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δ-opioid receptors protect from anoxic disruption of Na⁺ and K⁺ homeostasis via Na⁺ channel regulation

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Abstract

Hypoxic/ischemic disruption of ionic homeostasis, especially Na⁺ influx and K⁺ leakage, is a critical trigger of neuronal injury/death in the brain. There is, however, no promising strategy against such pathophysiological changes to protect the brain from hypoxic/ischemic injury. Here we present an exciting finding that activation of delta-opioid receptor (DOR), which is highly expressed in the cortex, reduced anoxic Na⁺ influx and K⁺ leakage in the cortex by restricting Na⁺ influx through voltage-gated Na⁺ channels. Furthermore, we show for the first time with direct evidence that DOR expression/activation indeed plays an inhibitory role in Na⁺ channel regulation by decreasing the amplitude of sodium currents and increasing activation threshold of Na⁺ channels. These first data have far-reaching impacts on understanding the intrinsic mechanism of neuronal responses to stress and provide novel insights into better solutions of hypoxic/ischemic encephalopathy and other neurological disorders such as epilepsy and pain.

Keywords

δ-opioid receptors; Na⁺ channels; anoxia; Na⁺ influx; K⁺ efflux; neuroprotection

INTRODUCTION

It has been well recognized that hypoxic/ischemic disruption of ionic homeostasis, especially massive Na⁺ influx and K⁺ leakage, is a critical trigger of neuronal injury/death in the brain (1–7), there is, however, no promising strategy against such pathophysiological changes to protect the brain from hypoxic/ischemic injury. Recently, we have shown that the activation of δ-opioid receptor (DOR) is neuroprotective from hypoxic/ischemic injury (8–11, Zhang et al, 1999 SfN Abstract), which is also documented by many other investigators (12–19). In the mechanistic exploration, our initial data suggest that the DOR-induced neuroprotection may be attributed to the stabilization of Na⁺-K⁺ homeostasis across the membrane under anoxic stress (20–22). Since Na⁺ channels are the major route of Na⁺ entry into neurons, our preliminary observations prompt us to raise an interesting question: Does

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DOR regulate Na⁺ channel function and thus protect the brain from anoxic disruption of Na⁺-K⁺ homeostasis? More specifically, the questions we might ask: Does DOR activation reduce anoxic Na⁺ influx and K⁺ leakage in the brain? Does DOR expression/ activation indeed affect Na⁺ channel function? There is, however, no information available in literature.

Our previous studies imply a potential interaction between DOR and Na⁺ channels in the cortex. For example, after being exposed to prolonged hypoxia during postnatal development, cortical neurons are more sensitive to subsequent stress, which is largely attenuated by tetrodotoxin (TTX), a Na⁺ channel blocker (23). Interestingly, an increased Na⁺ channel density (23) and decreased DOR density (Xia et al, 2001 SfN Abstract) occurred in the exposed brain. Furthermore, we observed that in a mutant brain with epileptic seizures, cortical neurons are hyper-excitable with an up-regulation of voltage-gated Na⁺ channels (24) and down-regulation of DOR expression (25). All these observations, though circumstantial, suggest that DOR may mediate an inhibitory regulation of Na⁺ channels in the brain under pathological conditions and then influence Na⁺ and K⁺ homeostasis in the brain. Since Na⁺ channel up-regulation has been proven to participate in pathological changes in several neurological disorders such as hypoxic/ischemic dysfunction/injury and epilepsy (23,24,26), the DOR-mediated inhibition on Na⁺ channels, if proven to be true, may open up a new field of research in pursuit of novel solutions for hypoxic/ischemic encephalopathy and other neurological disorders such as epilepsy.

We therefore performed this study to address three fundamental questions regarding the role of DOR in ionic homeostasis under anoxic condition. Firstly, we determined if DOR activation directly reduces anoxic Na⁺ influx in the cortex by examining the effect of DOR activation on anoxic Na⁺ influx measured with Na⁺-sensitive electrodes. Secondly, we tested whether anoxic K⁺ leakage is dependent on Na⁺ influx through Na⁺ channels in the cortex by examining the effect of Na⁺ channel blockade on anoxic K⁺ leakage measured with K⁺-sensitive electrodes. Thirdly, we investigated if DOR truly plays an inhibitory role in the regulation of Na⁺ channels, which was done in *Xenopus* oocytes with expression of Nav1.2 channels that are highly expressed in the cortex (24,27) by examining the effect of DOR expression and/or activation on Na⁺ channel currents.

