Review

Neuroprotection Against Hypoxic/Ischemic Injury: δ -Opioid Receptors and BDNF-TrkB Pathway

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Key Words

δ -opioid receptor • BDNF • TrkB • Brain • Hypoxia • Ischemia • Neuroprotection

Abstract

The delta-opioid receptor (DOR) is one of three classic opioid receptors in the opioid system. It was traditionally thought to be primarily involved in modulating the transmission of messages along pain signaling pathway. Although there were scattered studies on its other neural functions, inconsistent results and contradicting conclusions were found in past literatures, especially in terms of DOR's role in a hypoxic/ischemic brain. Taking inspiration from the finding that the turtle brain exhibits a higher DOR density and greater tolerance to hypoxic/ischemic insult than the mammalian brain, we clarified DOR's specific role in the brain against hypoxic/ischemic injury and reconciled previous controversies in this aspect. Our serial studies have strongly demonstrated that DOR is a unique neuroprotector against hypoxic/ischemic injury in the brain, which has been well confirmed in current research. Moreover, mechanistic studies have shown that during acute phases of hypoxic/ischemic stress, DOR protects the neurons mainly by the stabilization of ionic homeostasis, inhibition of excitatory transmitter release, and attenuation of disrupted neuronal transmission. During prolonged hypoxia/ischemia, however, DOR neuroprotection involves a variety of signaling pathways. More recently, our data suggest that DOR may display its neuroprotective role via the BDNF-TrkB pathway. This review concisely summarizes the progress in this field.

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Introduction

Hypoxic/ischemic injury remains the most dreaded cause of neurological disability and mortality. At present, however, clinical therapies against hypoxic/ischemic injury are

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still very limited. Since the brain is extremely sensitive to hypoxia and ischemia, protecting brain tissue from injury (simplified as “neuroprotection”) has therefore been a long sought after strategy in quelling physiological damage following stroke onset. Over the course of more than 30 years, abundant research efforts have been made to investigate over 1, 000 pharmacological neuroprotectants. Unfortunately, almost all attempts at protecting the brain from ischemic injury have failed at making a successful transition into clinical use. Therefore, it is important to better understand the mechanisms of neuroprotection and explore new strategies for the prevention and treatment of hypoxic/ischemic brain injury. As a result of this goal, we have recently found that the delta-opioid receptor (DOR) is an important neuroprotector against hypoxic/ischemic injury in the brain [1-4]

DOR is one of the three classic opioid receptors in the opioid system. Since its cloning in the early 1990s [5, 6], past functional studies were mostly focused on pain modulation and addiction [7-10]. Although scattered studies on other functions of DOR can be found in the literature, the outcomes and conclusions of these studies are inconsistent, and sometimes very contradictory. For instance, some studies suggested that opioid agonists were neuroprotective against hypoxic or ischemic injury [11-15], while others showed that opioid antagonists (e.g., naloxone, a non-selective opioid antagonist) had the same effect [16-19]. These controversies may be attributed to many factors including inappropriate methodologies and ligands.

In 1990s, we found that DOR density is much higher in the brain of a turtle that is highly tolerant to hypoxic/ischemic stress in comparison to the rat brain [20, 21]. Such distinctive distribution of DOR between species prompted us to believe that DOR has unique functions, including brain protection against hypoxia/ischemia. Since then, we have conducted serial studies with diversified approaches including biochemical, molecular, transgenic, and electrophysiological techniques to investigate the role of DOR in the brain under hypoxic/ischemic conditions and demonstrated that DOR is neuroprotective against hypoxic/ischemic stress. Our findings have been confirmed by many independent investigators.

More recently, our work suggests that DOR may display its protective role through neurotrophic factors, especially brain-derived neurotrophic factor (BDNF) via tropomyosin receptor kinase B (TrkB). This review concisely summarizes the progress in the research on the role of BDNF in the DOR neuroprotection.

Opioid receptors and endogenous opioids

Opioids have been widely exploited for their potent analgesic and ecstasy-inducing effects in both medical and non-medical settings throughout a long history.

