

Invited Review

A novel insight into neuroprotection against hypoxic/ischemic stress

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Abstract: The use of opioid analgesics has a long history in clinical settings, although the functions of opioid receptors, especially their role in the brain, are not well understood yet. Recent studies have generated abundant new data on opioid receptor-mediated functions and the underlying mechanisms. The most exciting finding in the past decade is probably the neuroprotection against hypoxic/ischemic stress mediated by δ -opioid receptors (DOR). An up-regulation of DOR expression and the release of endogenous opioids may increase neuronal tolerance to hypoxic/ischemic stress. The DOR signal triggers, depending on stress duration and severity, different mechanisms at multiple levels to preserve neuronal survival, including the stabilization of ionic homeostasis, an increase in pro-survival signaling (e.g., PKC-ERK-Bcl 2) and the enhanced anti-oxidative capacity. Recent data on DOR-mediated neuroprotection provide us a new concept of neuroprotection against neurological disorders and have a potentially significant impact on the prevention and treatment of some serious neurological conditions, such as stroke.

Key words: opioids; δ -opioid receptors; neurotransmitters; brain, ionic homeostasis; neuroprotection; hypoxia; ischemia

缺氧缺血时神经元保护的新观点

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摘要: 阿片类镇痛剂的临床应用具有悠久的历史。然而, 阿片受体的功能, 特别是其在脑内的作用, 尚未完全阐明。近年来, 有关阿片受体介导的功能及其作用机制的研究, 产生了大量新资料。其中, 最令人兴奋的发现或许是 δ 阿片受体 (δ -opioid receptor, DOR) 对缺氧缺血神经元的保护作用。DOR 表达的上调和内源性阿片释放可增加神经元对缺氧缺血应激的耐受性。根据应激的持续时间和严重程度不同, DOR 信号可在多个水平触发不同机制, 从而保护神经元, 使之得以在应激环境中生存。这些机制包括稳定离子内环境、增强促生存信号通路的转导 (如 PKC-ERK-Bcl2 通路) 和增加抗氧化损伤的能力。DOR 保护缺氧缺血神经元的最新资料, 给神经保护并籍以对抗神经系统疾患的研究提供了一个新观念, 对某些严重脑疾病 (如中风) 的预防和治疗可能具有较大的意义。

关键词: 阿片肽; δ 阿片受体; 神经递质; 脑; 离子稳态; 神经保护; 低氧; 缺血

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1 Introduction

Opioids have been widely used as analgesics throughout history to combat pain or induce ecstasy in medical and non-medical situations. Despite the flow of time and the energies of scientists around the world, the function of opioid receptors and their role in the brain still is not well understood to this day.

One of the most interesting findings on opioid receptor function in the past decade is probably the opioid receptor-mediated neuroprotection against hypoxic/ischemic injury. Hypoxia and/or ischemia lead to serious consequences in the clinical setting. Unfortunately, there have been no promising drugs for relieving hypoxic/ischemic injury in the brain despite considerable research efforts that have been made in past several decades. Because of the unique and complex features of neuronal responses to hypoxic/ischemic stress, neuroprotection against hypoxic/ischemic injury must be an exceptional and comprehensive process that is different from neuroprotective strategies against other insults. Accumulating evidence has shown that opioids may protect neuronal tissues/cells via activation of opioid receptors during hypoxic/ischemic stress, providing a novel insight on hypoxic/ischemic injury in the brain. Indeed, the role of opioids and other analgesics in neuroprotection has attracted more and more attention from clinicians and research scientists over the course of time.

2 Historical controversy

The release of opioids increases in the brain during hypoxia^[1-3]; however, the role of the opioid system in neuronal responses to hypoxic/ischemic stress remained unclear. Some *in vivo* studies^[4-9] showed that opioid receptor inhibition by intravenous injection of a high dose of naloxone, a non-specific opioid antagonist that interacts with DOR, μ -opioid receptors (MOR) and κ -opioid receptors (KOR), protects the brain from ischemia-induced injury, while others^[10-13] suggested that opioid receptor activation with opioid agonists protects the brain from ischemia or extends animal survival time during severe hypoxia. The major controversy that might result from methodological limitations can be laid at the doorstep of ligand selectivity, namely, a majority of these earlier studies were mainly based on the application of opioid ligands with relatively poor in low receptor selectivity and administered at high concentrations intravenously, which complicated the ensuing results; i.e., high doses of opioid ligands may lead to cross-reactivity with various opioid receptor

subtypes or induce non-opioid effects. Furthermore, systemic administration of opioids (e.g., DOR ligands)^[11,14] may induce multiple extraneous effects, including heart^[15] and blood vessels^[16], such that the resultant data were difficult to interpret as the results of neuronal action, since DOR protects animals from cardiac injury^[17,18] thereby extending survival during severe hypoxia.