RESULTS

1. DOR activation reduced anoxic Na⁺ influx in the cortex

To obtain the first evidence as to whether DOR activation reduces Na⁺ influx in anoxia, we measured the changes in extracellular [Na⁺] induced by anoxia in mouse cortical slices. When the slices were equilibrated in artificial cerebrospinal fluid (ACSF), the baseline of extracellular [Na⁺] was found to be around 152 mM at rest and was stable for at least 90 minutes (Figure 1). In response to anoxic stress, extracellular [Na⁺] displayed a sudden drop from its baseline to 45.93±3.67 mM (n=5). After re-introducing the slices to oxygen, extracellular [Na⁺] gradually recovered to the baseline in 7.7±0.9 min (n=5). The response of extracellular [Na⁺] to anoxia decreased when UFP-512 (1–5 μM), a specific and potent DOR agonist (28), was applied to the slices starting 20 min. before anoxia and lasting for the whole period of anoxic stress. For example, UFP-512 at 5 μM significantly attenuated the drop of extracellular [Na⁺] (62.47±4.84 mM vs. 45.93±3.67 mM in control, p=0.026). UFP-512 also accelerated the recovery of the anoxia-induced drop of extracellular [Na⁺] (3.7±0.6 min vs. 7.7±0.9 min. in control, n=5, p=0.0053). These results suggested that DOR activation inhibits anoxia-induced Na⁺ influx in the cortex.

2. DOR activation attenuated Na⁺-triggered K⁺ leakage in the cortex

Overloaded intracellular Na⁺ may trigger substantial K⁺ efflux (22,29) that plays a key role in neuronal injury (3–6). In our previous work, we have demonstrated that DOR-mediated inhibition of Na⁺ entry constitutes a major mechanism underlying the DOR-protection against anoxic K⁺ derangement in the cortex (22). Also as shown in Table 1, the similar treatment as in Figure 1 (i.e., DOR activation with UFP-512) decreased K⁺ leakage by 39.06±9.32% (p<0.001) and prolonged the anoxic response by 2.4±0.3 times (p<0.001, n=14), accompanying with the reduction of anoxic Na⁺ influx. Since the major route of Na⁺ influx is voltage-gated Na⁺ channels that are highly expressed in the cortex (30,31), we asked whether DOR targets at the Na⁺ influx through voltage-gated Na⁺ channels, thus attenuating K⁺ leakage. Therefore, we applied TTX, a potent and specific voltage-gated Na⁺ channel blocker, to the cortical slices and tested its effect on DOR protection from anoxic K⁺ leakage. With perfusion of TTX (1 μM), as shown in Table 1, anoxia-induced increase in peak extracellular [K⁺] decreased by 53.30±9.39% (p<0.001), and the latency of response to anoxia increased by 2.1±0.2 times (p<0.001). In the presence of TTX (1 μM), UFP-512 could not further reduce the anoxic increase in peak extracellular [K⁺] and also, could not further prolong the latency of response to anoxia. These results suggest that anoxic K⁺ leakage was greatly dependent on Na⁺ influx via the voltage-gated Na⁺ channels and DOR signal may target at the voltage-gated Na⁺ channels to restrict Na⁺ influx and thus K⁺ leakage in the cortex.

3. DOR expression and activation down-regulated Na⁺ channel function

Since the major subtype of the voltage-gated Na⁺ channels in the cortex is Na1.2 channels (24,32), we further asked whether DOR truly plays an inhibitory role in the regulation of Na1.2 channels. Because there is no specific activator/blocker for individual subtype of Na⁺ channels, we expressed Na1.2 channels in *Xenopus* oocytes and then tested the effects of DOR expression and activation on Na1.2 channel function.

3.1 Injection of Na⁺ channel cRNA induced TTX-sensitive inward currents—

First, we examined whether there was any endogenous inward current in the naive oocytes in our experimental system. As shown in Figure 2, a family of current traces was recorded in the oocytes in the solution with “normal” Na⁺ concentration. There were no recordable inward currents whatsoever (Figure 2B). The addition of TTX (1 μM) did not affect the recordings (Figure 2D). The similar recordings were also made in the buffer with 0 Na⁺ concentration (Figure 2C). Figure 2E (n=5) showed clearly that there was no appreciable inward current in the naive oocytes without injection of exogenous cRNA in any conditions.

Large inward currents could be recorded after the injection of Nav1.2 cRNA (Figure 3). Forty hours after the injection of 5 ng cRNA, the inward currents appeared from −30 to 30 mV in the depolarized mode. The mean value was about −1765 ± 290 nA with the peak current at the potential 0 mV (n=6) (Figure 3 A, D). Within the range of depolarization from −100 mV to 70 mV in 10 mV increments, the inward current opened to its max value at 50–60 ms during the simulated time frame. These inward currents could not be seen in the solution containing zero Na⁺ (n=6; Figure 3 B, D). The application of TTX in the “normal” solution (90 mM Na⁺) completely abolished the inward currents (n=6; Figure 3 C, D), suggesting that the inward currents were TTX-sensitive and Na⁺ channel-mediated currents.

