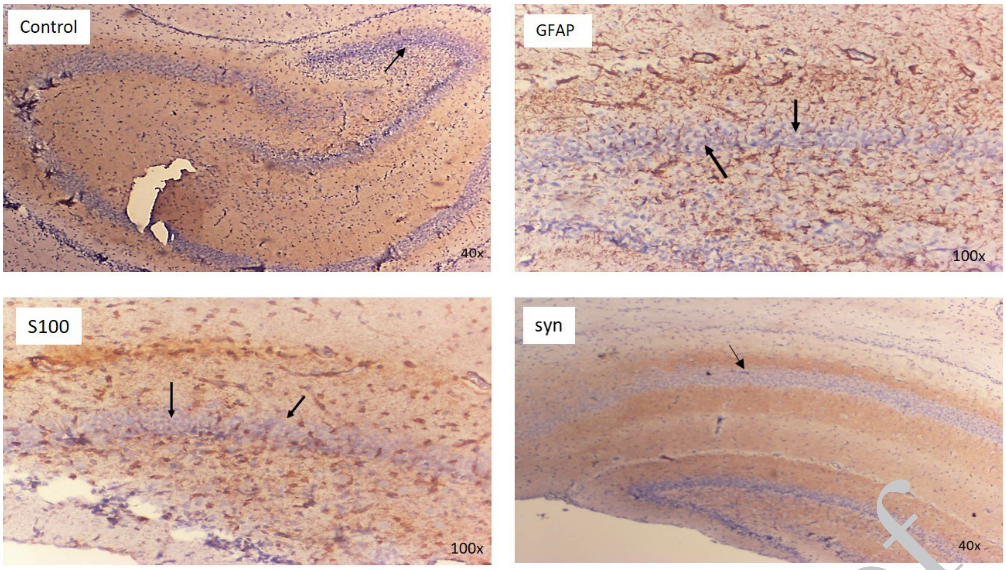

Figure 3. IHC staining using Glial fibrillary acidic protein (GFAP), S100, and synaptophysin (syn) antibodies shows that the cells in the neonatal rat hippocampus are not stained region are not stained, indicating damage
The control group is properly stained.

Discussion

Given the critical role of the hippocampus in cognitive and behavioral regulation, and the long-term consequences of its structural and molecular disruptions, this study aimed to investigate the impact of prenatal morphine exposure on hippocampal integrity. Our findings demonstrate that morphine administration during pregnancy leads to significant up-reglation of MDH2, Neurog1, and BDNF gene expression in the fetal brain, as confirmed by qRT-PCR analysis. Concurrently, histological examination revealed clear neuronal damage, including pyknotic nuclei and inflammatory cell infiltration within the hippocampus. Immunohistochemical analyses further indicated a complete loss of GFAP, S100, and synaptophysin immunoreactivity, suggesting severe astrocytic loss and synaptic disruption. Collectively, these molecular and structural alterations point to extensive neurodevelopmental impairments induced by prenatal morphine exposure, which may underlie long-term cognitive and behavioral dysfunctions. These results demonstrated that fetal exposure to morphine can have dual effects on nervous system development, with some evidence indicating a protective role of morphine under stress or acute injury, while other studies report its detrimental impact on brain maturation and neurological outcomes (1, 6). The up-reglation of MDH2, Neurog1, and BDNF may reflect a compensatory response to morphine-induced stress. Morphine modulates gene transcription via cAMP/CREB and NF-κB pathways (24) and may induce epigenetic modifications, such as histone acetylation and DNA methylation (25). The pharmacological action of morphine is associated with the stimulation of opioid receptors, the activation of which causes significant molecular changes inside the cell, such as inhibition of adenylate cyclase activity, activation of potassium channels, reduction of calcium conductance, effects on phospholipase C, mitogen-activated kinases (MAP kinases), or β-arrestin (26). These molecular effects could explain altered gene expression despite structural damage

Opioids disrupt astrocytic homeostasis by dysregulating glutamate and redox balance, triggering proinflammatory signaling and release of neurotoxic extracellular vesicles that promote neuroinflammation, the blood-brain barrier disruption, and synaptic dysfunction (27). Also, Morphine induces hippocampal apoptosis, inflammation, and mitochondrial oxidative stress via TRPM2 channel activation and nitric oxide signaling pathways (28). Elevated expression of neurodevelopmental genes alongside astrocytic loss likely represents an adaptive yet insufficient repair response. While genes such as BDNF and Neurog1 are up-reglated to counteract injury, persistent oxidative and inflammatory stress caused by morphine may override these compensatory effects, leading to glial apoptosis. Repeated opioid exposure may induce age-dependent changes in glial function through adaptations in the mesolimbic dopaminergic system (22).