In the middle of the 1970s, Hughes and other scientists discovered the existence of various types of endogenous opioids - a family of chemically distinct endogenous compounds with properties similar to morphine, including enkephalins, endorphins, and dynorphins [22-25]. In the early 1980s, opioid peptide precursor genes were identified, proving the existence of the preproenkephalin gene that encoded several copies of enkephalins [26], the preprodynorphin gene encoding dynorphins [27], and the proopiomelanocortin gene encoding β -endorphin [28].

Opioid receptors were first recognized in the 1960s during pharmacological studies that focused on the specific sites at which opiates exerted their actions. Martin et al [29]. provided the first evidence of opioid receptors *in vivo*. In the early 1970s, the receptors were first identified by the use of radio-binding assay [30-32]. In late 1992, two groups independently cloned DOR from the neuroblastoma x glioma cell line NG-108 by using an expression cloning strategy. Both reported the identification of a novel member of the 7-helix family of G-protein coupled receptors that had pharmacological properties typical of DOR [5, 6]. Almost one year later, mu-opioid receptors (MOR) [33, 34] and kappa-opioid receptors (KOR) [35, 36] were cloned as well.

These opioid receptors are members of the rhodopsin subfamily in the superfamily of seven-transmembrane nucleotide binding regulatory G-protein coupled receptors (GPCRs) [1, 37, 38]. Although homology is present, they recognize structurally diverse exogenous/endogenous peptide and non-peptide ligands [39-41]. They are distinctly distributed in both the central nervous system, especially in the cortical regions (Fig. 1), and peripheral organs [21, 25, 41-46]. Leu- and met-enkephalins constitute the main endogenous agonists. They are produced in many types of cells and preferentially bind to DOR in physiological concentrations [41, 47-49].

Pharmacological evidence indicated the existence of two distinct DOR subtypes: $\delta 1$ and $\delta 2$ [50-52]. However, there exists only a single DOR gene for distinct subtypes of DOR [39]. DOR subtypes likely originate from a single DOR gene, and differ due to multiple affinity states or to post-translational modifications at the protein level rather than at the gene level [39]. Several endogenous opioids (including enkephalins, endorphins and dynorphins that originate from the same or different precursor proteins) can activate DOR with different binding affinities. However, it is unclear yet if there is any endogenous opioid antagonist. In the past three decades, many exogenous opioid agonists and antagonists have been used for DOR research. However, they differ widely with respect to their efficacies, potency, side-effects, clearance, and passage through the blood-brain barrier; refer to our previous articles [41, 53]. To the best of our knowledge, UFP-512 (H-Dmt-Tic-NH-CH[CH₂-COOH]-Bid) is the most potent and specific DOR agonist, and Naltrindole is a well-accepted DOR antagonist [41, 53-58].

Previous studies on the function of DOR were mainly focused on pain modulation and addiction with little conclusive information on its other functions [7-10]. Our recent studies have gained substantial data to show that DOR is a critical neuroprotector in the brain under hypoxic/ischemic conditions.

Opioid receptors and neuroprotection: Major controversies in the past

Before our work in this area, there was a major controversy on the role of opioids/opioid receptors in the brain/neurons with hypoxia and/or ischemia.

Some studies suggested that opioid receptor inhibition with naloxone, a non-specific antagonist to DOR, MOR, and KOR [7, 41, 49], is neuroprotective against ischemic injury [16-19, 59].

Fig. 1. Differential distribution of MOR (left) and DOR (right) in the rat rostral brain. BL, basolateral amygdaloid nucleus; CA1, field CA1 of hippocampus; CA3, field CA3 of hippocampus; CPu, caudate putamen; GP, globus pallidus; Me, medial amygdaloid nucleus; VMH, ventromedial hypothalamic nucleus; VP, ventral posterior thalamic nucleus. Note that the distributional patterns of MOR and DOR are totally different with MOR having a high density in all major regions including subcortical areas and DOR having a much higher density in the cortex, caudate putamen and amygdala than most of the subcortical regions. Also note that there is a higher density of MOR in the middle layer than the outer and inner layers of the cortex, while the opposite is true for the density of DOR. The other cortical layers have an opposite pattern of expression. Scale bar = 2 mm. Cited from Xia and Haddad [21].