3.2 Na⁺ current size was dependent on the amount of injected cRNA and culture time—

We measured the sodium currents in the oocytes with various amounts of injected Nav1.2 cRNA after 40 h of incubation. As shown in Figure 4A, no inward currents were recorded in the group without injection of Na1.2 cRNA (n=5). Only one out of three oocytes showed inward currents with the peak value being less than 500 nA when injected

with 1 ng of Nav1.2 cRNA (Figure 4B) (n=3). At 5 ng of Nav1.2 cRNA, the sodium currents increased greatly with the average value of the peak currents being -1765.2 ± 290.5 nA (n=6) (Figure 4C). In the oocytes with injection of 10 ng Nav1.2 cRNA, the inward currents increased by several folds with the peak values being >6000 nA (-6604.8 ± 416.7 nA) (n=6) (Figure 4D). These results, as summarized in Figure 4E and 4F, suggest that sodium current amplitude was proportionally related to the amount of the Nav1.2 cRNA injected into the oocytes. On the other hand, the amplitude of sodium currents increased with the time after the injection. For example, in the oocytes with 5 ng of Nav1.2 cRNA, the current amplitude could double or more if the culture time was extended from 40 to 72 hours or longer (Figures 5–6).

3.3 Na⁺ currents were inhibited by DOR activation in oocytes with co-expression of DOR, but not in those without DOR expression—To determine the role of DOR in Na⁺ channel regulation, we co-expressed Nav1.2 channels and DOR in the oocytes and then recorded sodium currents. Because DOR density critically affects neuronal responses to hypoxia (11), we expressed DOR at different levels by injecting various amounts of DOR cRNA into the oocytes and then tested their effects on sodium currents. To maximize effects of DOR expression, we extended the culture time to 72 hours in this set of the experiments since the protein expression increases with time after the injection of cRNA as shown above.

In the group with the injection of 1 ng DOR cRNA, activation of DOR with 5 μ M of UFP-512 had little effect on sodium currents (n=5, data not shown). When the injected DOR cRNA was increased to 10 ng, DOR activation with the same amount of UFP-512 significantly reduced the sodium current by 27.4% ($p=0.01285$, n=4) (Figure 5A). When the injected DOR cRNA was increased to 20 ng, 5 μ M of UFP-512 decreased sodium current amplitude by 35.2% ($p=0.03042$, n=9; Figure 5C). In the oocytes with a large amount of DOR cRNA (10 and 20 ng), UFP-512 significantly inhibited sodium currents within a broad range of depolarization potential (-30 to $+40$ mV) (Figure 5A, 5C). On the other hand, UFP-512 (5 μ M) had no significant effect on sodium currents in the oocytes expressing Nav1.2 alone (5 ng Nav1.2 cRNA) ($p>0.05$, n=10, data not shown). Interestingly, our data suggest that DOR expression alone (without DOR activation) could reduce the size of sodium currents. As shown in Figure 6, the size of sodium currents was much smaller in oocytes with injection of 20 ng DOR cRNA than in those with 10 ng DOR cRNA.

To further characterize the DOR-induced inhibition of sodium currents, we analyzed the conductance/amplitude–voltage relationship by fitting with a Boltzmann equation in the oocytes with injection of 10 and 20 ng of DOR cRNA. Our results showed that DOR activation right shifted the curves (Figure 5B and 5D), suggesting that DOR activation not only reduced the size of sodium currents but also increased the threshold of sodium channel activation.

In general, the peak current was around 0 mV of the potential in the oocytes with 5 ng of Nav1.2 cRNA alone (Figure 3). With DOR co-expression, the evoked peak currents appeared at different depolarization potentials, typically 4 different types of recordings, i.e., 20 mV, 0 mV, -10 mV, and -30 mV. Despite of this difference, DOR activation could further inhibit sodium currents in all these oocytes with DOR expression (Figure 7).

3.5 DOR activation reduced Na⁺ channel currents in a dose-response manner—We performed a dose-response study to further clarify the DOR activation-induced inhibition of sodium currents. In the oocytes with co-expression of Nav1.2 channels (5 ng cRNA) and DOR (10 ng cRNA) under the same experimental conditions, we applied UFP-512 at concentrations of 0, 0.1, 0.5, 2.5 and 5 μ M and compared their effects on the

sodium currents (Figure 8). We found that 0.1 μM or less hardly inhibited sodium currents (Figure 8B). However, at 0.5 μM , UFP-512 induced significant inhibition on the sodium currents (Figure 8C). The maximal inhibition was seen at 2.5 μM (Figure 8D). When the concentration was increased to 5 μM , the DOR-mediated inhibition of sodium currents could not be further strengthened (Figure 8E, 8F), suggesting a saturable dose-response manner in terms of the DOR agonist induced inhibition on the sodium currents.

In control naive oocytes, the application of UFP-512 (5 μM) had no effect at all on the electrophysiological recordings that could not detect any inward current ($n=9$, data not shown).

DISCUSSION

We have made an exciting finding that DOR activation reduced anoxic Na^+ influx and K^+ leakage in the cortex by restricting Na^+ entry through voltage-gated Na^+ channels. Furthermore, we presented the first direct evidence that DOR indeed plays an inhibitory role in Na^+ channel regulation. Since hypoxic/ischemic disruption of ionic homeostasis, especially Na^+ influx and K^+ leakage, is a key trigger of neuronal injury/death, the present data are of major significance in understanding the intrinsic nature of neuronal response to environmental stress and provide novel insights into better solutions of hypoxic/ischemic encephalopathy.