To ascertain the role of opioid receptors in neuronal response to hypoxic stress, it is important to differentiate the role of opioid receptor subtypes in specific neurons/regions, since opioid receptors are differentially distributed in the brain. One earlier report using morphine, U50,488H and naloxone provided no significant protection in the hippocampus subjected to ischemia^[19]. Another study revealed that a variety of μ -opioid ligands at excessively high concentrations (0.1-3 mmol/L) reduced neuroexcitotoxicity in cortical neurons, which could not be blocked by naloxone, suggesting a “non-opioid effect”^[20]. Those results have added to the complexity of the role played by opioids on neurons during conditions of stress. To clarify the potential mode of action of opioids and their receptors in hypoxic/ischemic neuronal injury, we demonstrated that DOR is neuroprotective against hypoxic/excitotoxic stress in the brain, especially cortical neurons^[21-32].

3 δ -opioid receptor mediated neuroprotection

In the late 1980s, we pursued the mechanisms underlying the major differences in neuronal sensitivity to hypoxic stress between the turtle and rat. In early 1989, an observation based on receptor autographical analysis indicated that turtle brain had a higher density of DOR than rat brain, while MOR density was lower in the former than in the latter, which was confirmed in 1997 and eventually published in 2001^[33]. Since turtle brains are more tolerant to hypoxic/ischemic stress^[34,35], this unique phenomenon was linked to a potential mechanism of neuroprotection^[21,33].

The confirmation of that observation prompted the further elucidation of the role of DOR in neuroprotection against hypoxic/ischemic injury. To clarify the action of various opioid receptors, especially DOR in neuronal responses to excitotoxicity, opioid agonists and/or antagonists were directly applied to cultured neurons to investigate whether opioids protect from glutamate-induced^[21] neuronal injury and death due to the fact that DOR is highly distributed in the cortex^[36].

Initially, glutamate was added to cultured cortical neurons to mimic neuroexcitotoxicity since it is a key media-

tor of ischemic/hypoxic injury and induces neuronal injury and cell death in a dose- and time-dependent manner. After 8-10 days *in vitro*, neurons exposed to 100 $\mu\text{mol/L}$ glutamate for 4 hours daily showed substantial neuronal injury as assessed by morphologic determination and LDH release. Activation of DOR with 10 $\mu\text{mol/L}$ DADLE reduced glutamate-induced injury by almost half, and naltrindole (10 $\mu\text{mol/L}$) completely blocked this protective effect. In sharp contrast, administration of MOR and KOR agonists (5-10 $\mu\text{mol/L}$ DAMGO and U50,488H, respectively) did not induce appreciable neuroprotection, and MOR or KOR antagonists had no significant effect on glutamate-induced injury. These data demonstrate that activation of DOR, but not MOR and KOR, protected cultured neocortical neurons from glutamate excitotoxicity^[21,22]. DOR neuroprotection from hypoxic stress, ischemic insult or excitotoxic injury was shown by our studies^[21,22,24,37] and substantiated in other laboratories^[38-52].

Similarly, we observed that following the microinjection and expression of DOR mRNA into *Xenopus* oocytes, 1-5 $\mu\text{mol/L}$ of the selective and potent δ -agonist UFP-512^[53] not only significantly attenuated anoxic decrease in extracellular $[\text{Na}^+]$, but also enhanced the recovery in the anoxia-induced decline due to the change in $[\text{Na}^+]$, which was blocked by naltrindole^[32]. Furthermore, with the expression of DOR, UFP-512 reduced sodium currents, permitting us to conclude that DOR is directly involved in the inhibition of Na^+ channel regulation^[32].

Interestingly, we also observed that electroacupuncture attenuated ischemic injury via DOR system^[37], which is now well documented^[27-29,54]. In addition, we demonstrate that neuronal preconditioning induced neuroprotection is dependent on the activity of DOR^[25,55], which is now also confirmed in another model^[51].

Based on these accumulative data, the previous controversies that existed in the literature can now be addressed. The reason why μ -ligands had little or no protective effect on hippocampal neurons^[19] is likely due to the low density of MOR in the hippocampus, and that high concentrations of μ -ligands reduced neuroexcitotoxicity in cortical neurons^[20] might be, at least partially, attributed to activation of DOR, which is highly expressed in the cortex, through use of opioid ligands with limited receptor selectivity and specificity.

4 Mechanisms of DOR neuroprotection

Acute hypoxic/ischemic stress causes an immediate loss of ionic homeostasis based on the functional response of

inherent membrane proteins, while prolonged stress may lead to major alterations in signal transduction (especially death/survival signal systems), gene expression (e.g., DOR expression^[55]) and even cellular structure^[56-59]. DOR signals may trigger, depending on the duration of stress and different modes of action at multiple levels, a preservation of neuronal survival. Recent progress on the mechanistic exploration is briefly summarized below.