In contrast, other studies implied that opioid receptor activation induced neuroprotection. Hayward et al [11, 12]. reported the neuroprotective efficacy of a KOR agonist in two acute rat models of focal cerebral ischemia. However, Iwai et al. 's work did not support this notion [60]. They investigated the effect of opioids on delayed neuronal death in the hippocampus of male Mongolian gerbils subjected to transient forebrain ischemia, and found that treatment with the KOR agonist U-50488H, MOR agonist morphine, or naloxone did not induce any significant protection. However, Endoh et al [61]. reported that morphine had a protective action against acute hypoxia; i.e., increasing the survival rate of the mice subjected to acute hypoxia, while a high dose of naloxone decreased the survival rate. On the other hand, Mayfield et al [13-15]. showed that hypoxic conditioning increases survival time during subsequent lethal hypoxic conditions in mice; this protective effect was blocked by naloxone, suggesting an opioid-dependent mechanism. Furthermore, they reported that DOR-1 mediated the mechanism of hypoxic conditioning induced an increase in animal survival time; neither the MOR antagonist (beta-funaltrexamine), nor KOR antagonist (norbinaltorphimine) significantly changed survival time in sham or hypoxic conditioned mice. All these data contradicted the conclusion that "opioid receptor inhibition induced neuroprotection".

Closer examination of the literature caused even greater confusion. For example, some clinical studies [62, 63] showed that the efficacy of naloxone for the treatment of cerebral ischemia is inconclusive. Upon examination of the studies by Mayfield et al [13-15]., it was unclear if "neuroprotection" was involved in increased survival time because an increased animal survival time following systemic administration of opioid ligands does not necessarily indicate "neuroprotection". Since DOR is widely distributed in the central and peripheral systems including the heart and kidney [41, 44, 49], an intravenous ligand may elicit systemic effects through many complex and varied mechanisms. The change in survival time may have resulted from the diverse effects of opioid ligands on various organs and organ systems, especially in the heart and kidney [64-71].

In addition, limited data from some *in vitro* studies [72, 73] still did not clear up the confusion over the above-mentioned controversies. First, the results were not consistent with those of *in vivo* studies. Secondly, the limited studies contradicted each other - one claimed a "non-opioid" effect while the other argued for a KOR protective effect. Third and most importantly, there is no convincing data on the role of DOR in neuroprotection against hypoxic/ischemic injury.

The outcome of previous studies varied in all aspects, and depended on many factors including the conditions of subjects, dose and specificity of opioid ligands used, time point of assessment, accuracy of measurements, and etc.

δ -opioid receptor: A neuroprotector against hypoxic/ischemic injury

In our early studies on the differences in hypoxic/ischemic tolerance between mammalian and freshwater turtle brains [20, 74-76], we found that the turtle brain has a higher density of DOR and a higher binding affinity with its ligand than the rat brain [21]. In contrast, other membrane proteins, such as Na⁺ channels [77] and sulfonylurea receptors [42], did not show this phenomenon.

Based on the observations on the turtle brain that showed a much higher DOR density [21] and greater tolerance to hypoxic/ischemic insult than the rat brain [20, 74-76, 78], we believe that DOR plays a unique role in neuroprotection against hypoxic/ischemic injury.

We therefore attempted to determine if DOR is neuroprotective against hypoxic/ischemic injury with a reliable model. Since the cortex is rich in DOR expression compared to subcortical regions such as the hippocampus (Fig. 1) [21, 42], we specifically cultured cortical neurons and exposed them to hypoxia or glutamate excitotoxicity and then determined if DOR activation attenuates hypoxia- and glutamate-induced neuronal injury [4, 79, 80]. We applied [D-al², D-leu⁵] enkephalin (DADLE), a popular and commercially

available DOR agonist at that time, to evaluate its effect on the neurons. The data showed that DOR activation with DADLE indeed reduced hypoxia or excitotoxicity induced injury [4, 79, 80]. At the same time, Borlongan et al [81], also showed that DADLE protects against ischemia reperfusion damage in the striatum and cerebral cortex.

However, it was extremely difficult to come to a reliable conclusion about the role of DOR in neuroprotection simply based on the data of DADLE, because DADLE is not highly specific to DOR and might bind to other opioid receptors. For example, it binds to MOR sites and inhibits the binding of DMGO, a MOR ligand, in a competitive manner [82].