There are tremendous data that solidly demonstrate that Na^+ influx and K^+ leakage triggers neuronal injury or even death in the brain (1–7). The DOR-induced reduction of Na^+ influx and K^+ leakage is certainly beneficial to neuronal adaptation to anoxic stress. The significance of these observations is clearly self-explanatory. More importantly, our data, especially those obtained from *Xenopus* oocytes, reveal a novel mechanism underlying the reduction of Na^+ influx and K^+ leakage, i.e., DOR's inhibitory role in Na^+ channel regulation.

It has been shown that the pore-forming α subunits generate sodium currents with gating properties similar to native channels when expressed in *Xenopus laevis* oocytes (33). In our work, the oocytes with injection of Nav1.2 cRNA showed large voltage-dependent inward currents that could be completely inhibited by TTX or in a zero Na^+ solution, demonstrating that the inward currents we observed are indeed sodium currents. Therefore, it is reliable to use this model for investigating the response of Na^+ channels to DOR activation.

Historically, there is only one published study suggesting that high doses of SNC80, a putative DOR agonist, caused an opioid-independent effect on sodium channels in the rat hippocampus (34). However, we believe that the role of DOR in Na^+ channel regulation needs to be clarified with more direct evidence because the hippocampus may differ from other brain region such as the cortex in terms of Na^+ channel function and neuronal response to DOR activation. Our previous work has shown that the hippocampus has a very low density of DOR, while the cortex has a much higher density (35,36). This may lead to a major difference between the cortical and hippocampal neurons in neuronal properties including the responses of Na^+ channels to endogenously released opioids and exogenous opioid agonists/antagonists. For instance, applying low concentrations of DOR agonists to hippocampal neurons may induce an insignificant effect because of low DOR density. On the other hand, high concentrations of DOR ligands, either SNC80, naloxone or naltrindole, may induce a complicated, even adverse effect on neurons because of the non-selective action on multiple membrane proteins. Therefore, the differential distribution and density of DOR among cells/tissues may greatly affect neuronal tolerance to hypoxic/ischemic stress and the response of Na^+ channels to DOR activation. Indeed, there is evidence showing that

the cortical neurons are more tolerant to hypoxic stress than the hippocampal neurons (37,38). In addition, some experimental procedures such as mechanical trituration and chemical digestion (e.g., Pronase) (34) may affect the function of DOR and Na⁺ channels as well as their interactions, causing complex cellular changes in presence of DOR agonists/antagonists. To avoid these pitfalls and explore a “pure” interaction between DOR and Nav1.2 channels, we took the advantages of *Xenopus* oocytes in this work and made the exciting findings.

Sodium channels are critical to the generation and conduction of the action potential. Of all the existent Na⁺ channel isoforms, neuronal Nav1.2 channels are abundantly expressed in the central nervous system, especially in the cortex. To our knowledge, the present study represents the first demonstration that activation of DOR reduces the current amplitude and inhibits the excitability of Na⁺ channels, suggesting that DOR plays an inhibitory role in the regulation of Na⁺ channels. This is based on the following findings: (1) the amplitude of Nav1.2 currents was significantly reduced by the DOR agonist, UFP-512 (21,28) in a dose-dependent manner, which is a typical agonist-receptor reaction, and (2) activation of DOR by UFP-512 induced a right shift of the activation curve of Nav1.2 currents, which was seen in the oocytes co-expressing Nav1.2 channels and DOR but not in those without DOR expression. If this inhibitory effect was not mediated by DOR, we would see this phenomenon in the oocytes without DOR expression. Additional supporting evidence comes from the fact that an increase in DOR expression down-regulated Na⁺ currents.

Take together with the data obtained from slice recordings in the present work, we are confident that that DOR plays an inhibitory role in Na⁺ channel regulation in the cortex that has a high density of DOR (35,36). Our findings may have far-reaching significance in therapeutic potentials for certain neurological conditions such as hypoxic/ischemic injury, epilepsy and pain. Under acute pathophysiological conditions such as hypoxia/ischemia, excessive Na⁺ entry through Na⁺ channels (39,40) may trigger a serial process of neuronal injury and death (1–3,7,40). The DOR-induced inhibition of such pathophysiological response is able to rescue neurons from hypoxic/ischemic injury. On the other hand, an improved interaction between DOR and Na⁺ channels may improve the outcome of chronic neurological conditions. The inhibitory regulation of Na⁺ channels by DOR may contribute to proper control of neuronal excitability. An impairment of such mechanism may lead to neuronal dysfunction/injury and eventually neurological diseases. For example, sodium channel mutation has been casually linked to human epilepsy (41,42). Na⁺ channel up-regulation has been demonstrated to critically lead to epileptic hyper-excitability and seizures (24,26). Interestingly, we observed that in the mutant brain exhibiting spontaneous epilepsy, Na⁺ channel was up-regulated (24), while DOR was down-regulated (25), suggesting a potential role of DOR impairment in the pathophysiology of epilepsy associated with genetic abnormality. Since Na⁺ channel up-regulation contributes greatly to epileptic hyper-excitability and most of anti-epileptic drugs are actually inhibitors of Na⁺ channels (43), the DOR-mediated inhibition of Na⁺ channels may provide a novel clue for new solutions of epileptic seizures. In addition, the opposite changes in DOR and Na⁺ channel are also found in the brain and neurons exposed to prolonged hypoxia that have increased sensitivity to subsequent stress (23), while DOR activation attenuates Na⁺ channel dysregulation in hypoxia (Ma et al, 2004 SfN Abstract).