4.1 Up-regulation of DOR expression

DOR expression is influenced by multiple factors including development^[36], hypoxic stress^[25,55] and neurotransmitters. There is evidence showing that brain DOR is sensitive to hypoxia/ischemia and significantly decreases after a few hours of ischemic stress or prolonged hypoxia. For example, exposure to hypoxia for seven days down-regulated DOR, but not MOR and KOR^[60].

Since the hypoxia-resistant turtle has a high density of DOR in the brain, we asked whether DOR expression plays a role in neuroprotection against hypoxic/ischemic stress. Our recent studies provide strong evidence for the involvement of DOR expression in neuronal survival under hypoxic condition. In cortical neurons, rapid hypoxia preconditioning (HPC) increased DOR protein density with no appreciable changes in mRNA level of DOR^[55]. However, delayed HPC increased DOR expression at both mRNA and protein levels^[25]. The DOR up-expression is associated with an increase in neuronal survival^[25,55]. In sharp contrast to neuronal preconditioning with short-term hypoxia, prolonged hypoxia caused serious neuronal injury with a significant decrease in DOR protein and mRNA^[25].

Furthermore, we showed that transgenic over-expression of DOR alone made the cortex more tolerant to hypoxic stress^[26]. Indeed, an expression of DOR itself without DOR agonists inhibits Na^+ channel function^[32], which serves as a neuroprotective strategy against hypoxic stress^[32,65,66]. These data show that DOR expression is a critical factor determining neuronal tolerance to hypoxia/ischemia.

4.2 Stabilization of ionic homeostasis

Opioids may modulate electrical activity and excitatory transmission which are based on the changes in the activities of ionic channels and ion flux^[61,62]. Under hypoxia/ischemia, ionic homeostasis is greatly disrupted, which is characterized by the enhanced Na^+ , Ca^{2+} influx and K^+ efflux^[31,32]. Activation of DOR, but not MOR, remarkably attenuates K^+ leakage in hypoxia or simulated ischemia^[26,63-66], suggesting that the stabilization of K^+ homeostasis is an important mechanism of DOR neuroprotection, since the enhanced K^+ leakage is a characteristic response of neurons to

hypoxia/ischemia^[31,67] and initiates cellular apoptosis^[68-73]. Furthermore, data suggest that DOR activation attenuates anoxic K⁺ leakage partially through inhibition of Ca²⁺ loading^[63]. Additional results revealed that DOR-mediated inhibition on Na⁺ entry through voltage-gated Na⁺ channels and NMDA receptor channels constituted a major mechanism underlying the DOR protection against anoxic K⁺ derangement in the cortex because DOR protection against anoxic disruption of K⁺ homeostasis was largely abolished by low Na⁺ perfusion, either with impermeable *N*-methyl-*D*-glucamine or permeable Li⁺ substitution, Na⁺ channel blocker TTX, and NMDA receptor channel blocker MK 801^[65,66]. In support of this notion, activation of DOR indeed attenuated anoxic Na⁺ influx in the cortical slices, which can be abolished by naltrindole^[32]. Taken together, these results indicate that DOR signal stabilizes hypoxic/ischemic disruption of ionic homeostasis to protect neurons against hypoxic/ischemic insults.

4.3 Intracellular transduction

The post-receptor signaling following opioid receptor activation is an intricate dance among various intracellular signaling pathways. In hypoxic neurons, we found that DOR-mediated protection against hypoxic injury was inhibited by the treatment with a protein kinase C (PKC) inhibitor^[25], suggesting the involvement of PKC in the DOR protection. Furthermore, we found that DOR activation attenuated anoxic disruption of K⁺ homeostasis and this DOR-mediated event also relies on a PKC-dependent, but PKA-independent pathway^[64]. Down-stream, the extracellular signal-regulated kinase (ERK) stimulated by the mitogen-activated protein kinase kinase (MEK) is important in the signaling cascades of DOR neuroprotection because we observed that this DOR-mediated activity was largely inhibited by the treatment with an ERK inhibitor in the hypoxic neurons^[25]. Narita *et al.*^[41] also showed the importance of PKC and MEK in DOR-mediated neurogenesis and neuroprotection. Therefore, PKC-MEK-ERK signaling may form the central pathway for DOR protection.