Therefore, we further clarified the actions of DOR on neuronal responses to excitotoxicity by directly applying other various opioid agonists and/or antagonists to cultured cortical neurons to compare their effects on glutamate-induced neuroexcitotoxicity. We found that Naltrindole, a DOR antagonist, completely blocked the protective effect offered by DADLE. In contrast, administration of MOR agonist DAMGO had no protective effect, while MOR antagonists did not significantly affect glutamate-induced injury either. KOR agonist U50, 488H induced a slight, but not significant, reduction in neuronal injury, while KOR antagonism also had no appreciable effect on glutamate-induced injury [4, 79]. After this work, we were inclined to believe that activation of DOR, but not MOR nor KOR, induced neuroprotection against glutamate excitotoxicity in cortical neurons. Soon after, we observed similar protection in the cortical neurons exposed to hypoxia [80]. Consistently, our electrophysiological studies with UFP-512 (H-Dmt-Tic-NH-CH[CH₂-COOH]-Bid) [54], a more potent and specific DOR agonist, also showed that DOR is relatively specific in the protection against anoxic disruption of ionic homeostasis because MOR activation was not shown to induce any protective effect [55]. Furthermore, our transgenic studies showed that cortical DOR overexpression attenuated anoxia-induced disruption of ionic homeostasis, suggesting that an increase in DOR expression renders the cortex more tolerant to hypoxic stress [83].

Our strong evidence indicates that DOR is a unique neuroprotector against hypoxic/ischemic stress in the brain. Also, there have been abundant data thereafter showing DOR neuroprotection from many independent laboratories worldwide [84-105].

DOR neuroprotection via BDNF-TrkB pathway

Recent data from our laboratory and among others have presented some groundbreaking mechanisms involved in DOR neuroprotection against hypoxic/ischemic brain injury. During acute phases of hypoxic/ischemic stress, DOR protects the neurons mainly by the stabilization of ionic homeostasis, inhibition of excitatory transmitter release, and attenuation of disrupted neuronal transmission. During prolonged hypoxia/ischemia, however, DOR neuroprotection involves a variety of signaling pathways [2, 3, 106].

Neurotrophic factors constitute an important element in determining the fate of neurons under hypoxic/ischemic insults. Our recent studies suggest that some neurotrophic factors (NTFs) such as brain-derived neurotrophic factor (BDNF) are important for DOR-mediated neuroprotection.

Indeed, BDNF is co-localized with DOR in DOR-rich regions in the brain. Using fluorescence immunolabeling, we determined the localized distribution of DOR and BDNF in the cortex and striatum. The BDNF-labeled cells (exhibiting neuronal-like morphology) were found in the cortex and striatum with an abundant distribution in the frontoparietal cortex and lateral caudate putamen in the sham-operated group (Fig. 2A). Triple-labeled confocal images also showed that BDNF and DOR/MAP-2 protein were co-localized in the neuronal-like cells in the frontoparietal cortex (Fig. 2B, C) [107].

DOR upregulates BDNF and TrkB in hypoxia

Both *in vivo* and *in vitro* studies have demonstrated BDNF-induced neuroprotection against hypoglycemia, ischemia, and hypoxia [108, 109]. Since DOR is highly expressed in cortical and striatal regions [20, 21], we further investigated if DOR protects them from hypoxic injury through BDNF [110].

To determine if DOR-induced protection against prolonged hypoxia in cortical regions involves the BDNF pathway, we investigated the effect of DOR activation on the expression of BDNF and other proteins in the cortex of C57BL/6 mice exposed to hypoxia (10% of oxygen) for 1–10 days. We observed that 1-day hypoxia had no appreciable effect on BDNF expression, while 3- and 10-day hypoxia progressively decreased BDNF expression, resulting in a 37.3% reduction ($p < 0.05$) after 10-day exposure. DOR activation with UFP-512 (1 mg/kg, i.p., daily) partially reversed the hypoxia-induced reduction of BDNF expression in the 3- or 10-day exposed cortex.