The expression and functional properties of voltage-gated Na⁺ channels in peripheral sensory neurons can be dynamically regulated following axonal injury or peripheral inflammation, suggesting that specific voltage-gated Na⁺ channels may play crucial roles in inflammatory, and possibly neuropathic pain (44). Since DOR is inhibitory to Na⁺ channels, the anti-nociception effect of DOR may also be related to the inhibition of sodium channels.

More interestingly, our recent work suggests that electroacupuncture may protect the brain from ischemic injury via the DOR system (Zhao et al, 2002 SfN Abstract). Another independent group also observed this phenomenon (15). Since acupuncture or electroacupuncture can activate the opioid system in the brain (45), we believe that our finding in this study may help us better explore the mystery of acupuncture therapy on some neurological diseases.

In summary, we found that DOR activation reduced anoxic Na⁺ influx and K⁺ leakage in the cortex by restricting Na⁺ entry through voltage-gated Na⁺ channels. Moreover, we found that in oocytes with the co-expression of Nav1.2 channels and DOR, activation of DOR significantly decreased the amplitude of sodium currents in a dose-dependent fashion, and right shifted the activation curve of Nav1.2 channels. Furthermore, DOR expression itself reduces the amplitude of sodium currents. All these data represent the *first* findings showing that DOR expression/activation plays an inhibitory role in Na⁺ channel regulation, thus attenuating anoxic disruption of Na⁺ and K⁺ homeostasis in the brain. Our findings may have a far-reaching significance for novel solutions of certain neurological disorders such as hypoxic/ischemic injury, epilepsy and pain and for exploring the mystery of acupuncture therapy on neurological diseases.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). All procedures on the mice were performed in accordance with the guidelines of the Animal Care and Use Committee of Yale University School of Medicine, which is accredited by the American Association for Accreditation for Laboratory Animal Care.

Adult female *Xenopus laevis* with a body weight of 200–250 g were provided by Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and were housed in a tank with constantly filtered and re-circulated water maintained at 20°C. The *Xenopus laevis* were fed with beef and pig hearts. The experimental protocol was approved by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Chemicals and reagents

Drugs and chemicals used in the study were as follows: Na⁺ ionophore VI (Fluka 71739), 2-nitrophenyl octyl ether (Fluka 73732), potassium tetraphenylborate (Fluka 72018), hexamethyldisilazane (Fluka 52619), N-(trimethylsilyl)dimethylamine (Fluka 41420), valinomycin (Fluka 94675), potassium tetrakis (p-chlorophenyl) borate (Fluka 60591), 2,3-dimethylnitrobenzene (Fluka 40870), 3-(N-morpholino) propane sulfonic acid (MOPS, Amresco, USA), tetramethylammonium chloride (TMA, Shymax Chemical Co, China), mineral oil (Bio-Rad, USA), liberase blendzyme 3 (Roche, USA), ciprofloxacin (ICN Co., USA), tetrodotoxin (Sigma, USA). UFP-512, a specific and potent DOR agonist (28) was synthesized by our research team. RNA transcription kit, Transcription kit Sp6 and RNA Transcription kit T7 were the products from Ambion (USA).

Slice preparation

Cortical slices were prepared from 24–32 day old mice as described in our previous studies (20–22). In brief, the brain was rapidly removed from the skull after decapitation under inhalational anesthesia with ether and placed in chilled carbogen-saturated ACSF for 1–2 min. Transverse cortical slices (400 µm) were cut on a vibrotome containing carbogen saturated ice-cold ACSF. Slices were then transferred to an incubation holder placed in a

beaker containing 150 ml ACSF vigorously aerated with carbogen at ~35°C. Standard ACSF consisted of (in mM) NaCl 125, KCl 3.1, NaHCO₃ 26, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.25, and D-glucose 10 at pH 7.4. After an equilibration period of at least 90 min in carbogen saturated ACSF at ~35°C, slices were used for recording.