Our studies show that G proteins are an important component of signal transduction and the activation of the G protein-PKC-ERK pathway enhances the activity of Bcl-2 and suppresses cytochrome C release, thus protecting neurons from severe hypoxic stress^[25]. Interestingly, the cross-talk between ERK and p38 displays a "Yin-Yang" antagonism under the regulation of the DOR-G protein-PKC pathway^[25]. In addition, Narita *et al.*^[41] demonstrated that DOR signaling involves the activation of Trk-dependent tyrosine kinase, which could be linked to PI3K and CaMKII in ad-

dition to MEK and PKC. The neuroprotective property of DOR may be partly due to its ability to potently block Bax-related apoptotic processes^[74].

4.4 Attenuation of oxidative injury

Since ERK and cytochrome c are differentially involved in caspase signaling of oxidative injury that significantly contributes to neuronal damage in ischemia/reperfusion, the question arose if DOR activation protected the ischemic brain by attenuating oxidative injury. Recent data^[30] show that in the model of cerebral ischemia with middle cerebral artery occlusion, DOR activation increased the activity of major antioxidant enzymes, glutathione peroxidase and superoxide dismutase, and decreased malondialdehyde and nitric oxide levels in the cortex exposed to cerebral ischemia/reperfusion. Moreover, DOR activation reduced caspase-3 expression in ischemic regions, such as the hippocampus. PD98059, an inhibitor of MAPK extracellular signaling-regulated kinase, accelerated animal death during ischemia/reperfusion. Therefore, DOR activation greatly enhanced the activity of antioxidant enzymes and reduced free radicals, MDA and NO, thereby attenuating oxidative injury by enhancing antioxidant ability and inhibiting caspase activity in the brain after cerebral ischemia and reperfusion.

Taken together, the DOR-mediated signal is very likely a regulator at multiple levels in neurons and protects brains from hypoxic/ischemic stress through complex mechanisms. The major mechanisms are schematically shown in the accompanying Fig.1.

5 Concluding remarks

The research on the DOR-mediated neuroprotection provides us a new concept of how the brain responds to hypoxic/ischemic stress. The underlying mechanisms include the stabilization of ionic homeostasis in acute stage of hypoxic/ischemic stress, and up-regulation of DOR expression, survival molecules and anti-oxidative capacity, and the down-regulation of apoptotic signaling during the long-term stress of hypoxia/ischemia. These latest findings may have clinical importance because hypoxic/ischemic insult (e.g., stroke) is a major cause of neurological morbidity and mortality in the world as a consequence of neuronal dysfunction and death, while therapeutic modality directed against hypoxic/ischemic injury and death have had limited success.

At present, however, many questions remain unknown regarding the mechanism of DOR neuroprotection. For

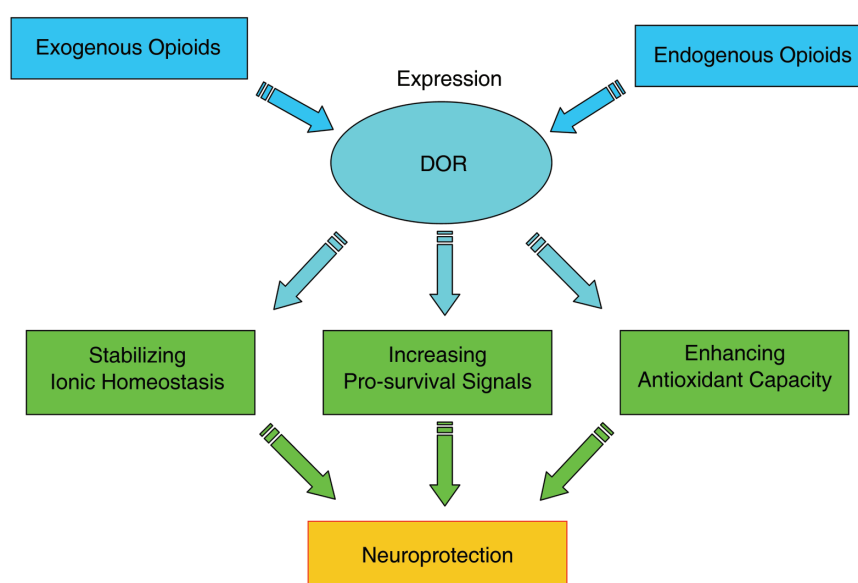


Fig. 1. Schematic diagram of major mechanisms for DOR neuroprotection.

example, does DOR activity affect cellular pH in hypoxia/ischemia? Since we have shown that DOR expression is down-regulated in the brain with null mutation of Na^+/H^+ exchanger 1^[75], and Na^+/H^+ exchangers are involved in the pH regulation in neurons^[76], it is likely that DOR plays a role in pH homeostasis and protein stability^[77] in hypoxic/ischemic conditions. It is our belief that more mechanistic research on DOR protection may eventually lead to novel clues for better solutions of hypoxic/ischemic injury in the brain.

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