Neuroscience Letters 636 (2017) 205–212

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

The role of morphine on rat neural stem cells viability, neuro-angiogenesis and neuro-steroidgenesis properties

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HIGHLIGHTS

- Morphine induced cell cytotoxicity, apoptosis and necrosis in rat neural stem cells.
- Naloxone could blunt the adverse effects of morphine on rat neural stem cells.
- Up-regulation of p53, aromatase and 5-alpha reductase genes occurred in morphine-primed cells while naloxone inhibited these effects.
- Morphine inhibited endothelial differentiation of rat neural stem cells.

ARTICLE INFO

Article history:
Received 21 August 2016
Received in revised form 2 November 2016
Accepted 10 November 2016
Available online 11 November 2016

Keywords:
Rat neural stem cells
Morphine
Aromatase and 5-alpha reductase expression
Endothelial differentiation
VE-cadherin

ABSTRACT

A lack of comprehensive data exists on the effect of morphine on neural stem cell neuro-steroidogenesis and neuro-angiogenesis properties. We, herein, investigated the effects of morphine (100 μM), naloxone (100 μM) and their combination on rat neural stem cells viability, clonogenicity and Ki-67 expression over a period of 72 h. Any alterations in the total fatty acids profile under treatment protocols were elucidated by direct transesterification method. We also monitored the expression of p53, aromatase and 5-alpha reductase by real-time PCR assay. To examine angiogenic capacity, *in vitro* tubulogenesis and the level of VE-cadherin transcript were investigated during neural to endothelial differentiation under the experimental procedure.

Cells supplemented with morphine displayed reduced survival ($p < 0.01$) and clonogenicity ($p < 0.001$). Flow cytometric analysis showed a decrease in Ki-67 during 72 h. Naloxone potentially blunted morphine-induced all effects. The normal levels of fatty acids, including saturated and unsaturated were altered by naloxone and morphine supplements. Following 48 h, the up-regulation of p53, aromatase and 5-alpha reductase genes occurred in morphine-primed cells. Using three-dimensional culture models of angiogenesis and real time PCR assay, we showed morphine impaired the tubulogenesis properties of neural stem cells ($p < 0.001$) by the inhibition of trans-differentiation into vascular cells and led to decrease of in VE-cadherin expression. Collectively, morphine strongly impaired the healthy status of neural stem cells by inducing p53 and concurrent elevation of aromatase and 5-alpha reductase activities especially during early 48 h. Also, neural stem cells-being exposed to morphine lost their potency to elicit angiogenesis.

1. Introduction

Today, opioids and non-steroidal anti-inflammatory drugs are increasingly used globally for pain relief [1]. In spite of potent activity for the attenuation of pain, some side effects such as nausea, respiratory failure, confusion, constipation and etc. have been, however, reported for a long-time application [2]. Amid opioids, morphine is a popular pharmacologically acting alkaloid possesses

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http://dx.doi.org/10.1016/j.neulet.2016.11.025
0304-3940/

analgesic and addictive properties [3]. Based to laboratory investigations, three types of opioid receptors, namely μ, δ and k, are the effective site of opioids, peculiarly morphine [4–6]. These receptors are extensively distributed in many parts of central nervous system (CNS) and peripheral tissues of mammalian organisms [7,8].

Nowadays, stem cells fields are touted as new therapeutic approaches with a great promise for the reconstitution of various injured tissues, particularly nervous system [9]. Neural stem cells (NSCs) are a group of generative cells in CNS niche with a potential limitless self-renewal and proliferative activity, which eventually trans-differentiate into consigned lineages such as neurons, astrocytes and oligodendrocytes [10]. Postnatal adult NSCs are being restricted in neurogenic regions of hippocampus and subventricular parts as well as non-neurogenic regions as spinal cord [11–13].