Induction of anoxia in cortical slices

A slice was transferred to the recording chamber (Model RC-22C, Warner Instrument Co, Hamden, CT) after equilibration with carbogen saturated ACSF. The chamber was perfused with carbogen saturated ACSF (35.5±0.5°C) with a flow rate of ~3 ml/min. Slices were completely submerged ~0.5 mm below the ACSF surface in the tissue chamber and kept under normoxic conditions for at least 15 min at ~35.5°C before experimental measurements were taken. Anoxia was induced by switching from the control superfusate (95% O₂, 5% CO₂) to one continuously bubbled with 95% N₂ and 5% CO₂. Each slice was subjected to a single period of anoxia that continued for about 1.5 min after the onset of anoxic depolarization (as assessed by a rapid increase in extracellular [K⁺] or sudden drop of extracellular [Na⁺] that usually occurs within 10 min after the onset of anoxia), or for a period of 20 min if anoxic depolarization did not occur.

Measurements of extracellular [Na⁺] and [K⁺]

Extracellular Na⁺ and K⁺ concentrations were measured using Na⁺ and K⁺ sensitive microelectrodes. K⁺ and Na⁺ sensitive microelectrodes were prepared as described previously (20,21,46). Glass capillary-pulled electrodes were silanized by exposure to N-(trimethylsilyl)dimethylamine for K⁺ electrode and hexamethyldisilazane for Na⁺ electrode, and subsequently baked at about 180°C for at least 2 h. The microelectrode tips were then broken back to ~2µm. The internal filling solution (10 mM KCl for K⁺ electrode; 150 mM NaCl + 10 mM HEPES for Na⁺ electrode) was injected from the back into the electrode. A column of optimized membrane phase (for K⁺ electrode: 5% wt. valinomycin, 2% potassium tetrakis (p-chlorophenyl) borate, and 93% 2,3-dimethylnitrobenzene; for Na⁺ electrode: 10% Na⁺ ionophore VI, 89.5% 2-nitrophenyl octyl ether, and 0.5% potassium tetraphenylborate), with height about 2 mm was sucked into the microelectrode tips. The reference electrode was a Ag/AgCl bridge electrode embedded in 2% agar in 3 M KCl. Calibrations were carried out by detecting the responses generated in KCl or NaCl solutions (1, 3.1, 5, 10, 20, 40, 80, 100, 160 mM KCl; 5, 10, 20, 50, 80, 100, 120, 150 mM NaCl) in triplicate. For each concentration, the average of voltage changes in three separate tests was used as the final voltage change. Over this range electrode response was near ideal, showing a logarithmic relationship to [K⁺] and [Na⁺].

Electrical signals were monitored on an oscilloscope and recorded by a DC amplifier (Model IE-210, LPF 200, Warner Instrument Co., Hamden, CT) and digitized by an Axon minidigitizer acquisition system (Model miniDigi 1A, Axon Instruments, Union City, CA) at a sampling rate of 100Hz. The following parameters were derived to assess K⁺ homeostasis: (1) the latency of anoxia-induced extracellular [K⁺] increase (Latency), which was defined as a time period from the beginning of anoxia to the time point when anoxia induced a K⁺ electrode voltage change greater than 1mV; (2) Maximal [K⁺] ([K⁺]_{max}), which was the peak change in extracellular [K⁺] induced by anoxia. For assessment of Na⁺ activities, the maximal drop in extracellular Na⁺ concentration induced by anoxia and the recovery duration of Na⁺ drop to basal level during reoxygenation were analyzed.

After recording of a stable baseline for at least 5 minutes, the slices were subject to experimental treatments. The electrophysiological recordings were continuously performed at least 60 or 75 min.

***Xenopus* oocytes preparation**

Females of the clawed toad *Xenopus laevis* under anesthesia were placed on a surgical tray buried in the ice. The ovary was gently pulled out from abdominal incision and 3 small pieces of the tissues were immediately removed from the ovary. Then, the pieces of ovaries were put into a dish filled with ORI solution (90 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 5 mM MOPS, pH 7.4 with Tris base), and the adventitia of ovaries was torn slightly to expose oocytes. After clearing away the dead oocytes and debris, oocytes were treated with 5 ml of ORI containing liberase blendzyme 3 (0.3 U/ml). After 4 hours of enzymatic digest on a shaker (40 rounds/min), the oocytes were moved into a centrifuge tube, and then gently washed three times with Ca²⁺-free ORI solution (90 mM NaCl, 2 mM KCl, 5 mM MOPS, pH 7.4 with Tris base), and five times with standard ORI solution. Large and healthy oocytes at stage V characterized by clear pigments in the animal poles were chosen for experiments.

Preparation of Nav1.2 cRNA and DOR cRNA

The increase, isolation and purification of the plasmids were performed according to the standard protocols (47). Nav1.2 cRNA was transcribed from Not I linearized rat Nav1.2 cDNA templates (generously provided by Dr. Goldin, Department of Microbiology and Molecular Genetics, University of California, USA) using RNA Transcription Kit T7 and was generously provided by Shanghai University Life-Science College. DOR cRNA was transcribed from linearized mouse DOR cDNA (kindly provided by Dr. Gang Pei, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences) using RNA Transcription kit SP6. The cRNA yield was estimated by agar gel analysis and spectrophotometer and then used for microinjection (see below).