The BDNF-mediated effect is mediated through activation of TrkB, a high-affinity tyrosine kinase receptor [111–113]. TrkB has two major types of isoforms: a full-length TrkB protein that possesses a tyrosine kinase domain, and a truncated isoform that lacks this domain [108]. Upon activation by BDNF, full-length TrkB undergoes autophosphorylation to regulate Erk/MAPK signaling, which may increase cAMP and activate CREB-regulated gene transcription, which further promotes transcription of BDNF. This is a positive feedback mechanism where BDNF induces the synthesis of BDNF itself (Yoshii and Constantine-Paton, 2010). We therefore determined if DOR activation affects TrkB. Our data showed that DOR activation partially reversed the hypoxia-induced reduction in functional TrkB (140-kDa) and attenuated hypoxia-induced increase in truncated TrkB (90-kDa) in the cortex exposed to 3- or 10-day hypoxia.

With the downregulation of BDNF and TrkB after prolonged hypoxia, the level of tumor necrosis factor- α (TNF- α) increased. After 10-day hypoxia, it significantly increased in the cortex, which was completely reversed following DOR activation (Fig. 3).

Our results suggest that prolonged hypoxia down-regulates BDNF-TrkB signaling, leading to an increase in TNF- α in the cortex, while DOR activation up-regulates BDNF-TrkB signaling, thereby decreasing TNF- α levels in the hypoxic cortex. Since TNF- α is known to induce neuroinflammation and neurotoxicity in the hypoxic/ischemic brain, DOR neuroprotection is likely mediated, at least partially, by upregulating BDNF-TrkB signaling and thus reducing TNF- α neurotoxicity.

Fig. 2. Co-localization of BDNF and DOR in cortical neurons. Top panel, Fluorescent micrographs of cortical BDNF positive cells. Bar = 30 μ m. Middle panel, Fluorescent micrographs of cortical BDNF/MAP-2 double-labeled positive cells. Bar = 15 μ m. Bottom panel, Fluorescent micrographs of cortical BDNF/DOR double-labeled positive cells. Bar = 15 μ m. Cited from Tian et al. [110].

Fig. 3. Effect of DOR activation on cortical TNF- α expression in hypoxia. Top panel, Representative blots of Western blot analysis of the cortical tissues. Bottom panel, Relative levels of TNF- α . C, Normoxic control. H, Hypoxia. C + U (UFP-512, a DOR agonist), DOR activation with UFP-512 in normoxic condition. H + U (UFP-512), DOR activation with UFP-512 in hypoxic condition. N = 4, 5 and 6 for 1, 3 and 10 days groups, respectively. * $p < 0.05$ vs. the control. # $p < 0.05$ vs. C + U (UFP-512). $\Delta p < 0.05$ vs. H + U (UFP-512). Note that 10 days hypoxia significantly increased the level of TNF- α , while DOR activation completely inhibited such increase. Cited from Tian et al. [110].

DOR activation reverses ischemic downregulation of TrkB

In another study [107], we further investigated if DOR modulates the BDNF-TrkB pathway in cerebral ischemia.

We exposed adult male Sprague-Dawley rats to focal cerebral ischemia, which was induced by middle cerebral artery occlusion (MCAO). DOR agonist TAN-67 (60 nmol) or antagonist Naltrindole (100 nmol) was injected into the lateral cerebral ventricle 30 min before MCAO. We measured ischemic injury and detected the expression of BDNF, full-length and truncated TrkB at 24 hours after MCAO. Our results showed that MCAO caused a large volume of ischemic infarct and significantly decreased full-length TrkB protein expression, while DOR activation with TAN-67 significantly reduced the size of ischemic infarct and largely reversed the decrease in full-length TrkB expression in the ischemic cortex (Fig. 4) and striatum (Fig. 5) without any appreciable change in cerebral blood flow. Meanwhile, the DOR antagonist, naltrindole, aggravated the ischemic injury.