Regarding to a great body of experiments, morphine and other opioids medicines reduce the growth in size of neurons, axon outgrowth, branching of dendrites and neurogenesis [2]. We previously showed that morphine reduced the number of formed neurospheres with a significant decline in viability rate of rat NSCs (rNSCs) in *in vitro* condition [14]. Noticeably, a vast array of extra CNS steroid hormones, including adrenal steroids, testosterone and estrogen, as well as peptide hormone prolactin, conceived as potential regulators of adult neurogenesis, could impress the dynamic status of adult NSCs neuro-regenerative capacity [15]. It was well-established that estrogen enhanced growth and differentiation of nerve cells during development, stimulated neurite outgrowth of hypothalamic neurons and increased dendritic length of embryonic neurons from the medial amygdala in *in vivo* models [16]. Estrogen exerts its neuro-protective activity by modulating protein levels of both anti and pro-apoptotic proteins [16]. For example, estrogen protects the dopaminergic neurons in hippocampal region against apoptosis by up-regulation of *Bcl-xl1* and *Bcl-2* genes [16]. Recent attempts have shown a critical role for androgens in NSCs biology further than reproduction, such as neurogenesis, resistance and survival of neural cells [17]. Testosterone (T) can be delivered by a certain population of motor neurons from axotomy induced death and glucose-deprived NCs [3]. An enzyme so-called 5-alpha reductase (5A-R) performs the conversion of T to dihydrotestosterone (DHT) (18). Another enzymatic pathway driven by p450-aromatase (AROM) enzyme transforms T to estradiol (E_2) [19]. Beside to be dynamic of extracellular neuro-steroids on stem cells integrity, the role of some genes, noticeably *p53* has been confirmed in neural, breast, hematopoietic, and embryonic stem cell self- renewability, symmetric division, quiescence, survival, and proliferation states [20]. *P53* regulates cell progression and apoptosis rate but also can be directly modulated transcription of genes that are specifically required for neural differentiation [20]. Interestingly, increase in the number of NSCs in the absence of p53 protein approved that this factor played as a negative regulator of self-renewal behavior of NSCs [21]. On the other hand, the ablation of *p53* in neurosphere attributed to increase self-renewal and proliferation properties whereas apoptosis rate declined [20].

Regarding morphine effect on angiogenesis, there have been difference of clinical and experimental reports on literature due to concentration and/or time of administration of morphine applied [22]. For instance, the induction of survival signal PKB/Akt activated by morphine at clinical relevant doses could contribute to enhanced angiogenesis rate in cancer milieu [23]. By contrast, an inhibited hypoxia-induced VEGF secretion happens in rat cardiomyocytes and human umbilical vein endothelial cells following the supplementation with morphine [24]. To our knowledge, no comprehensive experimental study exists addressing the question whether morphine supplementation affects the angiogenic behavior of rNSCs.

This *in vitro* study was further designated to evaluate whether morphine administration could in turn modulate the expression of genes related to 5-alpha reductase and aromatase in rNSCs through 72-h incubation period. In addition to monitoring *p53* transcript, we also investigated the tubulogenesis property and endothelial differentiation potential of rNSCs by the presence of vascular endothelial cadherin (VE-cadherin) and von Willebrand factor (vWF) under experimental condition (Table 1).

2. Material and methods

Detailed information is provided in the Supplementary materials section.

3. Result

3.1. Determination of typified NSCs based on morphology and relevant markers

A large number of floating neuroshperes were notified 2–3 days after initial seeding, which reached to their maximum size of diameter at day 7 (Fig. 1A) (14). The phenotypic characterization by flow cytometric assay revealed a 98.1 and 99.3% of cultivated rNSCs bearing Sox-2 and nestin factors, respectively (Fig. 1B). Both co-immune-reactivity directed against nestin and Sox-2 factors were also determined using immunofluorescence imaging (Fig. 1C). These results confirm the excellence of our procedure allows the appropriate isolation of rNSCs with stemness feature.

3.2. Morphine potentially decreased the rNSCs viability

A 72-h monitoring of rNSCs-being exposed to 100 μM morphine showed an apparently time-dependent decreased in viability rate as compared to parallel time-matched controls (Fig. 1D). An extra exciting finding of current investigation was that co-incubation of rNSCs with 100 μM morphine and naloxone showed the prohibitory action of naloxone in morphine-derived cytotoxic effects ($P_{\text{naloxone + morphineversusnaloxoneandcontrol}} < 0.001$). We, therefore, speculated that blockage of opioid receptors by an antagonist, naloxone, could attenuate side effects of morphine on viable rNSCs.

3.3. An inhibition of rNSCs clonogenicity was conferred by morphine priming

In line with results of cell viability assay, morphine attenuated rNSC clonogenic properties by declining the number of nervous-derived stem cells participating in neuroshperes at the end-stage of current experiment (Fig. 2A and B). Of note, the concurrent addition of naloxone with morphine increased clonogenicity rate as compared to rNSCs primed with morphine alone ($p < 0.001$) (Fig. 2B). Compared to control group, the average colonial diameter on morphine-containing was found to decrease from 179.05 ± 29.01 to 129.08 ± 13.68 μm ($P_{\text{morphineversuscontrol}} < 0.05$) (Fig. 2C). We did not observed any significant differences in colony size between control and other groups ($P < 0.05$). Commensurate with these findings, morphine restricted the rNSCs clonogenicity by decreasing proliferation rate.

3.4. The expression of Ki67 was significantly suppressed by morphine

To study the inherent modulatory effect of morphine on cell cycle status in rNSCs, the expression behavior ki-67 marker was also analyzed. Four experimental sets of rNSCs underwent a flow cytometric Ki-67 expression analysis through a period of 72 h (Fig. 3A). Indeed, our results showed downward changes in the expression of Ki-67 factor time-dependently in cells supplemented