Microinjection and Nav1.2 and DOR expression in *Xenopus* oocytes

The microinjection into the oocytes was made based on the methods described by Guille (48). Glass capillary-pulled electrodes were used as a microinjection needle, which was connected to a microsyringe pump controller (WPI, USA) and fixed on the micromanipulator. The manipulation was made on ice. Under the microscope, the electrode containing cRNA to be injected was moved onto the surface of one group of oocytes aligned in a dish, and inserted into the oocyte at its demarcation line between the animal and vegetal poles (about 300 µm in depth). The cRNA was injected with the desired amount in a rate of 5 nl/min controlled by the microsyringe controller. The amount of injected Nav1.2 cRNA in our experiments was 1, 5, and 10 ng per oocyte. For co-injection group, Nav1.2 cRNA at 5 ng and various amounts of DOR cRNA (1, 5, 10, 20 ng) were injected. A batch of non-injected oocytes and another batch of deionized water injected oocytes were used as the controls.

After a batch of oocytes was injected, they were moved to six-well culture plates filled with ORI solution containing ciprofloxacin (70 mg/L), and cultured in an incubator at 20 °C for 40–72 h. The culture media was changed every 20 h, and the dead oocytes were removed. After at least 40 hours of culture, oocytes were used for electrophysiological recording.

Two-electrode voltage-clamp recording

For each experiment, two-microelectrode voltage clamp was performed to record the current in the oocytes at room temperature. Both the potential and current electrodes were pulled on a Narishige PC-10 puller from filament glass pipettes (1.2 mm OD, 0.69 mm ID), and filled with KCl (3 mol/L) with the resistance of 1–5 MΩ. Recordings were conducted using a TURBO TEC-03 amplifier (NPI Electronic Co., Germany). After two-electrode configuration formed, oocytes were voltage-clamped at a membrane potential of −100 mV.

Sodium currents were elicited by applying a series of voltage steps from the holding potential of -100 mV to the test potential of $+70$ mV with an increment of 10 mV/step and an interval of 6 s among steps. Current signals were sampled at 10 kHz with TURBO TEC-03 amplifier (NPI electronic Co., Germany). Currents obtained from oocytes were digitized with an AD interface (Cellworks 5.5.1, NPI Electronic Co., Germany). The signals were displayed and stored on a PC computer using the data acquisition program Cellworks 5.5.1 (NPI electronic Co., Germany) with a simultaneous recording on chart paper by on-line recorder (Servogor SE102-2, KIPP & ZONEN B.V. Delft/Holland). Oocytes were perfused with normal ORI or 0 Na^+ ORI (90 mM TMA, 2 mM KCl, 2 mM CaCl_2 ; 5 mM MOPS, pH 7.4 with Tris base) at a rate of 3 – 5 mL/min. All drugs were prepared with ORI or ORI without Na^+ and administrated to the oocyte via perfusion.

Data analysis

All values were subjected to statistical analysis (one way analysis of variance, Student's t-test, Newman Keuls test, etc) with n representing the number of the cells/slices examined. For ionic electrode experiments, one way analysis of variance followed by Newman Keuls test was used for multiple pair-wise tests and two-tailed, unpaired Student's t-test was used for comparison of two experimental groups. For two-microelectrode voltage clamp experiments, all the data collection and analysis were carried out using Cellworks Reader 3.6 and Origin Pro 7.0 software (OriginLab Co., USA). Normalized currents were calculated by I/I_{70} , where I is the current amplitude measured during the test depolarization, I_{70} is the current at the last step of the test potential ($+70$ mV). The voltage-dependent activation was determined using standard protocol. The conductance (G) was calculated according to $G = I_{\text{max}}/(V - V_{\text{Na}})$, where I_{max} is the peak current amplitude, V is the command potential, and V_{Na} is the reversal potential which is determined by its I-V curve. G was then fitted by Boltzmann equation, $G/G_{\text{max}} = 1/(1 + \exp[(V - V_{50})/K_m])$, where G_{max} is the maximum Na^+ conductance, V_{50} is the voltage where G is half of G_{max} , and K_m indicates the slope of the relationship between channel activation and membrane voltage. Statistical significance was defined by $p < 0.05$.

Abbreviations used

ACSF	artificial cerebrospinal fluid
DOR	δ -opioid receptors
TTX	tetrodotoxin
MOPS	3-(<i>N</i> -morpholino) propane sulfonic acid
TMA	tetramethylammonium chloride

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Figure 1. Effect of DOR activation on anoxia-induced drop in extracellular $[Na^+]$ in cortical slices

Anoxia induced a sudden drop of extracellular $[Na^+]$ from its baseline of 152 mM to 45.93 ± 3.67 mM with 7.7 ± 0.9 min for recovery from peak drop to baseline after re-introducing oxygen ($n=5$) (upper trace). Activation of DOR with UFP-512 ($5 \mu M$) significantly attenuated this drop to 62.47 ± 4.84 mM ($p=0.026$) (lower trace) and accelerated the recovery of anoxia-induced drop of extracellular $[Na^+]$ from 7.7 ± 0.9 min. in control to 3.7 ± 0.6 min. ($n=5$, $p=0.0053$), suggesting that DOR activation reduced the anoxia-induced Na^+ influx in the cortex..