Interestingly, the levels of BDNF remained unchanged in the cortex, striatum, and hippocampus at 24 hours after MCAO and did not change in response to DOR activation or inhibition. This notion is slightly different from that under hypoxic conditions [110]. Since we did not observe any significant changes in BDNF in the cortex after 24 hours of hypoxic exposure, it is likely that BDNF, as a neuroprotective factor in the cortex, is maintained at a relatively stable level for the purpose of neuroprotection. Therefore, no appreciable changes were found in tissues sampled from the brain at 24 hours after ischemia. In contrast to BDNF, TrkB seems more sensitive to ischemic stress. This is based on the notion that full-length functional TrkB protein was significantly reduced in the cortex at 24 hours after ischemia, while it did not have a significant change in the same region after exposure to hypoxia for 24 hours [110]. All these data suggest that DOR activation rescues TrkB signaling by reversing ischemia/reperfusion induced decrease in the full-length TrkB receptor and reduces brain injury in ischemia/reperfusion.

DOR activation increases astrocytic BDNF expression

BDNF, nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) are expressed in both neurons and astrocytes. Both astrocytic and neuronal NTFs are thought to enhance the growth, functional maintenance, and phenotypic development of neurons and play a crucial role in brain protection against hypoxic/ischemic encephalopathy and neurodegenerative diseases. However, there was no published information available in

Fig. 4. Effects of DOR activation and inhibition on cortical TrkB at 24 hrs after MCAO. A, Representative Western blot images of TrkB expression in different groups. B, Quantitative analysis of 140 KDa full-length TrkB (n=4). S, Sham control. M, MCAO. *P<0.05 vs. the sham control. #P<0.05 vs. M + TAN67 (a DOR agonist). Note that MCAO significantly reduced the expression of 140 KDa TrkB in the cortex, while DOR activation with TAN67 largely reversed such ischemic reduction. Cited from Tian et al. [107].

the literature in terms of the interaction between DOR and NTFs in astrocytes before our work [114]. We therefore cultured pure astrocytes from the mouse brain and studied the effect of DOR activation by UFP-512, a potent DOR agonist, on mRNA expression of NTFs using quantitative RT-PCR. Our data revealed that DOR activation with UFP-512 enhanced mRNA expression of both BDNF and NGF, but not of GDNF. This effect could be largely attenuated by DOR antagonist, Naltrindole. A protein kinase C inhibitor, Calphostin C, completely blocked UFP-512 induced BDNF expression. In contrast, a protein kinase A inhibitor, H89, significantly suppressed UFP-512 induced NGF expression. Both Calphostin C and H89 had no appreciable effect on GDNF expression. These data suggest that the DOR activation up-regulates BDNF and NGF expression through differential protein kinase pathways in astrocytes, which may also form a component of molecular mechanisms underlying the DOR protection [114].

Conclusion

DOR was traditionally thought to be primarily involved in modulating the transmission of messages along pain pathways. Most previous studies focused on its function in pain modulation and addiction. Therefore, little was known regarding its neural

Fig. 5. Effects of DOR activation and inhibition on striatal TrkB at 24 hours after MCAO. Top panel, Representative Western blot images of TrkB expression in different groups. Bottom panel, Quantitative analysis of 140 KDa full-length TrkB (n=4). S, Sham control. M, MCAO. *P<0.05 vs. the sham control. #P<0.05 vs. M + TAN67 (a DOR agonist). Note that the MCAO-induced changes and DOR effects are the same as those of the cortex. Cited from Tian et al. [110].

function, along with major controversies on its role in the brain under hypoxic/ischemic conditions. Our serial studies have reconciled previous controversies, and demonstrated that DOR is a unique neuroprotector in the brain and plays an important role in neuroprotection against hypoxic and ischemic brain injury. Moreover, many other investigations have also shown DOR's neuroprotection against hypoxic/ischemic injury. Our mechanistic research has led us to paint a picture depicting cellular and molecular mechanisms underlying DOR neuroprotection. More recently, our data provide new evidence showing that DOR may display its protective role in the brain, especially the cortex and striatum, via neuronal and astrocytic BDNF-TrkB pathways. It is our belief that further comprehensive and in-depth research on DOR neuroprotection will eventually open a door for the prevention and treatment of stroke and other neurodegenerative diseases, such as Parkinson's disease, since previous clues and new molecular evidence [115-118] suggest that DOR activation may trigger an anti-parkinsonian effect.

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Disclosure Statement

The authors declare no conflicts of interest regarding the publication of this article.

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