Previous studies show that the impact of morphine depends on dose, duration, and route of administration (29). Morphine sulfate has been shown to reduce neural differentiation in mouse embryonic stem cells (30). There is evidence that prenatal exposure to morphine induces long-term effects on synaptic plasticity by altering the expression of genes involved in neuronal development and differentiation (31). A 2015 study reported that chronic prenatal morphine (days 11–18, twice daily) exposure in rats leads to reduced hippocampal levels of brain-derived neurotrophic factor (BDNF) (32). Similarly, another study found decreased expression of BDNF precursors following prolonged morphine exposure (through drinking water, days 1 to 13) during the embryonic period (33). Khayat *et al.* also reported that prenatal morphine exposure (5-10 mg/kg S.C. days 1 to 21) decreases NRG-1, ErbB-4, and BDNF expression in the offspring cortex, further disrupting pathways related to neurodevelopment, inflammation, oxidative stress, and neurotrophic signaling (34). The difference between the present study and other studies may be due to the limited use of morphine, its dosage, its method of administration, and the location of the brain sample. In another study, no significant change in hippocampal expression of Arc, BDNF, or NGF genes was observed after a course of morphine treatment, but subchronic morphine administration (15 and 20 mg/kg) increased Arc and BDNF gene expression in a dose-dependent manner (35). For the other two genes (MDH2 and Neurog1), no studies were found on the effect of prenatal morphine exposure. A systematic review by Balalian *et al*., based on 79 studies, reported that prenatal morphine disrupts neonatal growth, learning, and behavioral outcomes by altering the expression of key neurodevelopmental genes (36). The hippocampus, as a key structure involved in learning, memory, and cognitive processing, is one of the primary targets of opioid-induced damage (7, 8). Additional studies have revealed that co-administration of morphine and caffeine during early postnatal development increases apoptosis and neuronal damage compared to either drug alone, indicating a synergistic neurotoxic effect (37). Brazil *et al.* identified sex-dependent molecular responses to morphine (15 mg/kg, IP), including increased Bax levels and caspase-3 activation, with evidence of demyelination in females, suggesting distinct mechanisms of morphine tolerance and neurodegeneration between sexes (38). Bornavard *et al.* further demonstrated that maternal morphine exposure exacerbates hypoxic-ischemic injury in offspring by reducing total anti-oxidant capacity and BDNF, while increasing cerebral edema and infarct volume (39). Moreover, in vitro studies confirm that morphine exposure alters the expression of genes critical for neural differentiation and nervous system development (40). Consistent with our findings, Zhang *et al.* demonstrated that morphine impairs adult neurogenesis and contextual memory by inhibiting the maturation of neural progenitors and downregulating related gene expression (41). Similarly, Aghighi *et al.*, despite not evaluating gene expression, reported behavioral and electrophysiological evidence indicating hippocampal dysfunction in neonatal rats exposed to morphine via maternal transmission (42). Their findings contrast with our results, possibly due to differences in morphine administration protocols (acute vs. chronic) or variation in the anatomical regions sampled across studies.

This study is limited by the use of an animal model, which may not fully capture the complexity of human neurodevelopmental processes. In addition, the analysis was restricted to selected molecular markers, and no behavioral assessments were performed to correlate histological and molecular findings with functional outcomes. Nevertheless, the results underscore the detrimental impact of prenatal morphine exposure on neurodevelopmental pathways and its potential link to long-term cognitive and behavioral deficits. Future research should aim to identify neuroprotective interventions—such as environmental enrichment or targeted pharmacologic agents—and to elucidate sex-specific mechanisms underlying opioid-induced neurotoxicity.

Conclusion

This study provides evidence that short-term prenatal exposure to morphine induces significant molecular and histopathological changes in the developing hippocampus of neonatal rats. The up-reglation of MDH2, Neurog1, and BDNF, combined with astrocytic loss and synaptic disruption, suggests that morphine interferes with key neurodevelopmental processes. These findings highlight the potential risks of opioid exposure during pregnancy and emphasize the need for careful prescription practices and further investigation into protective strategies.

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Authors' Contributions

All authors of the present study participated in different stages and shared ideas, but responsibilities were divided as follows: S F contributed the design of the work, supervision, data collection and analysis, and writing of the article. P N handled project implementation and writing. Z D was responsible for writing the article. Z A contributed to design implementation and writing of the article.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration

We acknowledge using ChatGPT for grammatical editing.

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