Figure 2. Representative recording traces from the naive oocytes and the current–voltage relationship

The protocol of test potential is shown in A. B–D are families of representative currents traces that were recorded in the control oocytes in 90 mM Na⁺ solution (B), 0 Na⁺ solution (C), and 90 mM Na⁺ solution with 1 μM of TTX (D). E is the current-voltage (I–V) curve of the naive oocytes (n=5). Note that there was no appreciable inward current in naive oocytes.

Figure 3. Inward currents in *Xenopus* oocytes with Na1.2 channel expression

A, A family of representative current traces were recorded in the oocytes with microinjection of 5 ng Nav1.2 cRNA in 90 mM Na⁺ solution (A, n = 6), 0 mM Na⁺ solution (B, n=6), and 90 mM Na⁺ solution plus 1 μM of TTX. (C, n=6). The I-V curves of each group were summarized in D. Note that Nav1.2 channel expression caused inward currents, which disappeared in 0 Na⁺ solution and could be completely blocked by TTX, suggesting that the inward currents are sodium currents.

Figure 4. The relationship between the amplitude of sodium currents and the amount of the injected Nav1.2 cRNA

A family of currents were elicited from control (A, n=5), oocytes with injection of 1 ng (B, n=3), 5 ng (C, n=6), and 10 ng of Nav1.2 cRNA (D, n=6), respectively. The I-V curves of each group were summarized in E. F shows the summary of the relationship between the peak currents and the amount of the injected Nav1.2 cRNA. *p<0.05, **p<0.01 vs. control; #p<0.05, ##p<0.01 vs. 1 ng group; ♣♣p<0.01 vs. 5 ng group. Note that inward currents increased with the amount of injected Nav1.2 cRNA with no inward currents seen in naive oocytes.

Figure 5. DOR activation induced inhibition of sodium currents in oocytes co-expressing DOR and Nav1.2

The recordings were performed in the oocytes expressing Nav1.2 expression (5 ng of injected Nav1.2 cRNA) with co-expression of DOR (A, 10 ng of injected DOR cRNA, n=4; C, 20 ng of injected DOR cRNA, n=9). Five μ M of UFP-512 were applied to activate DOR in these oocytes. B and D are conductance/amplitude–voltage curves from A and C, respectively. * p <0.05, ** p <0.01 vs. control. Note that DOR activation inhibited the size of the sodium currents and rightly shifted the conductance/amplitude–voltage curves.

Figure 6. Effect of DOR expression on sodium currents

Five ng of Nav1.2 cRNA with 10 or 20 ng of DOR cRNA were injected into the oocytes and cultured for 72 hours. * $p < 0.05$ vs. 10 ng DOR cRNA. Note that the magnitude of sodium current was significantly smaller in the oocytes with 20 ng DOR cRNA and 5 ng Nav1.2 cRNA as compared to that of the oocytes with 10 ng DOR cRNA with 5 ng Nav1.2 cRNA in the range of -10 to 30 mV of test potential in the presence of $5 \mu\text{M}$ UFP-512, suggesting that an increase in DOR expression could inhibit sodium channel function.

Figure 7. Effect of DOR activation on peak sodium currents in the oocytes co-expressing sodium channels and DOR

The recordings were performed in the oocytes co-expressing sodium channels (5 ng Nav1.2 cRNA) and DOR (10 ng DOR cRNA). ** $P < 0.01$ ($n = 7$). Note that although the peak currents were elicited at different depolarization potentials, DOR activation with 5 μM of UFP-512 could induce a significant inhibition of peak sodium currents in all cases.

Figure 8. Dose-responses of the DOR agonist-induced inhibition of sodium currents

The recordings were performed in the oocytes co-expressing sodium channels (5 ng of Nav1.2 cRNA) and DOR (10 ng of DOR cRNA). The families of representative current traces show the inward currents recorded in control (A), 0.1 μ M UFP-512 (B), 0.5 μ M UFP-512 (C), 2.5 μ M UFP-512 (D), and 5.0 μ M UFP-512 (E), respectively. F shows the relationship curve of the currents size vs. concentration of UFP-512 (n=4–5). * p <0.05, ** p <0.01 vs. control; # p <0.05, ## p <0.01 vs. 0.1 μ M UFP-512. Note that the DOR agonist-induced reduction of sodium current amplitude was in a dose-response manner.

Table 1Effect of TTX on DOR protection against anoxic K⁺ derangement

	[K]max (% of control)	Latency (of control)	n
UFP-512	-39.06±9.32	2.4±0.3	14
TTX	-53.30±9.39	2.1±0.2	13
TTX+UFP-512	-58.57±10.33	2.3±0.2	14